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Photodynamic action of hypocrellin dyes: structure–activity relationships

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Abstract

Hypocrellin and its derivatives were compared for their photodynamic effects on human oral cavity epithelial carcinoma KB cell line. The amphiphilicity as well as the singlet oxygen generating quantum yield of the hypocrellin dyes affected their photodynamic activity. The most hydrophilic dyes exhibited the lowest phototoxic activity, whereas the hydrophobic and amphiphilic dyes with higher singlet oxygen-generating quantum yield, exhibited high photodynamic activity. Cysteamine mono- and di-substituted hypocrellin B and cysteine mono-substituted hypocrellin B, demonstrating strong red absorption in the domain of phototherapeutic window (600–900 nm), proper hydrophobic and amphiphilic properties and high photocytotoxicity to KB cells, might prove to be potential phototherapeutic agents.

Keywords: Photodynamic action; Hypocrellin dyes; Photocytotoxicity; Singlet oxygen; Amphiphilicity

1. Introduction

Photodynamic therapy (PDT) is a relatively new procedure for ablating tumors and other tissues. It is affected by inducing the tissues of interest to take up a photosensitizer and then irradiating them with visible or near infrared radiation [1–3]. It is believed that PDT usually involves the photochemical generation of singlet oxygen and the subsequent oxidation of the tissues by it.

The only photosensitizer to be approved by the United States FDA so far is Photofrin®. This photosensitizer is composed of a fraction of hematoporphyrin derivative and is a complex mixture [4]. Although promising results have been obtained with Photofrin®, it has substantial limitations. One is its tendency to localize in the skin and remain there for a prolonged period of time [4,5]. The limitations and the complex composition of Photofrin® have led to an examination of many possible alternative photosensitizers [6,7]. Among the latter, hypocrellins, including hypocrellin A (HA) and hypocrellin B (HB) have been proposed as second-generation photosensitizers for PDT mainly because of their high quantum yields of singlet oxygen, facility for site-directed chemical modification and fast clearance from normal tissues [8]. To improve the red absorption and amphiphilic of the parent hypocrellins, a series of hypocrellin congeners (Fig. 1) have been synthesized recently, and the photophysical and

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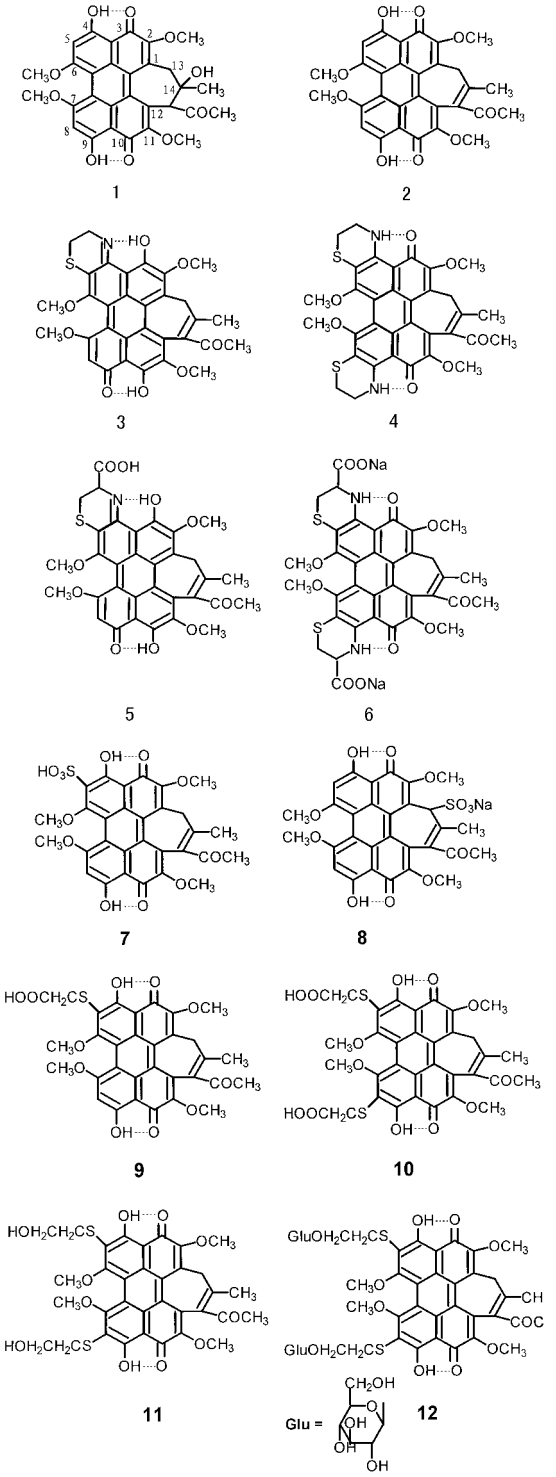

Fig. 1. Chemical structures of hypocrellins and their derivatives.

photochemical properties have been investigated [9–13]. In the present study we evaluated the photodynamic effects of these hypocrellin derivatives to human oral cavity epithelial carcinoma KB cell line and surveyed the structure–activity relationships, which will facilitate the selection among hypocrellin dyes suitable for PDT treatment.

2. Results and discussion

The structures of the compounds are shown in Fig. 1, and their relevant photophysical properties are summarized in Table 1. It is evident that the red absorption of compounds **3–6** was improved significantly and the longer band had extended into the phototherapeutic window (600–900 nm) due to the amination of the hypocrellin chromophore. Furthermore, compounds **3–6**, the aminated derivatives, almost maintained the $^1O_2$ generating efficiency, while the incorporation of aliphatic side chains (**7–12**) induced significant decrease of the $^1O_2$-generating quantum yield except for compound **7**. The most likely reason for this difference is that the triplet quantum yields differ in the similar way. The incorporation of aliphatic side chains enhanced the internal conversion process, resulting in the decrease of triplet quantum yield. The results demonstrated in Table 2 implied that the $^1O_2$-generating and the triplet quantum yields were not influenced significantly by the cyclic amination.

The amphiphilicity of hypocrellin dyes has been evaluated via the measurement of their partition coefficients between *n*-octanol and PBS buffer (pH 7.4). The results are shown in Table 2. The amphiphilicity of the hypocrellin derivatives (**3–12**) was enhanced as compared with that of the parent compounds (HA and HB). The incorporation of anionic groups (COOH, COONa, SO_3H, SO_3Na), in **5–10** and the incorporation of carbohydrate groups in **12** increased the hydrophilicity of them significantly. Compounds **6**, **8** and **12** can even be dissolved in water directly.

The cell survival was estimated with the MTT colorimetric assay, which measures the activity of mitochondrial hydrogenases [14]. This quick and reliable essay has been shown to correlate well

Table 1
UV–Vis absorption of hypocrellin dyes in chloroform on in ethanol and quantum yield of $^1O_2$ generation (Φ)

Compound	λ_{max} (log ϵ)	Φ
1	465 (4.33), 539 (4.07), 580 (4.08)	0.81
2	464 (4.34), 554 (4.07), 590 (4.03)	0.76
3	532 (4.25), 598 (4.03), 652 (4.06)	0.71
4	580 (4.13), 684 (3.95)	0.64
5	532 (4.24), 598 (4.03), 652 (4.06)	0.60
6[a]	580 (4.02), 684 (3.95)	0.54
7	464 (4.32), 570 (4.03)	0.62
8[a]	444 (420)	0.21
9	490 (4.32), 580 (4.04)	0.22
10	512 (4.27)	0.18
11	514 (4.28)	0.19
12[a]	512 (4.26)	0.19

[a] Measured in ethanol.

Table 2
Partition coefficients (PC) and half inhibition concentration (IC_{50}) (μg/ml) of hypocrellin dyes

Compound	PC	IC_{50}
1	41.6	0.28
2	46.4	0.95
3	34.4	0.09
4	30.6	0.27
5	7.2	0.87
6	0.05	> 20
7	4.3	3.81
8	0.2	> 20
9	7.8	3.02
10	2.7	> 20
11	20.6	1.58
12	1.3	> 20

with other cell-viability tests, including the clonogenic assay and [^{3}H]thymidine incorporation in the case of PDT inactivation of cells with haematoporphyrin derivative [15,16] and benzoporphyrin derivatives [17]. We evaluated the effects of the concentrations on the phototoxic potential of hypocrellin dyes. The ranges of photosensitizer concentrations required to exert 50% toxicity (or inhibition) (IC_{50}) under irradiation were estimated from the survival curves for each photosensitizer. Table 2 shows the IC_{50} values in μg/ml for hypocrellin dyes listed in Fig. 1; the lower the IC_{50} values, the more phototoxic the photosensitizer.

Of the perylenequinonoid derivatives in Fig. 1, three compounds demonstrated markedly improved phototoxicity with respect to the parent compound, HB. It is worth noting that **3** and **4** demonstrated even more efficient photodynamic activity to that of HA. The IC_{50} values of **7**, **9**, **10**, and **11** are higher than those of the parent compounds, HA and HB, indicating lower photodynamic activity under our experiment conditions, whereas the three water-soluble derivatives **8**, **10** and **12** demonstrated poor phototoxicity, with IC_{50} values higher than 20 μg/ml.

All of these compounds can photosensitize the generation of singlet oxygen (discussed above). Singlet oxygen is the main species in the phototoxicity of photosensitizers in PDT. Comparison between **5** and **9**, with similar partition coefficients, demonstrated that higher $^1O_2$-generating quantum yield promoted photocytotoxicity. However, comparison between **4** and **6**, with similar singlet oxygen quantum yield but with different partition coefficients and IC_{50} values, suggested that some factors other than $^1O_2$ quantum yield, such as partition coefficients, influenced the phototoxicity of photosensitizers. Comparison between **11** and **12** (or **8** or **10**) afforded a similar conclusion. Previous results on a related series of phthalocyanine-based photosensitizers suggested that hydrophobic and amphiphilic photosensitizers exhibited high cell uptake and efficient photodynamic activity, whereas hydrophilic photosensitizers demonstrated low photodynamic activity owing to the low affinity for the lipidic cell membrane. Intratumoral distribution studies suggested that the more hydrophilic photosensitizers localized in the extracellular space and associate with connective tissue in the tumor, whereas the amphiphilic and hydrophobic dyes penetrated and accumulated within the neoplastic cell themselves [18]. The phototoxic efficiency of porphyrins towards Mediterranean fruit fly demonstrated that the phototoxicity increased upon increasing the hydrophobicity of the porphyrin molecules and the highest photocidal activity is exhibited by a cationic amphiphilic porphyrin [19]. As compared with porphyrins and phthalocyanines, hypocrellins exhibited similar results. The most hydrophilic photosensitizers exhibited the lowest phototoxic activity toward KB cells while the hydrophobic

and amphiphilic photosensitizers exhibited the highest or acceptable phototoxic activity. The enhancement of the photodynamic activity of **3–5** with similar $^1O_2$-generating quantum yield as compared with HB suggested that they might become the most potential phototherapeutic agents among hypocrellin dyes. It can be seen that the photodynamic activity of hypocrellin dyes depends on their chemical structures.

3. Conclusions

Chemical modifications on hypocrellin B afforded variation of molecule structures, absorption properties, partition coefficients, quantum yield of $^1O_2$-generation and photodynamic activity to KB cells of different hypocrellin congeners. The photophysics and phototoxicity of these photosensitizers depended on the chemical structures. The amphiphilicity, as well as $^1O_2$-generating quantum yield of hypocrellin dyes, affected their photodynamic activity. The most hydrophilic dyes exhibited the lowest phototoxic activity whereas the hydrophobic and amphiphilic dyes with higher $^1O_2$-generating quantum yield exhibited high photodynamic activity.

Cysteamine mono- and di-substituted hypocrellin B (**3** and **4**) and cysteine mono- substituted hypocrellin B (**5**), which have strong red absorption in the range between 600 and 700 nm, appropriate hydrophobic and amphiphilic property and high photodynamic activity to KB cells, might prove to be potential phototherapeutic agents for PDT. The in vivo photodynamic activity of these hypocrellin dyes is currently being evaluated.

4. Experimental

4.1. Chemicals

Compounds were isolated, synthesized and purified according to published methods [9–13,20,21].

4.2. Singlet oxygen determination

The 9,10-diphenylanthracene (DPA) bleaching method was use to determine the quantum yields of singlet oxygen ($^1O_2$) production by hypocrellin dyes [22]. The photooxidation of DPA sensitized by hypocrellin dyes was carried out on a ''merry-go-round'' apparatus where the samples were illuminated by 436 nm light, obtained from the combination of a high-pressure mercury lamp (Beijing Lightsource Factory, Beijing, China) with a narrow-band interference filter (Oriel Corporation). The DPA concentration was maintained at 0.54 mM. The photooxidations were monitored by the absorbance decrease of DPA at 374 nm, and the quantum yields of $^1O_2$ production by hypocrellin dyes were calculated as described by Diwu and Lown [22].

4.3. Partition coefficients

Hypocrellin dyes (5 μM) were partitioned between *n*-octanol and 0.1 M phosphate-buffered saline (PBS) buffer (pH 7.4). Both solvents were previously equilibrated with each other. After shaking for 2 min at 20°C, the phases were separated by centrifugation and the dye concentration was spectrophotometrically measured in aliquots of both phases.

4.4. Cell culture

Human oral cavity epithelial carcinoma KB cells (Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijng, China) were maintained as monolayer cultures in RPMI-1640 medium containing 10% fetal bovine serum (FBS) (GIBCO-BRL products) at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and were used in the logarithmic phase of growth.

4.5. Assessment of cell survival

Hypocrellin dyes, in dimethyl sulfoxide (DMSO), were diluted to the desired concentrations in medium (plus 0.1% FBS). Exponentially growing KB cells in 75 cm^2 flasks (Coster, Camridge, MA, USA) were incubated with or without graded doses of hypocrellin dyes in RPMI-1640 medium (FBS-free) for 4 h at 37°C. The cells were detached using 0.2% trypsin/0.5 mmol dm^{-3}

EDTA solution in PBS buffer. After spinning at 1000 rpm for 5 min, trypsin/EDTA was removed and the remaining cell pellet was resuspended in RPMI-1640 medium (without phenol red and FBS). The cells were then transferred to 35 mm dishes (Costar), 1 ml in each dish containing 3×10^5 cells, and irradiated with 550 nm light for 10 min with an intensity of 24 mW cm^{-2} at the position of the samples. Immediately after irradiation, 2 ml RPMI-1640 medium containing 15% FBS were added to each dish. Cell survival was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay [14,17]. Cells were transferred into flat-bottomed 96-well plates (Nunc, Denmark), 100 μl in each well containing 1×10^3 cells. 10 μl of MTT solution (Sigma Chemical Company, St. Louis, MO, USA; 10 mg/ml in PBS) were added after 24 h to each well for 4 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The wells were then carefully drained and 150 μl DMSO were added to each well to stop MTT reduction and to dissolve the blue formazan crystals produced by mitochondrial hydrogenases in living cells only. The plates were then shaken at room temperature for 10 min and read immediately at 595 nm on a Bio-Rad model 3550 microplate reader (Richmond, CA). Samples were measured in 12 replicates and each experiment was repeated at least three times. Survival of PDT-treated cells was normalized against cells incubated with photosensitizer alone. The inhibition of cell survival was calculated via the formula $(Abs_{CTR}-Abs)/(Abs_{CTR}-Abs_{BLK})$ where Abs is the average absorbance of 12 replicates, Abs_{CTR} is the average absorbance of the control wells (cells without dye) and Abs_{BLK} the average absorbance of the blank wells (medium without cells). The molar concentration of each compound required to exert 50% inhibition (IC_{50}) was determined from survived curves.

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