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Full-length article

Synergistic effect of combining paeonol and cisplatin on apoptotic induction of human hepatoma cell lines[1]

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Key words

paeonol; cisplatin; drug interaction; human hepatoma cell line; apoptosis; cell cycle

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Abstract

Aim: To investigate whether paeonol (Pae) has synergistic effects with cisplatin (CDDP) on the growth-inhibition and apoptosis-induction of human hepatoma cell lines $HepG_2$ and SMMC-7721. **Methods:** The cytotoxic effect of drugs was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay. The coefficient of drug interaction was used to analyze the nature of drug interactions. Morphological changes were observed by acridine orange fluorescence staining. Cell cycle and the apoptosis rate were detected by flow cytometry. Bcl-2 and Bax expression were assayed by immunohistochemical staining. **Results:** Pae or CDDP had antiproliferative effect on the 2 cell lines in a dose-dependent manner, with different sensitivities to drugs. More interestingly, a synergistic inhibitory effect on the viability of the 2 cell lines was observed after treatment with a combination of Pae (15.63, 31.25, and 62.5 mg/L) with various concentrations of CDDP. Further study showed typical morphological changes of apoptosis if the cells were exposed to the two agents for 24 h. The apoptotic rate of the cells with combination treatment was significantly higher than that of cells treated with Pae or CDDP alone. The expression of Bcl-2 decreased and that of Bax increased in the treated groups, especially in the combination group, with the ratio of Bcl-2/Bax decreasing correspondingly. Additionally, a combination of Pae with CDDP resulted in a stronger S phase arrest, compared with Pae or CDDP alone. **Conclusion:** Pae, in combination with CDDP, had a significantly synergistic growth-inhibitory and apoptosis-inducing effect on the 2 human hepatoma cell lines, which may be useful in hepatoma treatment.

Introduction

Hepatocellular carcinoma (HCC) is a major contributor to cancer incidence and mortality in the world. The incidence of HCC is rising worldwide and 80% of the burden is borne by countries in Asia and sub-Saharan Africa[1]. Despite recent advances in diagnostic modalities for HCC, the disease often develops to an advanced stage before it is detected clinically, and 5 year survival is less than 10%[2,3]. No effective treatment is currently available. Therefore, there is a critical need to develop more effective strategies for the chemotherapy of hepatoma.

Chemotherapy is one of the commonly used strategies in HCC treatment, especially for unresectable patients. Conventional chemotherapeutic drugs such as cisplatin (CDDP), adriamycin, and 5-fluorouracil (5-FU) often have severe side effects that limit their efficacy. Combination therapy with multiple drugs or modalities is a common practice in the treatment of cancers, which can achieve therapeutic effects greater than those provided by a single drug or modality, and can reduce the side effects and resistance to drugs.

Chinese herbal medicines are now attracting great atten-

tion in the world. They have also shown promising effects when combined with chemotherapy and may benefit patients with HCC[4]. Paeonol (Pae), a major active component extracted from the herb *Pycnostelma paniculatum* (Bunge) K Schum, and the root cortex of Paeonia suffruticosa Andrews[5], possesses extensive pharmacological activities such as sedation, hypnosis, antipyresis, analgesic, anti-oxidation, anti-inflammation, and immunoregulation[6]. It is a white needle crystal with a relatively low-melting point of 51–52 °C and has a minimal systemic toxicity (LD_{50} 3430 mg/kg) when orally administered to mice[7]. In our previous study, the antineoplastic activity of Pae has been demonstrated both in cell lines, such as the human erythromyeloid cell line K562, the breast cancer cell line T6-17, the human hepatoma cell line Bel-7404, and cervical cancer cell line HeLa[8], and in animal models bearing HepA hepatocarcinoma[9,10]. A recent study showed that Pae in low concentration had synergetic effect with 5-FU, mitomycin C, and CDDP in inhibiting the proliferation of human colorectal cancer cell line HT-29[11].

The present study was designed to investigate the growth-inhibitory and apoptosis-inducing effect of Pae alone or combined with CDDP in order to develop an effective combination therapy for HCC.

Materials and methods

Cell culture Human hepatoma cells $HepG_2$ and SMMC-7721 were purchased from Shanghai Institute of Hepatocarcinoma (Shanghai, China), and cultured in Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 medium, respectively. Each was supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C in a humid atmosphere with 5% CO_2.

Drugs and chemicals The Pae injection was purchased from First Pharmaceutical Factory of Shanghai (Shanghai, China, Cat No 990402, 5 mg/mL); the CDDP injection was purchased from Nanjing Pharmaceutical Factory (Nanjing, China, Cat No 20050602, 1 mg/1 mL); DMEM and RPMI-1640 medium were from Gibco BRL Life Technologies (Grand Island, NY, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and acridine orange (AO) were from Sigma (St Louis, MO, USA); the DNA-Prep-Reagents Kit was purchased from Beckman Coulter (Miami, FL, USA, Cat No 760279K); rabbit polyclonal antibodies against human Bcl-2 and Bax were all purchased from Lab Vision Corporation (Fremont, CA, USA), and the streptavidin-biotin-peroxidase (S-P) reagents kit was obtained from Fuzhou Maxim Biotech (Fuzhou, Fujian, China).

***In vitro* cytotoxicity assay** $HepG_2$ and SMMC-7721 cells were seeded in 96-well plates at a density of 1×10^3–5×10^3 cells/well in 100 μL medium overnight. Then the cells were treated with various concentrations of Pae or CDDP alone or in combination. After drug exposure for 44 h, the MTT solution (5 g/L) was added to the plates. The cells were incubated at 37 °C for another 4 h. The formazan was dissolved in 150 μL/well DMSO, and the absorbance was detected at 490 nm using the ELx800 Strip Reader (Bio-Tek, Winooski, VT, USA). All MTT experiments were performed in triplicate and repeated at least 3 times. The percentage of cytotoxicity was calculated as follows: cytotoxicity (%) =(1–OD_{490} of experimental well)/OD_{490} of control well. The IC_{50} (defined as the drug concentration with which 50% cell growth was inhibited) was assessed from the dose-response curves.

Analysis of *in vitro* drug interaction The coefficient of drug interaction (CDI) was used to analyze the synergistically inhibitory effect of the drug combination[12]. CDI was calculated as follows: CDI=AB/(A×B). AB is the ratio of the 2-drug combination group to the control group in OD_{490}, and A or B is the ratio of the single drug group to the control group in OD_{490}. Therefore, CDI <1 indicates synergism, CDI <0.7 indicates a significantly synergistic effect, CDI =1 indicates additivity, and CDI >1 indicates antagonism.

AO fluorescence staining The cells were cultured overnight in 6-well plates containing cover slips. After treatment for 24 h, the cover slips were washed twice with PBS and fixed with 95% ethanol for 15 min. After being acidified with 1% acetic acid for 30 s, the cover slips were dyed with 0.1 g/L AO for 10 min. Then the slips were differentiated with 0.1 mol/L $CaCl_2$ for 2 min and washed with PBS 3 times. Finally, the cover slips were sealed and observed under fluorescence microscope (Olympus, Shinjuku-ku, Tokyo, Japan).

Flow cytometry assay The cells were cultured in 6-well plates and allowed to grow to 75%–80% confluency, in triplicate. Non-adhered cells were removed by gentle washing, and the medium was removed and replaced with fresh medium containing Pae and/or CDDP at the desired concentrations. After exposure to drugs for 24 h, the cells were collected and centrifuged at 1500 r/min in a 15 mL tube for 10 min. The cells were washed twice with PBS and resuspended in 50 μL DNA- Prep LPR (Lyse) at room temperature for 20 s. After that, 500 μL DNA-Prep Stain (propidium iodide+RNAse) was added and incubated in darkness at room temperature for 30 min (according to the procedure program of DNA-Prep Coulter Reagents Kit). A minimum of 1×10^6 cells treated for each group were analyzed using an EPICS XL-MCL model Coulter counter

(Beckman Coulter, Fullerton, CA, USA). Cell cycle distribution was analyzed using MacCycle software (Beckman Coulter, Fullerton, CA, USA).

Immunohistochemical analysis for Bcl-2 and Bax The cells were cultured overnight in 6-well plates containing cover slips. After incubation with various concentrations of Pae or CDDP alone or in combination for 24 h, the cover slips were washed twice with PBS and fixed in 4% paraformaldehyde for 25 min. Immunohistochemical staining for Bcl-2 and Bax was performed according to the standard S-P method described in the procedure program of the S-P Reagents Kit (Maxim, Fuzhou, Fujian, China). PBS 10 mmol/L was used as a negative control to replace the primary antibody.

Analysis of immunohistochemical results The immunohistochemical results were quantitatively analyzed by the Biological Image Analysis System (Yokohama, Kanagawa, Japan) which consisted of a Nikon ECLIPSE 80i biology microscope, Nikon Digital Camera DXM 1200F, and ACT-1 version 2.63 software (Yokohama, Kanagawa, Japan), and JEOA 801D Morphologic Biological Image Analysis software, version 6.0 (Jie Da Technologies, Nanjing, Jiangsu, China). The sample was observed on 6 randomly-selected optical fields by microscopy (×400), and an average *A* value was measured.

Date analysis Biostatistical analyses were done using SPSS 11.5 software package (SPSS, Chicago, Illinois, USA). The results of representative experiments are given as mean±SD, and mean±SEM for multiple experiments. The non-parametric Kruskal-Wallis test was used to detect differences among the different experimental groups. The Mann-Whitney U-test was subsequently used for statistical evaluation in 2-group comparisons. Pearson correlation coefficient was used to analyze continuous independent and dependent variables. A level of $P<0.05$ was accepted as statistically significant.

Results

Inhibitory effect of Pae and CDDP on hepatoma cell proliferation $HepG_2$ and SMMC-7721 cells incubated with various concentrations of Pae or CDDP alone for 48 h showed a dose-dependent reduction of cell viability (Figure 1). The *r* values of the dose-effect curves for single-agent Pae on both $HepG_2$ and SMMC-7721 cell lines were 0.959 and 0.984 ($P<0.01$), respectively. Similarly, the *r* values for CDDP were 0.924 and 0.949 ($P<0.01$), respectively. However, the sensitivity of the cells to Pae and CDDP was considerably different. The cells were more sensitive to CDDP than to Pae, when

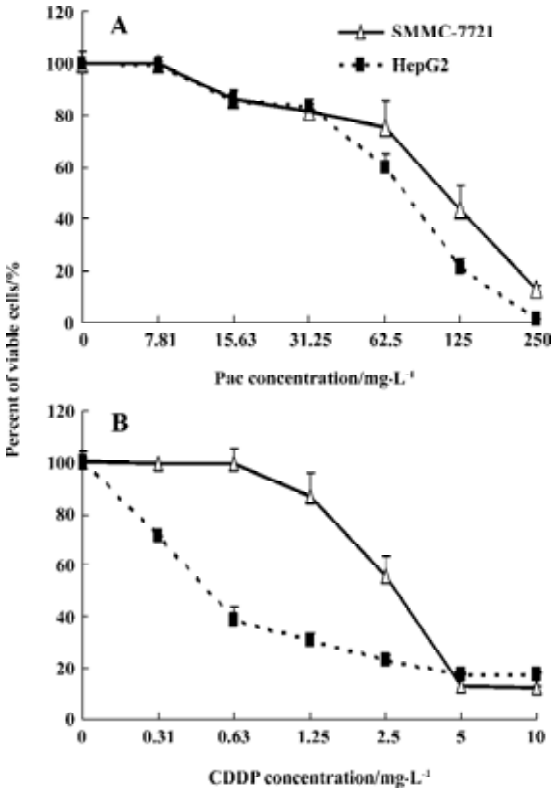

Figure 1. Dose-dependent cytotoxicity of Pae and CDDP on human $HepG_2$ and SMMC-7721 cells. (A) cells were incubated with Pae for 48 h. (B) cells were incubated with CDDP for 48 h. Data are presented as mean±SD (error bar) of triplicate cultures.

comparing the IC_{50} of CDDP to that of Pae. The IC_{50} of Pae on $HepG_2$ and SMMC-7721 cells was [104.77±7.26, 95% confident limits (CI): 86.74–122.80 and (128.47±9.29) (95% CI: 113.69–143.25 mg/L], respectively (Figure 1A), which was much more than that of CDDP (0.584±0.060, 95% CI: 0.443–0.726 mg/L) and 2.889±0.204 (95% CI: 2.380–3.398 mg/L) as shown in Figure 1B.

Synergistic cytotoxicity of Pae combined with CDDP To investigate the synergistic inhibitory effects of Pae and CDDP, 3 doses of Pae (15.63, 31.25, and 62.5 mg/L) were used in combination with different concentrations of CDDP mixed at a fixed ratio (1:1, *v/v*). The results showed that Pae increased the cytotoxicity of CDDP on $HepG_2$ and SMMC-7721 cells. For example, in the presence of 15.63, 31.25, and 62.5 mg/L Pae, the IC_{50} of CDDP reduced from 0.584±0.060 mg/L to 0.366±0.011, 0.161±0.018, and 0.007±0.002 mg/L, respectively for $HepG_2$ cells ($P<0.01$, Figure 2A).

CDI was used to evaluate the nature of the interaction. There are 2 situations (CDI < or =0.7) shown in Figure 2A under which the combination makes sense in $HepG_2$ cells. One is the combination of 0.31 mg/L CDDP with 62.5 mg/L

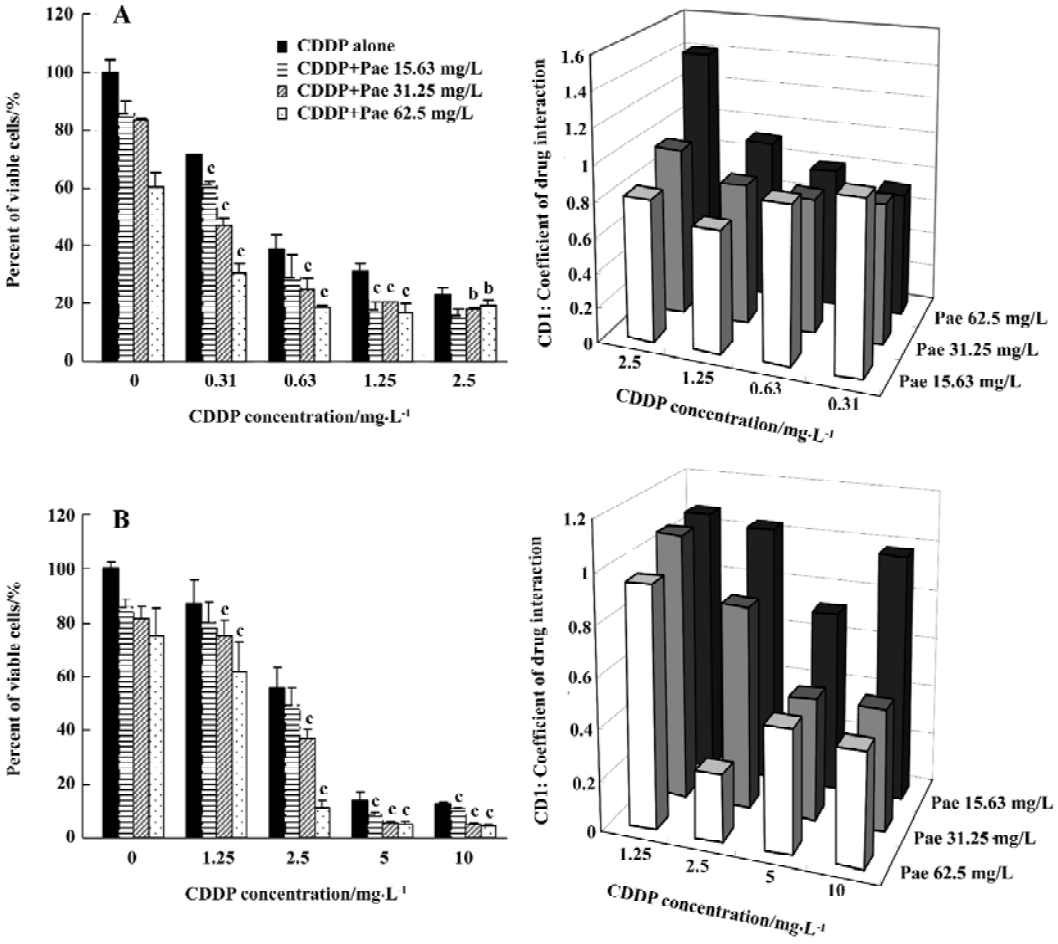

Figure 2. Synergistic effect and CDI of Pae and CDDP on $HepG_2$ and SMMC-7721 cells. (A) $HepG_2$ cells were treated with 15.63, 31.25, and 62.5 mg/L Pae and 0.31, 0.63, 1.25, and 2.5 mg/L CDDP for 48 h. (B) SMMC-7721 cells were treated with 15.63, 31.25, and 62.5 mg/L Pae and 1.25, 2.5, 5, and 10 mg/L CDDP for 48 h. Data are presented as mean±SEM (error bar) of triplicate cultures. $^bP<0.05$, $^cP<0.01$ *vs* CDDP treated alone.

Pae; the other is the combination of 1.25 mg/L CDDP with 15.63 mg/L Pae. Unexpectedly, the synergistic inhibitory effects of Pae and CDDP on SMMC-7721 cells required a large amount of those two agents (Figure 2B) which had the strongest synergism when 62.5 mg/L Pae was combined with 2.5 mg/L CDDP. This suggests that the synergistic inhibitory effects of Pae and CDDP depend on cell lines.

Cell apoptosis induced by Pae and CDDP We then examined whether the synergistic effect of Pae combined with CDDP also applied to the induction of apoptosis. All cells incubated with AO had green nuclei and yellow chromatin. Both of the 2 cell lines treated with Pae and CDDP showed typically apoptotic changes, such as chromatin condensation and deformed and fragmented nuclei, especially in the combination groups (Figures 3A, 4A). The ratio of apoptosis of 500 cells was calculated. In $HepG_2$ cells (Figure 3B), when Pae was employed at 62.5 mg/L, the number of apoptotic cells was only slightly above that of the control. However, the apoptotic rate rose greatly when treated in combination with 2.5 mg/L CDDP (Figure 3B). Similar results were found in SMMC-7721 cells (Figure 4B).

The induction of apoptosis by the treatment groups was also evident from the FCM(Flow cytometry) assay (Figures 3C, 4C). The sub-G_1 peak, which appeared before the G_1 phase that represents apoptotic cell population, was observed clearly in the 2 cell lines treated with CDDP alone. The apoptotic peak was dramatically increased when the cells were exposed to Pae combined with CDDP.

Cell cycle perturbation caused by Pae and CDDP Mcycle software was used to analyze the kinetic changes of cell

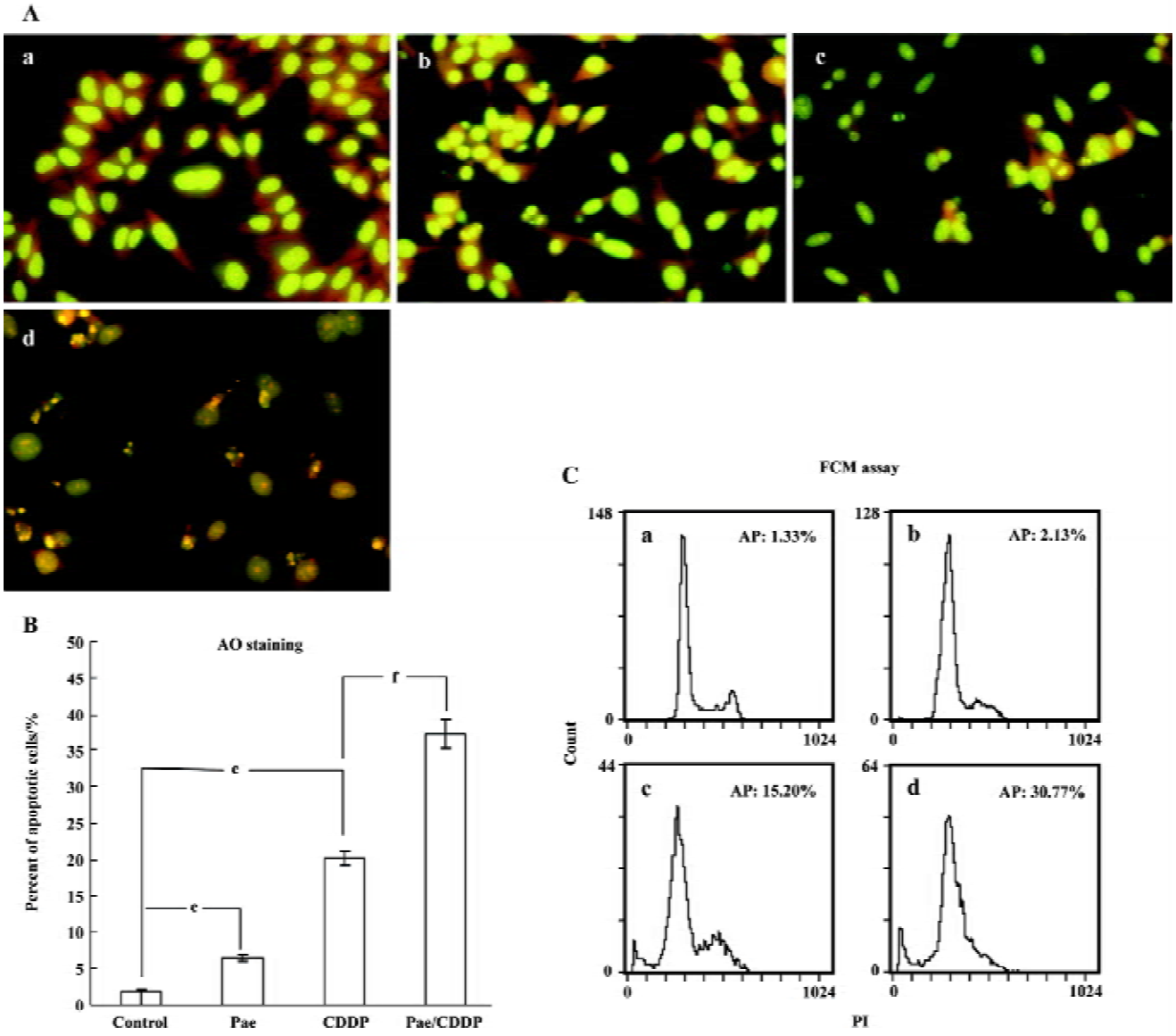

Figure 3. Effect of Pae and/or CDDP on apoptosis of $HepG_2$ cells. (A) morphological changes of $HepG_2$ cells treated with Pae and CDDP. (a) untreated cells; (b) 31.25 mg/L Pae; (c) 1.25 mg/L CDDP; (d) 31.25 mg/L Pae plus 1.25 mg/L CDDP. AO stain ×320. (B) percentage of apoptotic cells by AO staining. Data are presented as mean±SD (error bar) of triplicate cultures. $^{e}P<0.01$ *vs* control. $^{f}P<0.01$ *vs* CDDP treated alone. (C) apoptotic cells determined by FCM assay. (a) untreated cells; (b) 31.25 mg/L Pae; (c) 1.25 mg/L CDDP; (d) 31.25 mg/L Pae plus 1.25 mg/L CDDP.

cycle distribution. The $HepG_2$ cells exposed to Pae (31.25 mg/L) or CDDP (1.25 mg/L) alone appeared to move out of the G_0/G_1 phase and into the S phase, where the S-phase fraction increased while the G_0/G_1 fraction decreased. When treated with the combination of the 2 agents, the G_0/G_1 fraction of $HepG_2$ cells decreased from 71.79%±0.76% to 49.59%±0.89%, and the total S-phase fraction increased from 21.04%±0.58% to 47.07%±1.39% (Table 1). The phenomenon indicates that the combination of the 2 agents may arrest the cell cycle at the S phase, which may prevent cells from entering the M phase. The SMMC-7721 cells exposed to the combination group could also arrest cells in the S phase.

Effect of Pae and CDDP on the expression of Bcl-2 and Bax The S-P method was used to examine the expression of Bcl-2 and Bax. The standard positive Bcl-2 and Bax expressions were stained brown or yellow mainly in the cytoplasm or membrane. Bcl-2 and Bax were both expressed in the 2 cell

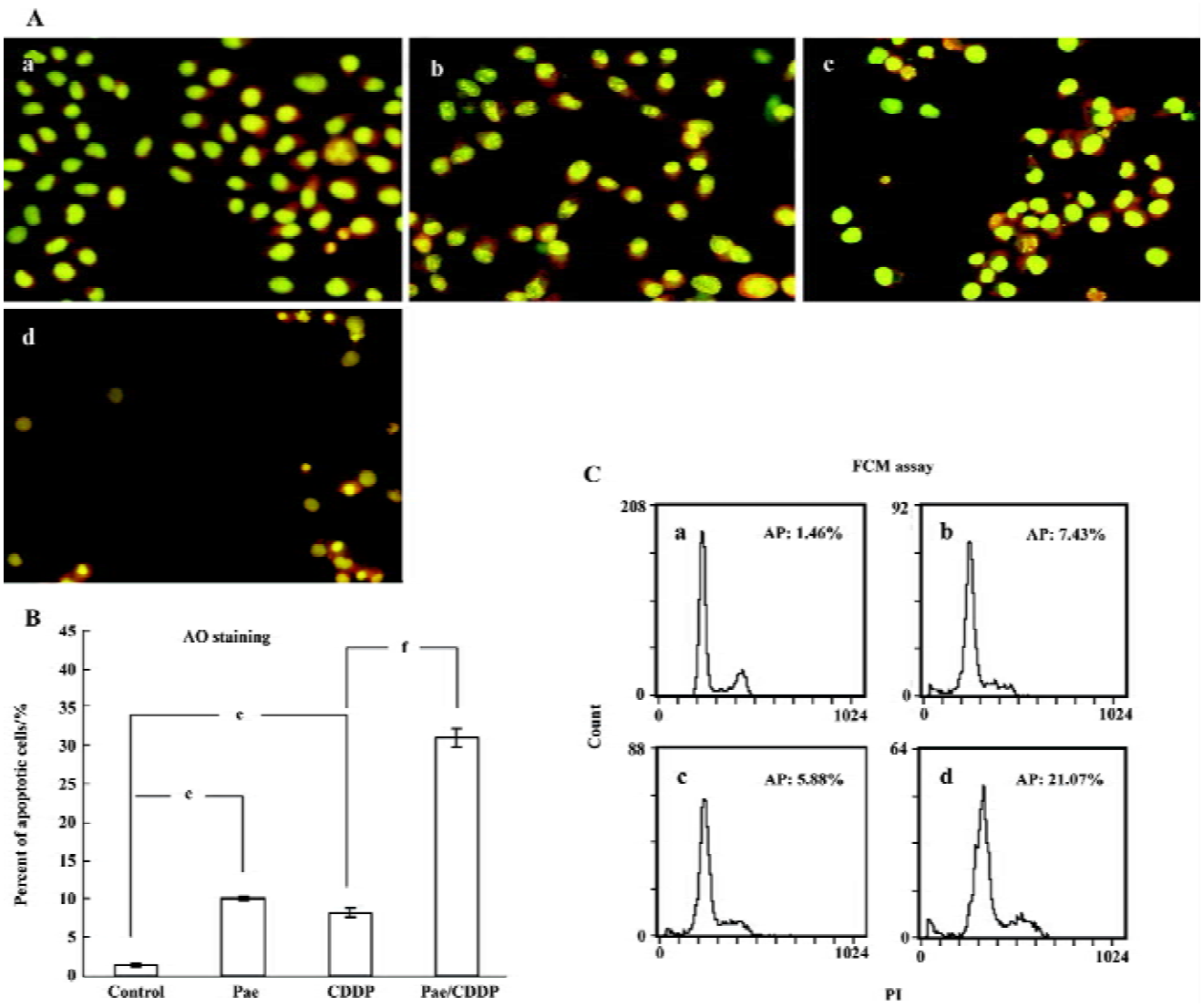

Figure 4. Effect of Pae and/or CDDP on apoptosis of SMMC-7721 cells. (A) morphological changes of SMMC-7721 cells treated with Pae and CDDP. (a) untreated cells; (b) 62.5 mg/L Pae; (c) 2.5 mg/L CDDP; (d) 62.5 mg/L Pae plus CDDP (2.5 mg/L). AO stain ×320. (B) percentage of apoptotic cells by AO staining. Data are presented as mean±SD (error bar) of triplicate cultures. $^{c}P<0.01$ *vs* control. $^{f}P<0.01$ *vs* CDDP treated alone. (C) FCM assay. (a) untreated cells; (b) 62.5 mg/L Pae; (c) 2.5 mg/L CDDP; (d) 62.5 mg/L Pae plus 2.5 mg/L CDDP.

lines. The expression of Bcl-2 decreased in the treatment groups, especially in the combination group (Figures 5A, 6A). In contrast, there was a significant increase of Bax expression in the combination group compared to the control (Figures 5B, 6B). The results were quantitatively analyzed by the Biological Image Analysis System. The expression of Bcl-2 was downregulated and that of Bax was upregulated by Pae and/or CDDP. Correspondingly, the ratio of Bcl-2/Bax decreased, especially in the combination group (Figures 5C, 6C).

Discussion

The findings of the present study demonstrated that Pae and CDDP, used as a single agent, possessed growth inhibition to $HepG_2$ and SMMC-7721 cells in a dose-dependent manner. There was a synergistic interaction between Pae and CDDP in the 2 cell lines. The cytotoxity of the combination group was significantly higher than that treated with Pae or CDDP alone in appropriate concentrations. We also found that the interaction between Pae and CDDP was specific to each cell line. In $HepG_2$ cells, at lower concentrations

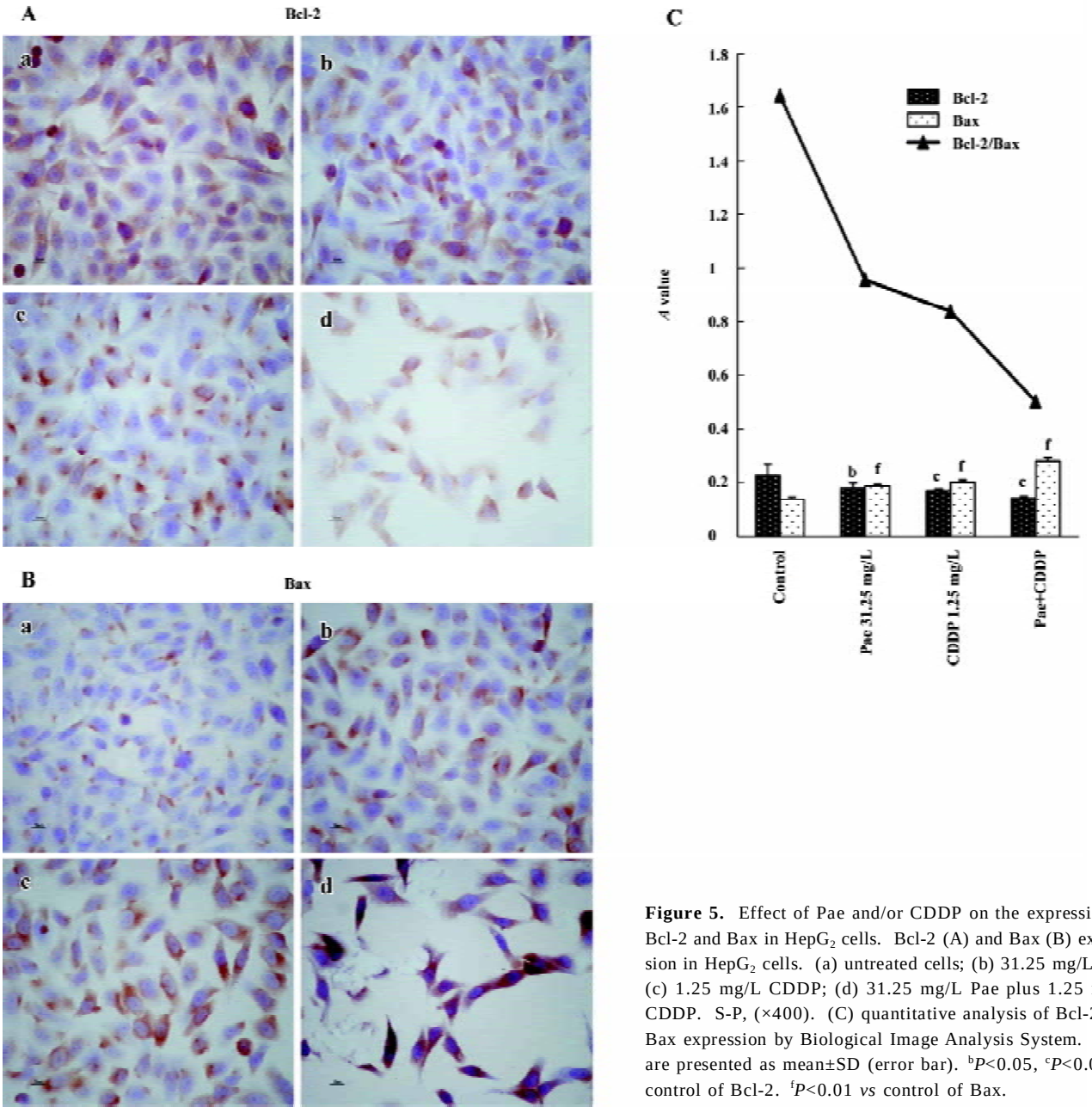

Figure 5. Effect of Pae and/or CDDP on the expression of Bcl-2 and Bax in $HepG_2$ cells. Bcl-2 (A) and Bax (B) expression in $HepG_2$ cells. (a) untreated cells; (b) 31.25 mg/L Pae; (c) 1.25 mg/L CDDP; (d) 31.25 mg/L Pae plus 1.25 mg/L CDDP. S-P, (×400). (C) quantitative analysis of Bcl-2 and Bax expression by Biological Image Analysis System. Data are presented as mean±SD (error bar). $^{b}P<0.05$, $^{c}P<0.01$ *vs* control of Bcl-2. $^{f}P<0.01$ *vs* control of Bax.

of Pae and CDDP, the combination was synergistic. The synergistic effect was the most prominent (CDI <0.7) when 15.63 mg/L Pae was combined with 1.25 mg/L CDDP in the $HepG_2$ cells. Treatment with a combination of chemotherapeutic agents resulted in an improved response as well as the ability to use less toxic concentrations of the drugs, which indicates that the combination of CDDP and Pae in certain concentrations could result in a synergistic effect. Our results are in accordance with the findings of Ji *et al*[11] who demonstrated that Pae in low concentrations had a synergetic effect with 5-FU, MMC, and CDDP in inhibiting the proliferation of the human colorectal cancer cell line HT-29. Similar results were observed in the SMMC-7721 cells, but with different sensitivity. As many reports have shown, different cell lines are different in their susceptibility to drugs[13–16]. In this study, the 2 cell lines are both adherent, epithelial-like cells, but from different origins. The $HepG_2$ cell was established from the tumor tissue of a 15-year-old Argentine boy with hepatocellular carcinoma in 1975, which was reported to produce a variety of proteins such as alpha-

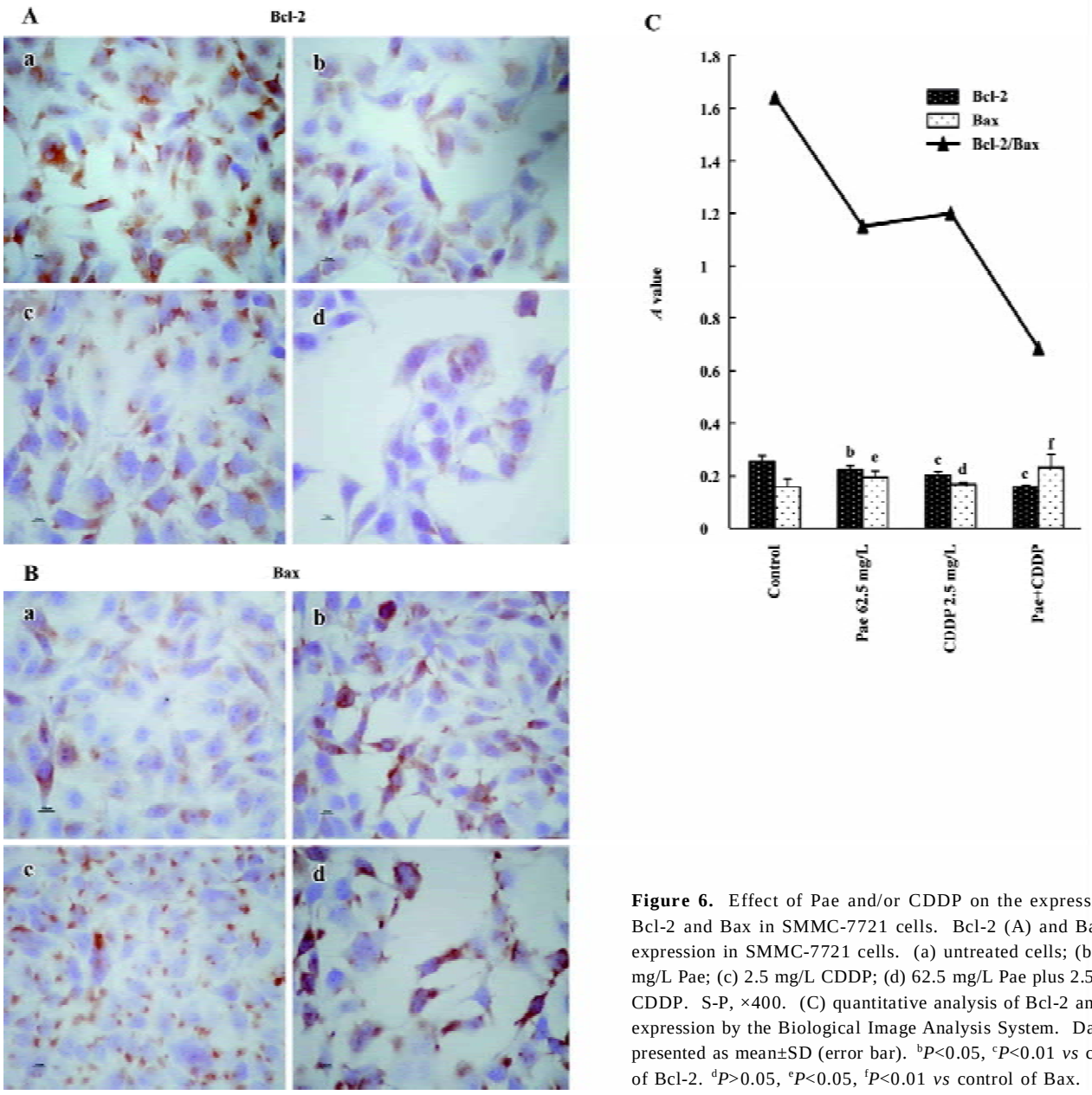

Figure 6. Effect of Pae and/or CDDP on the expression of Bcl-2 and Bax in SMMC-7721 cells. Bcl-2 (A) and Bax (B) expression in SMMC-7721 cells. (a) untreated cells; (b) 62.5 mg/L Pae; (c) 2.5 mg/L CDDP; (d) 62.5 mg/L Pae plus 2.5 mg/L CDDP. S-P, ×400. (C) quantitative analysis of Bcl-2 and Bax expression by the Biological Image Analysis System. Data are presented as mean±SD (error bar). $^{b}P<0.05$, $^{c}P<0.01$ *vs* control of Bcl-2. $^{d}P>0.05$, $^{e}P<0.05$, $^{f}P<0.01$ *vs* control of Bax.

fetoprotein, albumin, alpha2-macroglobulin, alpha1-antitrypsin, and transferrin. The SMMC-7721 cell was established from the tumor tissue of a 56-year-old Chinese man with HCC in 1980. The different origins and different biological activities may be one of the reasons for the different susceptibility to the drugs.

Although the exact mechanism of the cytotoxicity of Pae against tumor cells is not entirely clear, many potential mechanisms have been proposed for the growth inhibition by Pae on cultured cells and animal models. These mechanisms include the induction of apoptosis[17,18] and immunoregulation, such as promoted lymphocyte proliferation, interleukin-2 production by splenocytes, and TNF-α production by PMϕ (peritoneal macrophages) from model mice[9,10]. Apoptosis is a mechanism by which cells undergo death to control cell proliferation or in response to DNA damage. The hypothesis that failure to undergo apoptosis contributes to the development of resistance to anticancer agents has been the subject of extensive research[19,20]. Therefore, agents that facilitate apoptosis should improve therapeutic efficacy. Previous studies have demonstrated that Pae could induce apoptosis in K562[17] and HT-29

Table 1. Effect of Pae and/or CDDP on cell cycle of $HepG_2$ and SMMC-7721cells. n=3. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control. $^fP<0.01$ *vs* CDDP.

$HepG_2$			
Treatment	G_0/G_1 (%)	S (%)	G_2/M (%)
Control	71.79±0.76	21.04±0.58	7.16±0.57
Pae 31.25 mg/L	69.74±1.26^b	24.62±0.91^b	5.64±0.52^b
CDDP 1.25 mg/L	64.16±1.18^c	19.44±0.10^c	16.40±1.09^c
Pae+CDDP	49.59±0.89^{cf}	47.07±1.39^{cf}	3.34±0.59^{cf}
SMMC-7721			
Treatment	G_0/G_1 (%)	S (%)	G_2/M (%)
Control	73.62±0.63	19.03±0.41	7.32±0.40
Pae 62.5 mg/L	71.30±0.07^b	24.06±0.52^c	4.63±0.53^c
CDDP 2.5 mg/L	61.38±0.85^c	34.67±1.01^c	3.95±0.35^c
Pae+CDDP	55.39±1.18^{cf}	35.69±1.17^c	8.91±0.31^b

Pac: paeonol; CDDP: cisplatin

cells[18]. To investigate the apoptosis-inducing effect of Pae as a single agent and combined with CDDP in hepatoma cells, the morphological changes and apoptotic rate were detected. The cells treated with the drugs showed the typical characteristics of apoptosis, which were more prominent in the combination group. Similarly, an apoptotic peak appeared before the G_1 phase when treated with Pae or CDDP alone, and a significant synergistic effect on the induction of apoptosis was observed in the combination group.

We also found that the $HepG_2$ and SMMC-7721 cells exposed to Pae alone for 24 h showed depletion of the G_1 fraction and accumulation in the S phase. Accumulation in the S phase has also been reported by Liu *et al*[18], in which Pae could induce cell cycle perturbation and HT-29 cells attracted in the S phase increased, while cells of the G_0/G_1 and G_2/M phases decreased. The cytotoxic effect of CDDP is generally considered to be non-cell-cycle specific[21]. CDDP can cause perturbations in cell cycle distribution[22] and it is most specific to G_1-phase cells, but also has strong effect on cells in the S phase[23]. Our data also suggested that the combination group exhibited enhanced S-phase arrest, along with depletion of the G_0/G_1 fraction, which may be one of the mechanisms related to these interactions.

To examine the mechanism of apoptosis, we examined the expression of the Bcl-2 protein family, which is an important regulator of apoptosis[24]. The Bcl-2 family includes pro-apoptotic members such as Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim, and Hrk, and anti-apoptotic members such Bcl-2, $Bcl\text{-}X_L$, Bcl-W, Bfl-1, and Mcl-1[25]. These effects are more dependent on the balance between Bcl-2 and Bax than on the Bcl-2 quantity alone[26–28]. In the present study, treatment with Pae and/or CDDP decreased the expression of Bcl-2 and increased the expression of Bax, especially in the combination group. Furthermore, a significant decrease in the ratio of Bcl-2/Bax was observed when Pae was treated in combination with CDDP, which correlated with the incidence of apoptosis. One possible explanation for the synergistic interaction could be suggested. The upregulation or downregulation of the Bcl-2 protein family by Pae and/or CDDP might be the mechanism to introduce apoptosis.

In summary, the results obtained in the present study indicate that Pae in combination with CDDP has significantly synergistic growth-inhibitory and apoptosis-inducing effect on the human hepatoma cell lines $HepG_2$ and SMMC-7721, which may be related with cell cycle arrest and the upregulation of the Bcl-2 family. Pae is expected to be effective and useful as a new agent in HCC treatment in the future.

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