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Chrysin and its phosphate ester inhibit cell proliferation and induce apoptosis in Hela cells

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Abstract—To improve the biological activities of chrysin (CR), we synthesize Diethyl Chysin-7-yl phosphate (CPE: $C_{19}H_{19}O_7P$) and tetraethyl bis-phosphoric ester of chrysin (CP: $C_{23}H_{28}O_{10}P_2$) through a simplified Atheron–Todd reaction. The interactions of the CR and CPE with lysozyme were explored by electrospray ionization mass spectrometry (ESI) and fluorescence spectrometry method. Experimental results indicate that CPE could form the noncovalent compound with lysozyme, while the interaction of the CR with lysozyme was not detected. In addition, whether and how the compounds CPE and CP affect proliferation and apoptosis in human cervical cancer Hela cells were investigated. Moreover, the effects of CPE and CP in Hela cells were compared with that of the nonmodified CR compound. The Hela cells were co-cultured with CR, CP, and CPE as experimental groups, respectively, and corresponding control groups treated without CR, CP, and CPE. The proliferation and apoptosis were detected using MTT assay, HCl denatured-methyl green-pyronin staining, PCNA immunohistochemistry and TUNEL techniques. The cell growth IC_{50}, relative absorbance (RA), proliferating index (PI), PCNA-IR (immunoreactivity IR) integration value (IV), and apoptosis index (AI) were calculated and their correlation was analyzed in each group. The results show that all CR, CP, and CPE could inhibit proliferation and induce apoptosis in Hela cells. Moreover, the effects of CP and CPE were more potent than that of CR. The CP and CPE were proved to be a kind of stronger apoptosis inducers than nonphosphated CR. There was a negative correlation between proliferation and apoptosis. In conclusion, the CR, CP, and CPE could effectively inhibit growth by down-regulated expression of PCNA, and induce apoptosis in Hela cells. The efficiency of the modified CP and CPE preceded nonmodified CR compounds. The CP and CPE may be a new potential anti-cancer drug for therapy of human cervical carcinoma.

1. Introduction

Flavonoids are polyphenolic compounds existing ubiquitously in plants. They may be used as cancer-preventive diet components rich in fruits and vegetables. The effects of flavonoid on anti-proliferation,[1–3] cell cycle arrest in G0/G1 or G2/M,[4,5] induction of differentiation and apoptosis in different cell lines[6–8] were reported. Chrysin (CR), one kind of widely distributed flavonoids in nature, has been reported to have anti-cancer effect.[9] In order to improve its biological activity, a number of its derivatives have been prepared.[10,11] In this experiment, we synthesized Diethyl Chysin-7-yl phosphate (CPE: $C_{19}H_{19}O_7P$) and tetraethyl bis-phosphoric ester of chrysin (CP: $C_{23}H_{28}O_{10}P_2$) through a simplified Atheron–Todd reaction. Our previous research work showed that CP (tetraethyl bis-phosphoric ester of chrysin $C_{23}H_{28}O_{10}P_2$) possessed relatively stronger binding affinity towards proteins such as myoglobin, insulin, and lysozyme and were easier to form the noncovalent compounds with them.[12] However, the evidence about the phosphorylated flavonoids effect on the Hela cell biological character needs to be explored. In this paper, the interactions of the CR and CPE with protein (lysozyme) were investigated by ESI and fluorescence spectrometry methods. Furthermore, the biological effect of CP and CPE in comparison with that of CR was studied with MTT as survival biomarker, HCL-denatured MGP staining as a proliferation index (PI), the proliferative cell nuclear antigen immunoreactivity (PCNA-IR) as a proliferation/malignancy biomarker and TUNEL

Keywords: Chrysin; Phosphate ester of chrysin; Hela cell line; Proliferation; Differentiation; Apoptosis.
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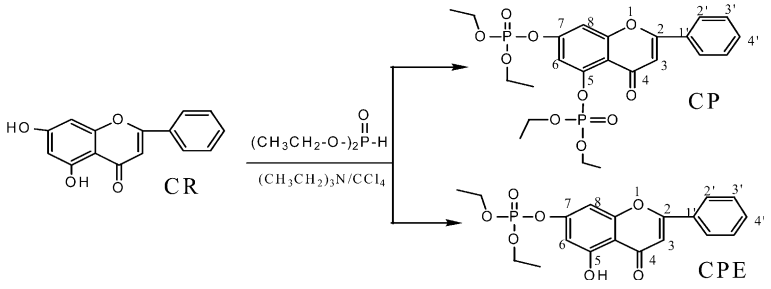

Scheme 1.

as apoptosis biomarker in Hela cells. It may have important implications for cancer prevention as well as other pharmacological effects of these compounds.

2. Materials and methods

2.1. The test compounds

Diethyl Chysin-7-yl phosphate (CPE: $C_{19}H_{19}O_7P$) and tetraethyl bis-phosphoric ester of chrysin (CP: $C_{23}H_{28}O_{10}P_2$) through a simplified Atheron–Todd reaction were synthesized (Scheme 1).

2.2. Mass spectrometric conditions

Solutions of the complexes were analyzed on a Brukes–Esquire 3000 mass spectrometer fitted with an ionspray source working in the positive ion mode. Nitrogen was used as drying gas at a flow rate of 4 μL/min. The neublizer pressure was 17 psi and the dry gas flow rate was 9.00 L/min. The capillary was typically held at 4 kV. Six spectra were averaged, and the rolling average was seven. The ICC was set at 30,000. The samples dissolved were continuously infused into the ESI chamber at a flow rate 4 μL/min using a Cole–Parmer Instrument Co. 744900 syringe pump.

2.3. Fluorescence spectra

The binding reactions between lysozyme and CPE in an aqueous phase were studied using fluorescence spectrometry. Fluorescence spectra were taken with a F4500 spectrofluorometer using excitation wavelength of 280 nm and the emission range set between 290 and 450 nm. Generally speaking, the excitation wavelength for proteins is about 280 nm, so this wavelength was selected as the excitation wavelength in the experiment to study the CPE–lysozyme interaction. No fluorescence was emitted by CPE under this excitation wavelength. The quenching experiment was performed for the same lysozyme concentration with different quantity CR and CPE at the temperature 24 and 37 °C.

2.4. Cell viability assay

The test compounds (CR, CP, and CPE) dissolved in a DMSO (dimethyl sulfoxide $\leqslant$0.1%) were co-cultured with HeLa cells (Shanghai Institutes of Biological Sciences). RPMI-1640 (GIBCO) supplemented with 10% fetal bovine serum (FBS) was used as the cell culture medium. The cell viability in the presence or absence of the experimental agents was examined by using the Mosmans's MTT assay.[13] Briefly, the cells harvested in the log phase of growth were inoculated in 96-well plates with the final concentration of 3×10^3 cells in each well. After 24 h of incubation at 37 °C, under 5% CO_2, the cell cultures were treated with 5, 10, 15, 20, 25, and 30 μM concentrations CR, CP, and CPE. The MTT absorbance value was detected at 492 nm with ELISA reader (Star) and the mean value was obtained from four replicate wells after exposure of the agents for 24, 48, and 72 h, respectively, and the control group treated with no any agents was performed simultaneously. The results were expressed as RA and IC_{50} values (drug concentrations inhibiting relative MTT absorbance, RA, by 50%), using the equation: RA (%) = ($A_{\text{treated group}}$/$A_{\text{control group}}$) × 100%.

2.5. Preparations of slide specimens

Based on above MTT assay, CR, CP, and CPE screened in the low physiological 10 μM concentration were added into Hela cell cultures for 24, 48, and 72 h as the experimental groups. The slide specimens, including the experimental and the control groups, were fixed with 4% paraformaldehyde for 20 min at room temperature, followed by washing in PBS, stored at −20 °C.

2.6. HCl-denatured methyl green-pyronin (MG-P) staining

The HCl-denatured-methyl green-pyronin (BDH) technique was based on method of Iseki[14] and modified by Sen.[15] In brief, the slides were post-fixed in ice-cold Carnoy's solution (6:3:1 v/v mixture of alcohol, chloroform, and iced acetic acid) for 10 min and then hydrolyzed with 0.1 N HCl in 80% alcohol for 5 min, and rinsed in distilled water. The slides were then stained using a freshly prepared MG-P solution (0.5% of methyl green and pyronine in a 100 mM sodium acetate buffer, pH 4.8) for 6 min at room temperature and the slides were differentially stained with acetone and D.H_2O, respectively.

2.7. Immunocytochemistry

The paraformaldehyde-fixed slides were treated with 0.5%Triton X-100/PBS solution for 30 min and 3% H_2O_2 for 5 min. The slides were heat renatured in

0.05 mM citric acid buffer, pH 6.0 for antigen retrieval, and blocked with 1:10 diluted normal goat serum in PBS for 30 min. After removal of excess serum, the monoclonal antibody against PCNA (Zymed) freshly diluted into 1:100 was incubated overnight at 4 °C. The biotin-labeled goat anti-mouse serum and streptavidin conjugated peroxidase were incubated at 37 °C, for 20 min subsequently. The 0.05% 3,3′-diaminobenzidine and 0.01% H_2O_2 were used as the substrate to develop a positive immunoreactivity (IR)/signal in brownish color. The substitution of PBS for the specific primary antibody was performed as the negative control.

2.8. TUNEL

Tumor cell apoptosis was identified by the TUNEL method.[16–18] The slides were first treated with 50 mM imidazole solution in 95% alcohol for 15–20 min and 0.3%Triton X-100/PBS solution for 10 min, respectively. After the slides were incubated with 5 μg/mL proteinase K (Promega) for 10 min at room temperature, and washed with 1 × TBS (20 mM Tris, pH 7.5, 140 mM NaCl), then followed by incubation with 8 μL TdT (terminal deoxynucleotidyl transferase) buffer containing 1 μL TdT enzyme (Promega) and 1 μL biotin-16-dUTP (Sigma) in a humidified chamber at 37 °C for 2 h. After washing, the slides were incubated with steptavidin–alkaline phosphatase conjugate at 37 °C for 20 min. The slides were washed thoroughly with TBS pH 7.5 three times, and then with TBS pH 9.5 two times. The 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium (BCIP–NBT) was used as the substrate to develop a positive signal in bluish-violet color. The TdT enzyme removed in the TdT buffer was performed in the negative control specimen.

2.9. Statistical analysis

The integration value (IV) of PCNA–IR according to the relative intensity of signals and the percentage of the proliferating and apoptosis cells were calculated in each slide under oil-emersion microscope. The statistical significance was analyzed with SSPS 10.0.

3. Results

3.1. Mass spectrometric

Figure 1 shows an ESI mass spectrum obtained from a mixed solution of lysozyme and CPE. Besides the expected multiply protonated molecule ions, a mass spectrum revealed several groups of new protonated ions, corresponding to several kinds of highly charged multiple adducts, respectively, for example, ions at *m/z* 1336.6, 1372.1, and 1407.2, corresponding to $(\text{lysozyme} + \text{CPE})^{11+}$, $(\text{lysozyme} + 2\text{CPE})^{11+}$, and $(\text{lysozyme} + 3\text{CPE})^{11+}$; ions at *m/z* 1470.2, 1509.3, and 1548.3, corresponding to $(\text{lysozyme} + \text{CPE})^{10+}$, $(\text{lysozyme} + 2\text{CPE})^{10+}$, and $(\text{lysozyme} + 3\ \text{CPE})^{10+}$; ions at *m/z* 1633.4, 1676.8, 1720.3, and 1763.7, corresponding to $(\text{lysozyme} + \text{CPE})^{9+}$, $(\text{lysozyme} + 2\text{CPE})^{9+}$, $(\text{lysozyme} + 3\ \text{CPE})^{9+}$, and $(\text{lysozyme} + 4\text{CPE})^{9+}$; ions at *m/z* 1837.5, corresponding to $(\text{lysozyme} + \text{CPE})^{8+}$. For comparison, 0.4 mM CR was also mixed with 0.04 mM lysozyme, and infused to ESI. Even though different parameters were tried, no corresponding noncovalent lysozyme–CR complex was detected. The experiments described herein suggest that the CPE (phosphorylated CR) could form noncovalent complex with lysozyme more easily than the nonphosphorylated compound CR. This suggests that phosphate esters of chrysin could enhance the interaction with the protein.

3.2. The effects of CPE on the emission spectra of lysozyme–CPE system

The interaction between lysozyme and CPE is shown in Figure 2. When CPE concentration was increased, the emission peak of lysozyme decreased in each case, and at the same time the maximum emission wavelength lengthened by about 10 nm.

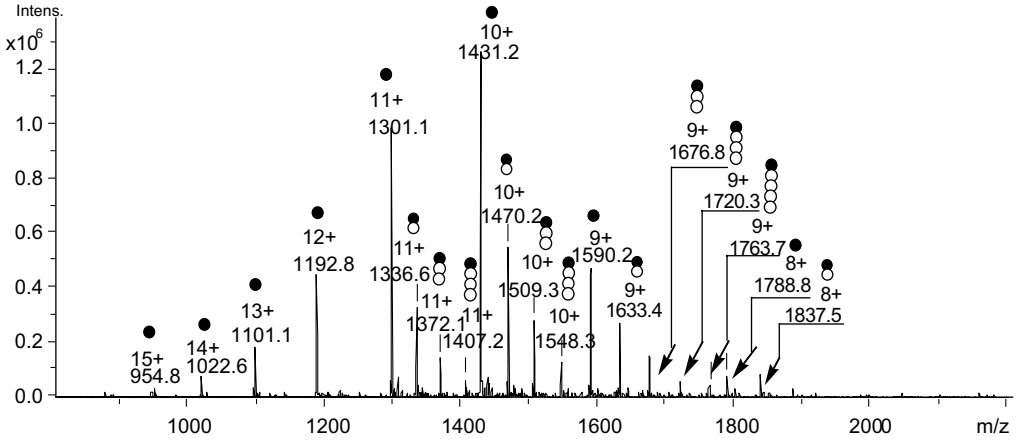

Figure 1. Electrospray ionization mass spectrum of lysozyme with CPE. The solution was prepared by mixing equal volumes of a 0.4 mM methanol solution of CPE and 0.04 mM lysozyme. ● Multiply charged ion peaks of HEWL; ●○ multiply charged ion peaks of lysozyme–CPE complex; ●○○ multiply charged ion peaks of lysozyme–2CPE complex; ●○○○ multiply charged ion peaks of lysozyme–3CPE complex; ●○○○○ multiply charged ion peaks of lysozyme–4CPE complex.

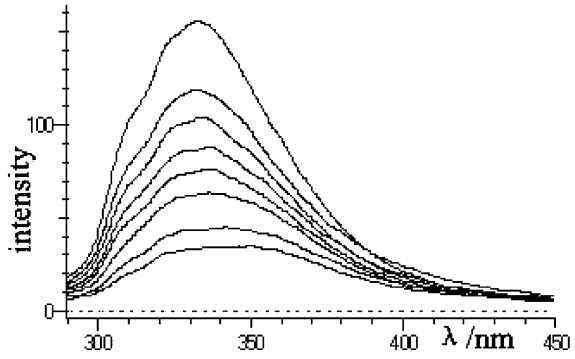

Figure 2. The effect of CPE on fluorescence spectra of lysozyme.

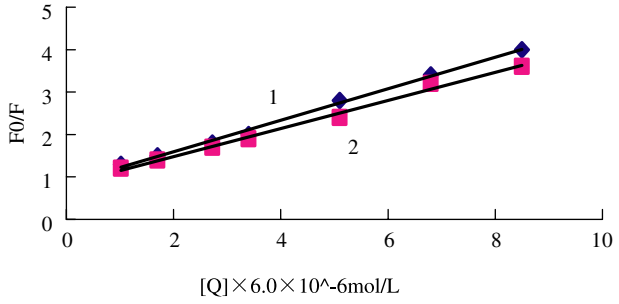

Figure 3. Stern–Volmer plots of CPE and lysozyme: 1—24 °C; 2—37 °C.

In order to make clear the style of the quenching process, a first approach to describe the fluorescence behavior is Stern–Volmer[19] equation, given by

$$F_0/F = 1 + K_q[Q]$$

where F_0 is the emission intensity in the absence of quencher, F is the emission intensity in the presence of quencher, K_q is the quenching constant and $[Q]$ is the quencher concentration. The shape of Stern–Volmer plots can be used to characterize the quenching as being predominantly dynamic or static. Plots of F/F versus $[Q]$ appear to be linear and K depends on temperature. Draw $F_0/F - [Q]$ lines at temperature 24 °C and 37 °C, respectively (Fig. 3; Line 1—24 °C, line 2—37 °C). The experiments demonstrated that the higher the temperature was, the lower the slops of quenching curve of lysozyme was in presence of different amounts of CPE. It was confirmed that the combination for CPE with lysozyme is a single static quenching process. So the quenching data were analyzed according to other equations, given by

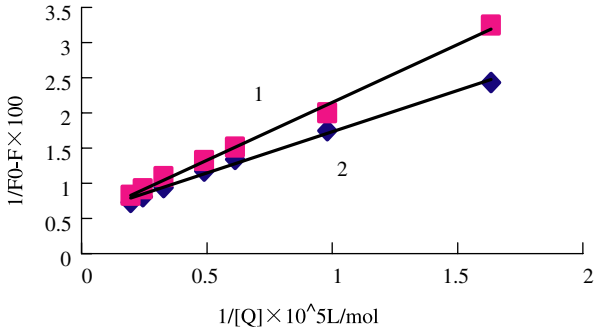

Figure 4. Lineweaver–Burk plots of CPE versus lysozyme: 1—24 °C; 2—37 °C.

$$(F_0 - F)^{-1} = F_0^{-1} + K^{-1}F_0^{-1}[Q]^{-1}$$

Draw Lineweaver–Burk plots (Fig. 4 line 1–24 °C; line 2–37 °C). The linearly dependent coefficients are 0.99 and 0.998. According to this equation the binding constants at different temperature could be calculated to be $k_{37\,°C} = 3.512 \times 10^4$ L/mol, $k_{24\,°C} = 5.485 \times 10^4$ L/mol, respectively. These results showed that the CPE formed noncovalent complexes and showed high binding affinity with lysozyme. However no interaction could be detected for lysozyme–CR system through using fluorescence spectrometry described above.

3.3. Cell viability assay

Cultured human (HeLa) cell lines were treated by CR, CP, and CPE with 10 μM for 24, 48, and 72 h. According to the data from the MTT reduction assay shown in Table 1, CR, CP, and CPE effects on cell viability exhibited markedly declined and time-dependent fashion, when compared to the control group. The cell viability induced by CR, CP, and CPE for 72 h under different concentrations of 5, 10, 15, 20, 25, and 30 μM displayed dose-dependent fashion (Fig. 5). The IC_{50} values were

Table 1. RA and IV in different groups for 24, 48 and 72 h

	Relative absorbance: RA (%)			PCNA-IR integration value: IV		
	24 h	48 h	72 h	24 h	48 h	72 h
CON	—	—	—	530 ± 12.9	512 ± 7.1	496 ± 8.9
CR	91.4 ± 1.4	87.4 ± 1.6[a,e]	59.1 ± 1.2[a,e]	480 ± 9.1	402 ± 9.7[*,a,e]	308 ± 7.6[*,a,e]
CPE	88.5 ± 1.2	73.8 ± 1.8[b,e]	52.6 ± 1.3[b,e]	450 ± 13.4[*]	348 ± 10.6[*,b,e]	198 ± 10.1[*,b,e]
CP	87.1 ± 1.9	67.5 ± 2.2[b,c,e]	45.6 ± 2.5[b,c,e]	450 ± 13.4[*]	327 ± 10.6[*,b,e]	185 ± 10.1[*,b,d,e]

Values are means ± SEM; CON, control; CR, 10 μM CR group; CPE, 10 μM CPE group; CP, 10 μM CP group.
[*] Significant difference from CON at $P < 0.01$.
[a] Significant difference from CPE at $P < 0.01$.
[b] Significant difference from CR at $P < 0.01$.
[c] Significant difference from CPE at $P < 0.05$.
[d] Significant difference from CPE at $P < 0.05$.
[e] Significant difference from 24 h at $P < 0.01$.

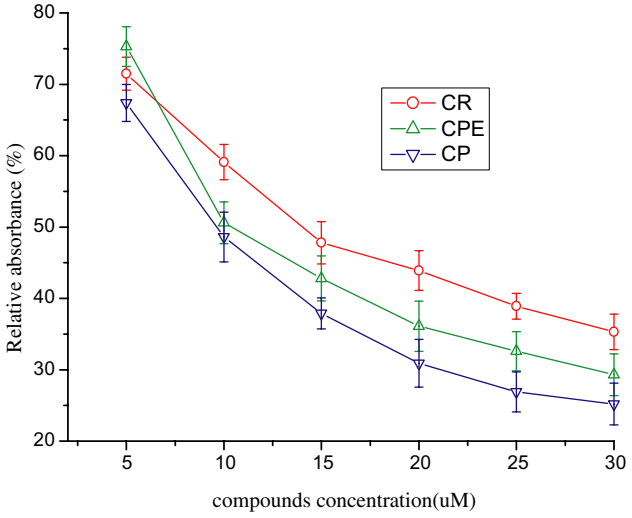

Figure 5. The effect of all test compounds with different concentrations in Hela cells following treatment for 72 h was shown. All data points represent mean and SEM values. Both of CP and CPE are significantly different from the RA of CR using the different compounds concentration ($P < 0.01$). There is significant difference between CP and CR ($P < 0.05$).

estimated 14.2 μM for CR, 10.3 μM for CPE and 9.8 μM for CP, respectively. The results indicated that the survival inhibition effect of CPE and CP was higher than that of CR, whereas CP was more potent than that of CPE. The following order of decreasing potency on Hela cell viability was found to be CP > CPE > CR.

3.4. HCl-denatured methyl green-pyronin (MG-P) staining

In cytochemistry the DNA can be stained with methyle green in bluish green color, while the RNA can be stained with pyronin in reddish color. As the intranuclear DNA of the nonproliferating cells is more susceptible to acid denaturation than that of the proliferating cells, the proliferating cells are mainly stained in bluish-green color, whereas the nonproliferating cells appeared reddish-violet colored. The proliferating cells in the control group were stained in bluish-green color (Fig. 6a). The nonproliferating cells (probably undergoing differentiation) in the CR, CP, and CPE treatment groups were mainly stained in red-violet color (Fig. 6b–d). The PI was declined gradually from control, CR, CPE to CP groups. After 48 h The PI values were about 85.4, 58.7, 49.1 and 44.9, respectively. The PI (showed in Table 2) in CR, CP, and CPE groups exhibited markedly declined and time-dependent fashion, as compared to the control group. The results showed that the proliferation was inhibited in sequence of CP > CPE > CR in respective treatment groups.

3.5. The expression of PCNA by immunocytochemistry

The PCNA immunoreactivity (IR)/signals in brownish-colored granules were mainly localized in the nuclei. The inactivated PCNA was primarily located in the cytoplasm, once activated would translocated into the nuclei. The PCNA-IR intensity was evaluated as the integration value (IV): 327 in CP group, 348 in CPE

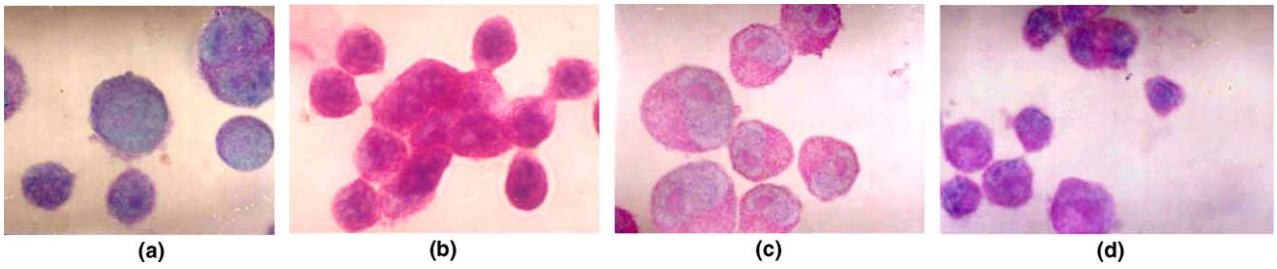

Figure 6. HCl-denatured methyl green-pyronin staining in Hela cells after treatment for 48h. (a) Control group: the majority of bluish-green colored cells were in the control group, 1000×; (b) CP group: the reddish colored cells were distinct in CP group, 1000×; (c) CPE group: the bright red-violet colored cells were in the CPE group, 1000×; (d) CR group: the red-violet colored cells were in the CR group, 1000×. Proliferating index (PI) = (number of proliferating cells/total number of cells counted) × 100.

Table 2. PI and AI in different groups for 24, 48 and 72 h

	The differentiation index: PI			The apoptosis index: AI		
	24 h	48 h	72 h	24 h	48 h	72 h
CON	87.2 ± 1.2	85.4 ± 0.6	83.50.7	5.6 ± 1.3	7.1 ± 1.8	8.5 ± 1.8
CR	71.7 ± 1.4[*]	58.7 ± 1.6[*,a,d]	40.7 ± 1.2[*,a,c,d]	12.2 ± 1.3	24.4 ± 1.8[*,a,c,d]	50.3 ± 1.7[*,a,c,d]
CPE	64.2 ± 1.2[*]	49.1 ± 1.6[*,b,c,d]	16.3 ± 1.3[*,b,c,d]	13.1 ± 0.9[*]	32.6 ± 2.1[*b,c,d]	65.5 ± 2.4[*,b,c,d]
CP	60.6 ± 2.3[*,b]	44.9 ± 2.9[*,a,b,d]	9.9 ± 1.9[*,a,b,d]	17.8 ± 2.1[*,b,a]	38.5 ± 2.1[*,b,a,d]	70.3 ± 2.1[*,b,a,d]

Values are means ± SEM; CON, control; CR, 10 μM CR group; CPE, 10 μM CPE group; CP, 10 μM CP group.
[*] Significant difference from CON at $P < 0.01$.
[a] Significant difference from CPE at $P < 0.01$.
[b] Significant difference from CR at $P < 0.01$.
[c] Significant difference from CP at $P < 0.05$.
[d] Significant difference from 24 h at $P < 0.01$. There were negative correlations AI versus PI, AI versus IV, AI versus RA. There were positive correlations RA versus IV, RA versus PI, IV versus PI.

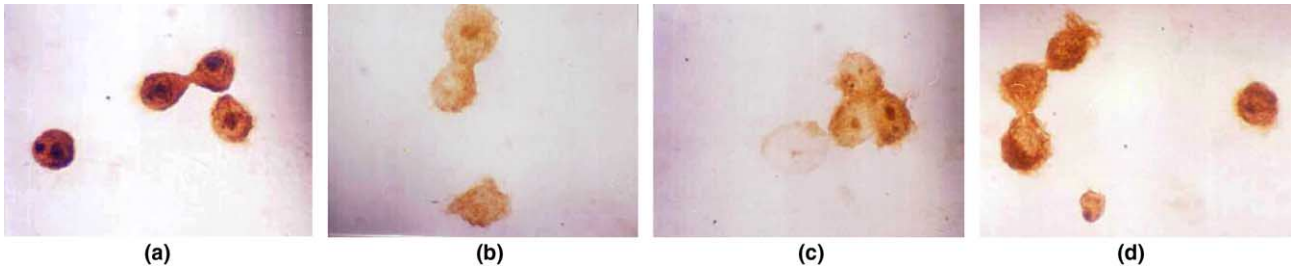

Figure 7. PCNA-IR in brownish color was demonstrated in Hela cells after treatment for 48 h. (a) Control group: the intense PCNA-IR was localized in the nuclei, (b) CP group: PCNA-IR was markly attenuated, 1000×; (c) CPE group: PCNA-IR was attenuate, 1000×; (d) CR group: PCNA-IR was attenuated and inactive PCNA-IR was also located in the cytoplasm, 1000×.

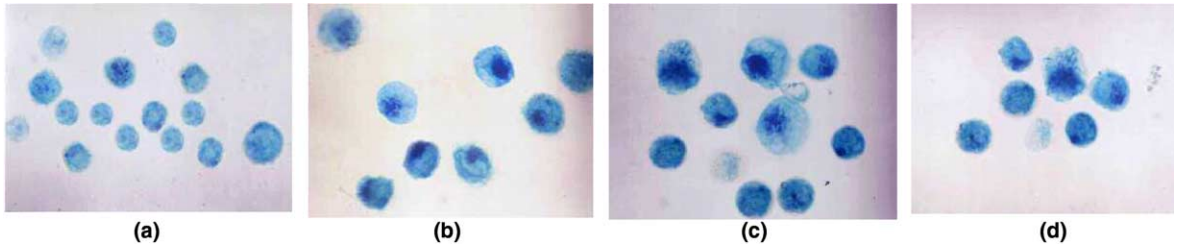

Figure 8. Demonstration of TUNEL as apoptosis biomarker in Hela cells after treatment for 48 h. (a) Control group: few apoptosis were found, methyle green counter-stained, 1000×; (b)–(d) CP group, CPE group, CR group: more apoptotic cells were found, methyle green counter-stained, 1000×; apoptotic index (AI) = (the number of apoptosis cells/total number of tumor cells) × 100.

group, 402 in CR group and 512 in the control group after 48 h. There was significantly difference between the treatment groups and the control group $p < 0.05$ (Table 1, Fig. 7). The results further showed that the inhibited proliferation activities of CP, CPE, and CR treatment groups were in accordance with the same order of PCNA expression signal intensity in Hela cells.

3.6. TUNEL

The TUNEL signal, as an apoptosis marker, appeared as bluish-violet colored and the denser nuclei frequently moved towards to the cell periphery (Fig. 8). The apoptosis percentage was 7% in the control group. More apoptosis was detected in the treatment groups. After 48 h, the AI was promoted up to 24% in CR, to 32% in CPE and up to 38% in CP groups. The AI in CR, CP, and CPE groups displayed sequentially increased and time-dependent manner, when compared to the control group $p < 0.01$ (Table 2). The Hela cell apoptosis effect induced by the compounds was in sequence of CP > CPE > CR.

4. Discussion

It was reported that most nontoxic dietary flavonoids could be used as general cell growth inhibitors in different kinds of cultured human cancer cell lines. Kandaswami[20] demonstrated anti-proliferative effects of four citrus flavonoids (quercetin, taxifolin, nobiletin, and tangeretin, at 2–8 mg/mL for 3–7 days) on squamous cell carcinoma HTB43. Fotsis[21] reported that flavonoids could inhibit cell proliferation and angiogenesis in six different cancer cell lines, and noted that the IC_{50} of active flavonoid was available in physiological concentration, in the low micromolar range. Our experimental results indicated that both CR, and phospholated CR (CP and CPE synthesized phosphoric ester of chrysin in our lab) could significantly decreased cell viability detected by using MTT assay in a dose- and time-dependent manner in Hela cells. The survival inhibition effect of CPE and CP was higher than that of CR, and that of CP was more potent than that of CPE. The decreased potency of cellular viability was CP > CPE > CR. Generally, the chemical-induced decrease in MTT absorbance could represent decrease in cell viability due to mitochondrial dysfunction, and indicate the cell proliferation inhibition.[13] In order to study the influence of the potency of the compounds on cell growth and viability, we further observed anti-proliferation and apoptosis effects of them on Hela cells by HCl-denatured MG-P staining, immunocytochemistry PCNA and TUNEL methods.

The HCl-denatured methyl-green and pyronin staining could identify the proliferating cells in bluish-green color distinct from the differentiated or nonproliferating cells in red colored staining, since the proliferating cells with nuclear dsDNA more tolerable to acid denaturation at a certain extent. Although HCl-denatured MG-P staining have proved to be useful for a well-characterized cell differentiation,[14,15] the cancer cell differentiation in vitro need to be evaluated by additional factors, such as protein kinase, the expression of oncogenes and tumor suppressor genes and so on.[22] Herein the red colored cells stained with pyronin could be evaluated as the nonproliferating cells. It has been reported

that PCNA is markedly expressed in proliferating cells and most of malignant tumor cells.[23,24] Hence, PCNA could be used as a proliferative/malignancy biomarker in the cancer. Our results showed that both PCNA-IV by immunocytochemistry and PI value by using HCL-denatured MG-P all declined in CP, CPE, and CR groups, when compared to the control group, and there was a positive correlation between PI value and PCNA-IV. Both alteration effects of PCNA and PI value of the CP and CPE group were more marked than that of the CR. The experimental results indicated that anti-proliferation effect of CP, CPE, and CR was consistent with the diminished MTT absorbance and an inhibition of PCNA activity of Hela cells. PCNA is required for DNA polymerases δ and ε in DNA synthesis. [25] The decreased PCNA expression in Hela cells could affect DNA synthesis. Moreover, there were some reports as to the positive correlation existed between the tumor malignancy and the PCNA expression level, and the PCNA could be used as a biomarker for evaluating both prognosis and therapy efficiency.[26,27] The changes of PCNA expression imply that the CP and CPE may be a kind of new potential anti-cancer drug for therapy of human cervical carcinoma.

The observed anti-proliferative properties of CR, CP, and CPE suggest that these compounds may inhibit the cell cycle progression or induce apoptosis. TUNEL, as apoptosis biomarker, is especially used to detect cell programmed cell death. Induction of tumor cell apoptosis has already been used as an important indicator to detect the ability of chemotherapeutic drugs to inhibit tumor growth.[28] It is possible to treat the cancer by means of increasing the proportion of tumor cell apoptosis. The nuclei with the characteristic TUNEL labeling as the typical morphological feature for apoptosis were observed in CR, CP and CPE treated specimens in comparison with the control group. The order of potency of the CP, CPE, and CR inducing Hela cellular apoptosis met with the result of MTT assay. The AI in CP and CPE groups was higher than that in CR, further suggesting that the CP and CPE may proved to be a potential anti-cancer drug for human cervical cancer therapy. Besides, there was a negative correlation between proliferation and apoptosis. Apoptotic Hela cancer cells were consistently negative for PCNA.[29] The cell growth of cells is controlled by proliferation, differentiation and programmed cell death (apoptosis). It can be reasonably assumed that CR and CPE could affect both growth inhibition and apoptosis cell using MTT assay, HCl-denatured MG-P staining, immunocytochemistry PCNA and TUNEL methods. The down-regulated PCNA expression and apoptosis induced by the CP, CPE, and CR may be molecular mechanism for inhibition of tumor growth.

In order to understand the molecule mechanism of the putative anti-cancer activity of flavonoids, Wenzel[30] reported that the core structure of the flavones, 2-phenyl-4H-1-benzopyran-4-one (flavone) could induce effectively growth inhibition, differentiation and apoptosis in HT-29 human colon cancer cells. The flavone in HT-29 cells affected altered mRNA levels of cell cycle and apoptosis-related genes, including cyclooxygenase-2 (COX-2), nuclear transcription factor B (NF-kB), and bcl-XL. Chrysin, containing the same core of flavones, may have similar molecular effect on growth inhibition, differentiation and apoptosis. Moreover, the position of hydroxyl group on the rings in the flavone could play a role in anti-cancer effect.[31,32] Since the effects of both CP and CPE are more pronounced than that of CR, it seems that phosphorylation at hydroxyl group of ring A could confer a more potent anti-cancer effect. Furthermore, these properties can vary with the number and/or the position of phosphoryl groups on the CR nucleus. The lack of the phosphoryl group in the 5-position seems to correspond to a lower effect of CPE than that of CP on Hela cells. The effect of phosphorylated flavonoids seems to positively correlate with the numbers of phosphoryl group.

It is an established fact that cellular function was often triggered by weak noncovalent interactions between enzyme–substrate, protein–ligand and antibody–antigen interactions.[33] Many chemicals exert their anti-tumor effects through binding to protein and inhibiting the growth of the tumor cells, which is the basis of designing new and more efficient anti-tumor drugs; and their effectiveness depends on the mode and affinity of the binding.[34] Similarly, interaction of CP and CPE with protein is very important in understanding the molecule mechanism of the activity. We therefore explored the difference in their binding modes and affinities to protein of CPE and CR. It is well known that ESI-MS has already served as a powerful tool in providing evidence in support of the existence of noncovalently associated macromolecular complexes in the gas phase.[35–39] In the work described in this paper, the ESI results demonstrated that CPE could form noncovalent complexes and showed stronger binding affinities with the protein than with CR in the gas phase. Furthermore the interaction between the lysozyme and CPE was explored using fluorescence spectrometry in an aqueous phase. Our results showed that the CPE could form noncovalent complexes lysozyme and showed higher binding affinity with the protein than CR did. The combination of CPE with lysozyme was proved to be a single static quenching process. Our previous research work show that CP possessed relatively stronger binding affinity towards proteins and was easier to form the noncovalent compounds with them by using ESI/MS and fluorescence spectrometry. Phosphate esters of flavonoids could enhance the interaction with proteins.[12] The preliminary biological activity screening tests indicated that CP and CPE indeed had stronger activity against HeLa tumor cells than CR in vitro. It might be deduced that anti-cancer effect of CPE might be associated with the CPE–protein interaction. In our previous experiment the relationship between a series of phosphorylated flavones binding with different numbers of phosphate groups and the anti-cancer effects were studied. The results showed that the more phosphate groups the flavone had, the higher anti-cancer effects displayed. The effect of diphosphate ester of chrysin (CP: tetraethyl bis-phosphoric ester of chrysin $C_{23}H_{28}O_{10}P_2$) was more prominent than that of monophosphate chrysin (CPE:

Diethyl Chysin-7-yl phosphate $C_{19}H_{19}O_7P$); and however, our experiment proved that the phosphorylated flavones, including CPE, exhibited no toxic effect on the cultured mouse normal fibroblast detected by MTT assay (unpublished). Indeed, CP and CPE can bind proteins with certain sites, such as the cell surface receptors, as what another our experiment showed that the gene expression of TPK, tyrosine-protein-kinase, was down-regulated by CR, CPE, and CR, especially the former, by RT-PCR technique. But the detailed molecule mechanism in the regulated network within the cancer cells for CP, CPE, and CR remains further studies.

In conclusion, this study of detecting the proliferation and apoptosis of Hela cells showed significant inhibition of Hela cell growth by CP, CPE, and CR, with its mechanism mainly through inducing tumor cell apoptosis and down-regulating PCNA expression (tumor malignancy). Furthermore, the anti-cancer effect of CP and CPE was more prominent than that of non-phosphorylated CR. Moreover, the effects of CP were more potent than that of CPE. The results suggest that the effects of CP and CPE might provide insight to be a new potential anti-cancer drug.

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The new compound CPE was characterized by detailed spectroscopic analysis: mp 61–63°C. Anal Calcd % for $C_{19}H_{19}O_6P$: C 58.30 (58.47), H 4.90 (4.91), P 7.90 (7.94); ^{1}H NMR (400 MHz, CD_3Cl) δ: 7.53 (m, 3H, 3′-H, 4′-H), 7.92 (m, 2H, 2′-H), 6.64 (s, 1H, 8-H), 6.99 (s, 1H, 6-H), 6.71 (s, 1H, 3-H), 4.26 (m, 2H, CH_2), 1.39 (t, 6H, CH_3); ^{31}P NMR (400 MHz, D_2O), δ: −9.32; ESI-MS/MS, *m/z* 391 $[M + H]^+$, 363 $[M - C_2H_4 + H]^+$, 335 $[M - 2C_2H_4 + H]^+$. IR 1265(PO).