

Photodynamic inhibitory effects of three perylenequinones on human colorectal carcinoma cell line and primate embryonic stem cell line

Lan Ma, Hong Tai, Cong Li, Yu Zhang, Ze-Hua Wang, Wei-Zhi Ji

Lan Ma, Tai Hong, Graduate School of the Chinese Academy of Sciences, Beijing 100871, China

Lan Ma, Yu Zhang, Ze-Hua Wang, Monoclonal Antibody Biotechnology Center, Yunnan University, Kunming 650091, Yunnan Province, China

Tai Hong, The First People's Hospital of Yunnan Province, Kunming 650032, Yunnan, China

Cong Li, Chemistry, Yunnan University, Kunming 650091, Yunnan Province, China

Lan Ma, Tai Hong, Wei-Zhi Ji, Kunming Institute of Zoology, Chinese Academy of Science, Kunming 650223, Yunnan Province, China

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Correspondence to: Wei-Zhi Ji, Kunming Institute of Zoology, Chinese Academy of Science, Kunming 650223, Yunnan, China. wji@mail.kiz.ac.cn

Telephone: +86-871-5139413 **Fax:** +86-871-5139413

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Abstract

AIM: To investigate the photodynamic inhibitory effects of Elsinochrome A (EA), Hypocrellin A (HA) and Hypocrellin B (HB) on human colorectal carcinoma Hce-8693 cells and rhesus monkey embryonic stem R366.4 cells, via inducing apoptosis.

METHODS: EA, HA and HB were extracted from metabolites of *Hypomyces* (Fr) Tul.Sp. R366.4 cells or Hce-8693 cells were cultured with different concentrations of EA, HA or HB respectively, irradiated and incubated with fresh medium for 2 h. Cell cycle analysis was performed by flow cytometry (FCM). Data were expressed as means \pm SD and analysis of variance and Student's *t*-test for individual comparisons.

RESULTS: The photodynamic bioactivity of EA was first reported in this study. After irradiation for 5 min, 6 min, 10 min or 20 min, photoactivated EA at lower concentrations, which were 10^{-7} Mol/L, 10^{-6} Mol/L, 10^{-5} Mol/L respectively, had no cytotoxic effects on R366.4 ES cells. Whereas, all of the three perylenequinones could induce apoptosis with a dose-dependent manner when Hce-8693 cells were incubated with photoactivated EA, HA and HB respectively. When Hce-8693 cells were incubated with EA at 10^{-6} Mol/L and irradiated 5 min, 6 min, 10 min and 20 min respectively, the rates of EA-induced apoptosis were 0, 0, 13.4 % and 40.5 %. While the rates of HA-induced apoptosis were 29.5 %, 32.0 %, 40.2 % and 22.6 %. And the rates of HB-induced apoptosis were 0, 0, 0 and 13.7 % respectively. Meanwhile, after 10^{-5} Mol/L treatment, the rates of EA-induced apoptosis were 32.7 %, 19.3 %, 26.4 % and 52.7 %, the rates of HA-induced apoptosis were 47.2 %, 39.1 %, 45.2 % and 56.6 %, and the rates of HB-induced apoptosis were 0, 0, 20.0 % and 13.9 % respectively.

CONCLUSION: EA, HA and HB have significant anti-cancer activity. The order of photodynamic inhibitory effects on tumor cells would be approximately HA>EA>HB. The molecular mechanisms of apoptosis may not be induced by reactive oxygen species and are worth further investigation.

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INTRODUCTION

Perylenequinones are a type of photosensitive pigments widespread in nature, which have been isolated from fungi, as well as other organisms^[1-5]. These lipid-soluble 4,9-dihydroxy-3, 10-perylenequinone derivatives are efficient producers of singlet oxygen (1O_2) in visible light^[6-11]. Due to their excellent photosensitive properties, they are expected to be developed as new phototherapeutic medicines^[8,12-17]. Among them, Elsinochrome A (EA) was first reported in 1966 by Chen CT *et al.*, who isolated EA from *Elsinoe* spp. I^[1-2]. And Meille SV *et al.* reported the structure of EA^[18]. Since then, there are no more related reports about EA. Hypocrellins are well-known photosensitizers, including hypocrellin A (HA) and hypocrellin B (HB), isolated from natural fungus sacs of *Hypocrella bambusae* growing in north western region of Yunnan Province in China^[19]. Hypocrellins were potent inhibitors of protein kinase C (PKC)^[20], and could inactivate some types of viruses in the presence of visible light and oxygen. These processes appeared to be mediated predominately by 1O_2 . This was further supported by the extremely high quantum yield of 1O_2 generation by hypocrellin^[21-23]s. Many investigations demonstrated that hypocrellins had a strong photodynamic effect on tumours^[24] and impressive antiviral activity against human immunodeficiency virus type 1 (HIV-1)^[25]. Recently, it has been reported that hypocrellin can photosensitize apoptotic cell death^[26]. The above investigations collectively provide a compelling rationale for the development of hypocrellin and its derivatives as PDT photosensitizers.

Our group has recently isolated a filamentous fungal strain from western region of Yunnan Province in China and identified it as *Ascomycetes Hypocreales Hypocreaceae Hypomyces* (Fr) Tul.sp based on the taxonomic study. *Hypomyces* (Fr) Tul.Sp. was found for the first time to produce Elsinochrome A (EA), Hypocrellin A (HA) and Hypocrellin B (HB), under solid-phase fermentation conditions. Colorectal cancer is common in China. Since EA and Hypocrellins could be a potential tumor photopreventive and phototherapeutic agents, it is worthwhile to investigate the photodynamic effects of these photosensitizers. In this study, we examined the relative potency of EA, HA and HB against two cell lines, human colorectal carcinoma Hce-8693 cells and rhesus monkey embryonic stem (ES) R366.4 cells, and attempted to correlate anticancer activity with chemical structure and quantum yield of 1O_2 .

MATERIALS AND METHODS

Synthesis

The fungal metabolites were isolated from solid-substrate fermentation cultures of *Hypomyces* (Fr) Tul.Sp. and evaporated to dryness. The powder of *Hypomyces* (Fr) Tul.Sp. was extracted with acetone at room temperature and then evaporated to dryness in vacuo. The recrystallized crude product was purified by silica gel column chromatography with a mixed solvent of petroleum ether:EtOAc:EtOH (4:2:1). The purified crystallized products were characterized with element analysis measurement (PE 2 400), UV-visible spectrophotometry (PE UV/V is Lambda Bio), fluorescence spectra instruments (Hitachi-850), FT-IR (PE 1 000), ^1H , ^{13}C -nuclear magnetic resonance (Bruker AM-400). The results were consistent with literature data.

Each of the above products was dissolved respectively in dimethylsulfoxide (DMSO) at 1 M and stored at 4 °C in dark conditions. Under these conditions the solutions were stable for 2 months. The stock solutions were diluted 10^3 to 10^7 fold and in the final experimental conditions, the final DMSO concentration (0.1 %) did not affect the viability of the culture cells, as demonstrated in control experiments.

Cell lines

Rhesus monkey embryonic stem cell line R366.4 was kindly provided by Dr James A Thomson (The Wisconsin Regional Primate Research Center, University of Wisconsin, US). Cells were plated in mouse embryonic fibroblasts (previously exposed to 3 000 rads γ -radiation) in medium consisting of 85 % Dulbecco's Modified Eagle medium (4 500 mg of glucose per liter, with L-glutamine, without sodium pyruvate; GIBCO) with 15 % fetal bovine serum (HyClone), 1×10^{-7} Mol/L 2-mercaptoethanol (Sigma) and 1 % nonessential amino acid stock (GIBCO). Human colorectal carcinoma Hce-8693 cells were obtained from ATCC. The cell lines Hce-8693 were maintained in Dulbecco's Modified Eagle medium (GIBCO) supplemented with 10 % new born calf serum (HyClone). All cell lines were grown at 37 °C under a water-saturated sterile atmosphere containing 5 % CO_2 (Forma Scientific Incubator). All cell manipulations in the presence of EA, HA and HB were performed under subdued light conditions.

Light irradiation

Cells incubated with EA, HA and HB were irradiated with a water-cooled 1 300 W tungsten-bromine lamp. All cells proliferated as monolayers attached to the plastic bottom of the plate which was completely transparent for the excitation light. Temperature recorded in tissue culture plate did not exceed room temperature during the irradiation period. Immediately after irradiation, cells were rinsed three times with PBS and grown in a fresh medium for 2 hours.

Flow cytometry

Cells were incubated with various doses of EA, HA or HB, irradiated, incubated for additional 2 h and then harvested, washed with phosphate-buffered saline (PBS) three times and fixed with 700 mL \cdot L $^{-1}$ ethanol at 4 °C overnight. Fixed cells were washed three times with PBS and stained with 800 μL propidium iodide and 200 μL deoxyribonuclease-free ribonuclease A in PBS. The fluorescence intensity of propidium iodide-stained nuclei was detected with flow cytometer (EPICS-XL, Coulter, USA) and 10 000 cells were analyzed with Multicycle software.

Photocytotoxicity studies in R366.4 cell lines

R366.4 cells growing in sub-confluent culture were used to assess photocytotoxic effects of EA via flow cytometric assays. Graded doses of EA (1×10^{-7} Mol/L, 1×10^{-6} Mol/L, 1×10^{-5} Mol/L, 1×10^{-4} Mol/L, 1×10^{-3} Mol/L) dissolved in DMSO were mixed

into the medium overlying 5.0×10^4 cells in 6-well plates. Following 2 h incubation, the cells were irradiated for 5 min, 6 min, 10 min and 20 min respectively (or not in case of darkness). After the drug-containing medium was removed, the cells were washed with phosphate-buffered saline (PBS) three times and the fresh ES culture medium was put on the cells prior to incubation for 2 h at 37 °C in saturated humidified air with 5 % CO_2 . Finally, the cell proliferation was determined by flow cytometric assay.

Inhibitory effect of EA, HA and HB on the proliferation of Hce-8693 cells by inducing apoptosis

Hce-8693 cells growing in confluent culture were used to assess inhibitory effects of EA, HA and HB via flow cytometric assays. For each compound, graded doses (1×10^{-6} Mol/L, 1×10^{-5} Mol/L, 1×10^{-4} Mol/L, 1×10^{-3} Mol/L) dissolved in DMSO were mixed into the medium overlying 5.0×10^4 cells in 6-well plates. Following 2 h incubation, the cells were irradiated for 5 min, 6 min, 10 min and 20 min respectively (or not in case of darkness). After the drug-containing medium was removed, the cells were washed with phosphate-buffered saline (PBS) three times and the fresh culture medium was put on the cells prior to an incubation for 2 h at 37 °C in saturated humidified air with 5 % CO_2 . Finally, the cell proliferation was determined by flow cytometric assay.

Statistical analysis

Student's *t* test was used to assess statistical significance of differences. If $P < 0.01$, the difference was considered very significant.

RESULTS

Synthesis

The structures of the compounds are shown in Figure 1, and their relevant photochemical properties are summarized in Table 1.

Table 1 The photochemical properties of the perylenequinones

Structure	UV λ_{max} (log ϵ)*	λ_{max} (log ϵ)*	$\phi \cdot ^1\text{O}_2$
EA	459(1.60), 528(0.84), 568(1.04)	460(3.78), 531(3.13), 571(3.60)	0.94
HA	468(1.88), 542(0.83), 582(0.90)	417(5.51), 542(1.02), 582(7.70)	0.83
HB	470(0.27), 540(0.12), 583(0.13)	471(4.39), 543(3.01), 583(3.36)	0.76

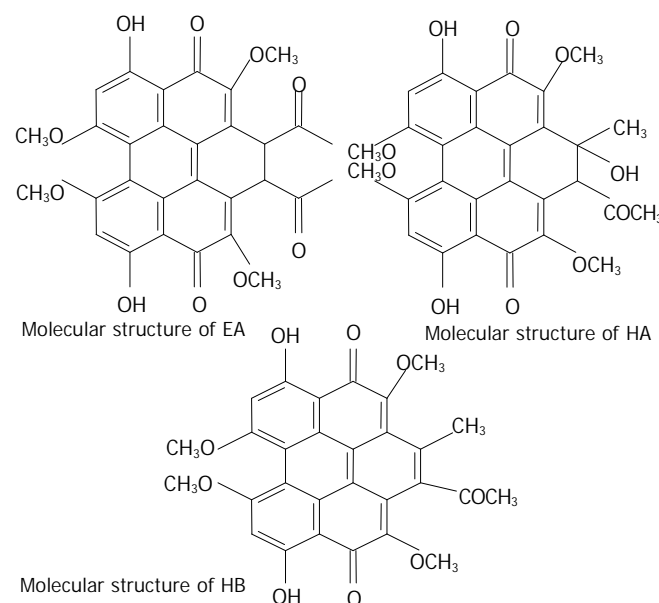


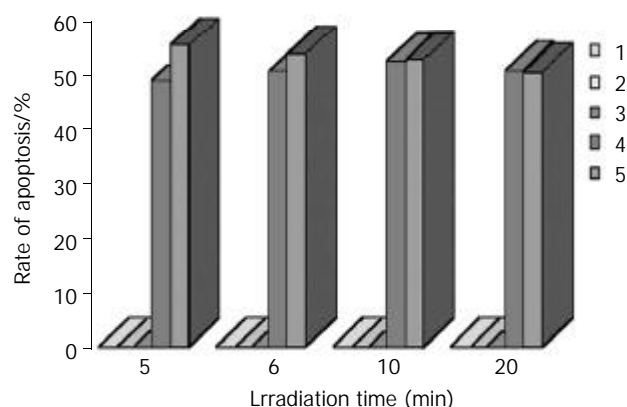
Figure 1 Structures of the three perylenequinones for photo-dynamic activity.

Photodependent cytotoxicity studies in R366.4 cell lines

Embryonic stem (ES) cells are derived from preimplantation embryos, have a normal karyotype, and are capable of indefinite, undifferentiated proliferation^[27]. Recently, *in vitro* mouse ES cell culture method has been used to test mutagenic, cytotoxic and embryotoxic effects of chemical substances^[28-30]. In this study, rhesus monkey ES R366.4 cells were first used to measure the photocytotoxicity of EA by judging the apoptosis of ES cells. After treated the R366.4 ES cells with EA at various concentrations, with or without light irradiation, the rate of apoptosis obtained by FCM were shown in Table 2 and Figure 2. The data illustrated that photoactivated EA had no cytotoxic effects on the R366.4 ES cells at low concentrations, which were 10^{-7} Mol/L, 10^{-6} Mol/L, 10^{-5} Mol/L respectively. Whereas, all of photoactivated EA at higher concentrations (10^{-4} Mol/L and 10^{-3} Mol/L respectively) exhibited a potent cytotoxic effects on R366.4 cells. In general, no large differences in the photodependent cytotoxic effects of EA were found between the different irradiation time. In the case of the photocytotoxic EA no cytotoxic effect was observed in dark conditions.

Table 2 EA-induced apoptosis in R366.4 ES cells with FCM assay (means \pm SD, $n=3$)

Group	Rate of apoptosis/%			
	5 min	6 min	10 min	20 min
Control	0	0	0	0
10^{-7} Mol/L	0	0	0	0
10^{-6} Mol/L	0	0	0	0
10^{-5} Mol/L	0	0	0	0
10^{-4} Mol/L	48.8 \pm 4.50 ^b	50.3 \pm 4.14 ^b	52.1 \pm 2.35 ^b	50.5 \pm 3.68 ^b
10^{-3} Mol/L	54.9 \pm 2.99 ^b	53.4 \pm 4.01 ^b	52.4 \pm 3.50 ^b	50.2 \pm 4.93 ^b

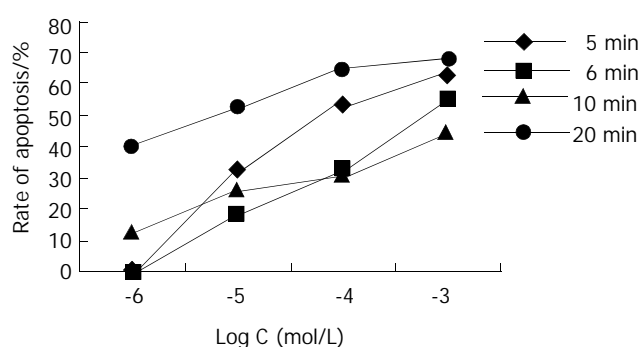
^b $P<0.01$, vs EA control.**Figure 2** Photodependent cytotoxic effects of EA on R366.4 ES cells at various concentrations: (1) 10^{-7} Mol/L, (2) 10^{-6} Mol/L, (3) 10^{-5} Mol/L, (4) 10^{-4} Mol/L, (5) 10^{-3} Mol/L respectively. Results are means \pm SD of three independent experiments.**Inhibitory effect of EA on the proliferation of Hce-8693 cells by inducing apoptosis**

In order to investigate the antiproliferative effect of EA, Hce-8693 cells were incubated with different concentrations of EA under dark conditions and subjected 2 hours to different irradiation time (5, 6, 10 and 20 min respectively). The cells were then further incubated for an additional 2 hours in the dark without photosensitizer and measured via FCM assay. The rates of apoptosis induced by EA are shown in Table 3 and Figure 3. For each irradiation time, the data showed that there was dose-dependent relationship between EA doses and

rate of Hce-8693 cell apoptosis. On the contrary, no large differences in the antiproliferative effect of the photoactivated EA was found between the different irradiation time.

Table 3 Hce-8693 cell apoptosis induced by photoactivated EA (means \pm SD, $n=3$)

Group	Rate of apoptosis/%			
	10^{-6} Mol/L	10^{-5} Mol/L	10^{-4} Mol/L	10^{-3} Mol/L
Control	0	0	0	0
5 min	0	32.7 \pm 7.56 ^b	53.6 \pm 6.62 ^b	63.4 \pm 10.24 ^b
6 min	0	19.3 \pm 4.16 ^b	32.8 \pm 7.38 ^b	55.5 \pm 7.00 ^b
10 min	13.4 \pm 3.25 ^b	26.4 \pm 4.89 ^b	31.3 \pm 5.39 ^b	44.9 \pm 5.46 ^b
20 min	40.5 \pm 8.58 ^b	52.7 \pm 11.82 ^b	65.2 \pm 11.22 ^b	68.0 \pm 5.93 ^b

^b $P<0.01$, vs EA control.**Figure 3** Dose-dependent relationship between EA doses and rate of Hce-8693 cell apoptosis. Cells were incubated for 2 h with 1×10^{-6} Mol/L, 1×10^{-5} Mol/L, 1×10^{-4} Mol/L, 1×10^{-3} Mol/L EA photosensitizer respectively and then irradiated. Results are means \pm SD of three independent experiments.**Inhibitory effect of HA on the proliferation of Hce-8693 cells by inducing apoptosis**

In order to investigate the antiproliferative effect of HA, Hce-8693 cells were incubated with different concentrations of HA under dark conditions and subjected 2 hours to different irradiation time (5, 6, 10 and 20 min respectively). The cells were then further incubated for additional 2 hours in the dark without photosensitizer and measured via FCM assay. The rates of apoptosis induced by HA were shown in Table 4 and Figure 4. For each irradiation time, the data showed that there was dose-dependent relationship between HA doses and rates of Hce-8693 cell apoptosis. On the contrary, no large differences in the antiproliferative effect of the photoactivated HA was found between the different irradiation time.

Table 4 Hce-8693 cell apoptosis induced by photoactivated HA (means \pm SD, $n=3$)

Ggroup	Rate of apoptosis/%			
	10^{-6} Mol/L	10^{-5} Mol/L	10^{-4} Mol/L	10^{-3} Mol/L
Control	0	0	0	0
5 min	29.5 \pm 2.29 ^b	47.2 \pm 8.79 ^b	48.4 \pm 4.66 ^b	58.8 \pm 8.40 ^b
6 min	32.0 \pm 5.64 ^b	39.1 \pm 6.41 ^b	43.2 \pm 8.84 ^b	66.4 \pm 8.02 ^b
10 min	40.2 \pm 6.23 ^b	45.2 \pm 8.40 ^b	45.5 \pm 8.38 ^b	53.4 \pm 8.77 ^b
20 min	22.6 \pm 3.39 ^b	56.6 \pm 3.86 ^b	62.8 \pm 4.23 ^b	68.4 \pm 8.85 ^b

^b $P<0.01$, vs HA control.

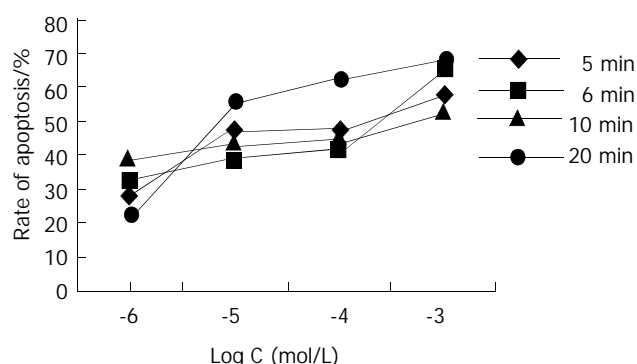


Figure 4 Dose-dependent relationship between HA doses and rate of Hce-8693 cell apoptosis. Cells were incubated for 2 h with 1×10^{-6} Mol/L, 1×10^{-5} Mol/L, 1×10^{-4} Mol/L, 1×10^{-3} Mol/L HA photosensitizer respectively and then irradiated. Results are means \pm SD of three independent experiments.

Inhibitory effect of HB on the proliferation of Hce-8693 cells by inducing apoptosis

In order to investigate the antiproliferative effect of HB, Hce-8693 cells were incubated with different concentrations of HB under dark conditions and subjected 2 hours to different irradiation time (5, 6, 10 and 20 min respectively). The cells were then further incubated for additional 2 hours in the dark without photosensitizer and measured via FCM assay. The rates of apoptosis induced by HB were shown in Table 5 and Figure 5. For each irradiation time, the data showed that there was dose-dependent relationship between HB doses and rate of Hce-8693 cell apoptosis. On the contrary, no large differences in the antiproliferative effect of the photoactivated HB was found between the different irradiation time.

Table 5 Hce-8693 cell apoptosis induced by photoactivated HB (means \pm SD, $n=3$)

Group	Rate of apoptosis/%			
	10^{-6} Mol/L	10^{-5} Mol/L	10^{-4} Mol/L	10^{-3} Mol/L
Control	0	0	0	0
5 min	0	0	28.1 ± 6.21^b	64.8 ± 11.78^b
6 min	0	0	17.3 ± 3.68^b	32.0 ± 7.57^b
10 min	0	20.0 ± 4.21^b	20.5 ± 4.57^b	71.0 ± 10.57^b
20 min	13.7 ± 3.02^b	13.9 ± 2.87^b	19.1 ± 4.06^b	29.4 ± 6.56^b

^b $P < 0.01$, vs HB control.

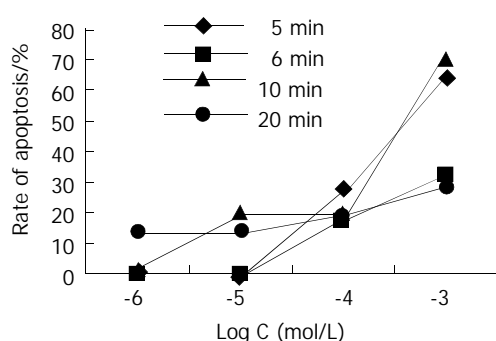


Figure 5 Dose-dependent relationship between HB doses and rate of Hce-8693 cell apoptosis. Cells were incubated for 2 h with 1×10^{-6} Mol/L, 1×10^{-5} Mol/L, 1×10^{-4} Mol/L, 1×10^{-3} Mol/L HB photosensitizer respectively and then irradiated. Results were means \pm SD of three independent experiments.

Inhibitory effect of EA, HA and HB on the proliferation of Hce-8693 cells by inducing apoptosis

From the results of photodependent cytotoxicity studies in R366.4 cell lines, it seemed that photoactivated EA had no cytotoxic effects at 10^{-6} Mol/L and 10^{-5} Mol/L concentrations. On the contrary, EA, HA and HB exhibited more or less antiproliferative effects on human Hce-8693 cells at this range of concentrations. Thus, the photodynamic effects of the photosensitizers could be compared from the rate of apoptosis (Table 6). The order of efficiency would be approximately $HA > EA > HB$ (Figure 6).

Table 6 Comparison of antiproliferative effects of EA, HA and HB

Group	Rate of apoptosis/%					
	10^{-6} Mol/L			10^{-5} Mol/L		
	HA	EA	HB	HA	EA	HB
5 min	0	0	0	47.2 ± 8.79	32.7 ± 7.56	0
6 min	32.0 ± 5.64	0	0	39.1 ± 6.41	19.3 ± 4.16	0
10 min	40.2 ± 6.23	13.4 ± 3.25	0	45.2 ± 8.40	26.4 ± 4.89	20.0 ± 4.21
20 min	22.6 ± 3.39	40.5 ± 8.58	13.7 ± 3.02	56.6 ± 3.86	52.7 ± 11.82	13.9 ± 2.87

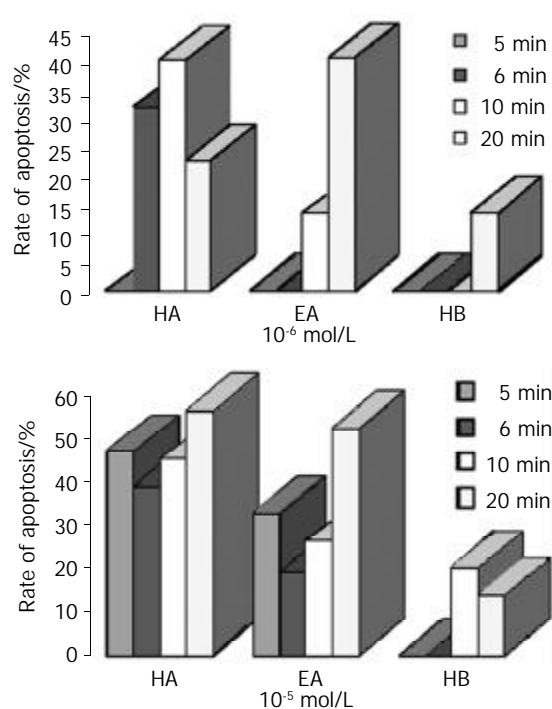


Figure 6 (a)-(b) illustrated the different antiproliferative effects of photoactivated HA, EA and HB on human Hce-8693 cells.

DISCUSSION

Photodynamic therapy (PDT) is a medical treatment based on the use of a sensitizer to promote photoinduced damage to biological molecules including lipids, proteins and DNA^[31,32]. It can be used to eradicate early localized tumours and for palliation of more advanced disease when metastasis has occurred. This treatment modality involves the use of light in combination with a photosensitizing compound. Following excitation of photosensitizers to long-lived excited singlet and/or triplet states, the tumour is destroyed either by reactive oxygen species (Type II mechanism) and/or by radical products (Type I mechanism)^[33-37].

Hypocrellins are efficient singlet oxygen generators during

photochemical reactions and may also exert photosensitization via radical mechanisms, which may confer a degree of independence from classical oxygen-dependent photochemical mechanisms. This feature is important in the context of impaired radiosensitivity and chemosensitivity of hypoxic human tumour cells. However, the precise mode of action of these molecules at the cellular level is not clear and seems to go far beyond Type I and Type II photoprocesses^[38-42]. An additional mechanism involving protons released in the excited state and leading to cellular pH drop has also been proposed for the related pigments hypocrellin and hypericin^[43].

Apoptosis is a complex and programmed process which is regulated by a variety of factors. Recently, it has been reported that hypocrellins and their derivatives can photosensitize apoptotic cell death^[44,45]. However, the molecular mechanisms of tumor cell apoptosis induction by Hypocrellin A and B are poorly understood. The antiproliferative actions of hypocrellin may be due, in part, to the ability of hypocrellin A to induce reactive oxygen species (Type II mechanism)^[46,47]. In addition, hypocrellin A-mediated apoptosis is increased when antisense bcl-2 retrovirus vector is transfected into human gastric adenocarcinoma MGC803 cells^[48]. And also, Ali *et al.* reported that caspase-3 and hydrogen peroxide may play an important role in HA and HB-induced apoptosis^[49, 50].

According to the photochemical properties, the quantum yields of EA, HA and HB are 0.94, 0.83, 0.76 respectively. From the results of inhibitory effect of EA, HA and HB on the proliferation of Hce-8693 cells, it seems that the order of efficiency would be approximately HA>EA>HB. In this way, the molecular mechanisms of Hce-8693 cell apoptosis induction by EA, HA and HB may not be induced by reactive oxygen species (Type II mechanism). It is also noteworthy that photoactivated EA, HA and HB can selectively inhibit the growth of human colorectal carcinoma cells but not rhesus monkey embryonic stem R366.4 cells at lower concentrations. Thus, the molecular mechanisms of apoptosis induced by photoactivated EA, HA and HB are worth further investigation.

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