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Hypocrellins as photosensitizers for photodynamic therapy: a screening evaluation and pharmacokinetic study

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Abstract Hypocrellin compounds were selected as potential photosensitizers for photodynamic therapy (PDT) owing to their high quantum yields of singlet oxygen ($^1O_2$), and facility for site-directed chemical modification to enhance phototoxicity, pharmacokinetics, solubility, and light absorption in the red spectral region, among other properties. Parent hypocrellins A and B share an absorption peak at 658 nm. These molecules may therefore be considered useful progenitors of derivatives which absorb more strongly in the red, considering that the ideal sensitizer should absorb in the 650–800 nm range, beyond the absorption range of hemoglobin and melanin, and where light penetration in tissues is maximized through reduced scattering. A series of pure, monomeric hypocrellin derivatives was tested for properties of dark cytotoxicity and photosensitizing potential by clonogenic assay in monolayer cultures of EMT6/Ed murine tumor cells. Their respective toxicities are reported on a molar basis. The in vitro screening assay has, to date, resulted in the selection of four hypocrellin derivatives for further development as photosensitizers for PDT. Cellular uptake for photosensitizing doses of selected compounds was determined by fluorimetry. Dose escalation studies in rodents indicate that potentially photosensitizing doses promote no demonstrable systemic toxicity.

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Introduction

Treatment for cancer has traditionally encompassed three main strategies: surgery, chemotherapy, and radiotherapy. Although considerable progress in these areas has been attained, the search for effective and safe alternative treatments continues. Considerable interest has evolved during the past two decades in the use of hematoporphyrin derivatives (HpD) for tumor localization and treatment by photodynamic therapy (PDT) of tumors, particularly those of esophagus, bronchi, and bladder. Lipson et al. [20] were the first to use PDT in 1966 at the Mayo Clinic. By 1972, widespread interest in PDT had prompted animal experiments which demonstrated that the combination of a photosensitizer (fluorescein), light, and cellular oxygen could inhibit tumor growth [12]. The first animal study utilizing HpD was reported in 1975 [13].

Since the advent of HpD and its more purified version, Photofrin, PDT of tumors has progressed to phase III clinical trials. Although HpD has been an extremely useful tissue photosensitizer, there are some problems associated with its use, including prolonged cutaneous phototoxicity. These limitations have prompted a search for alternative photosensitizers for PDT. Current candidates include the promising sensitizer, benzoporphyrin derivative monoacid ring A [26] and 5-aminolevulinic acid/protoporphyrin IX [17], both of which are in clinical trials. Further studies involve merocyanine 540 [28], phthalocyanine (CASPc) [29], mono-L-aspartyl chlorin e_6 (MACE) [1], Nile blue [3], anthrapyrazoles [18], anthracenediones [19], anthracyclines [23], and hypocrellins [5]. This screening study focused on the hypocrellins as potential photosensitizers for PDT.

Hypocrellins derive their name from *Hypocrella bambusae sacc*., a parasitic fungus of *Sinarundinaria* species, which grows abundantly in the northwestern region of Yunnan Province and the southeastern region of Tibet Autonomous Region of the People's Republic of China, and also in certain parts of Sri Lanka [4]. They belong to the general class of perylenequinonoid pigments, which include hypocrellins A (HA), B (HB), and deacetylated hypocrellin A (DAHA). Originally, hypocrellin was utilized as a potent therapeutic agent for white lesions of the vulva, keloid, vitiligo, psoriasis, tinea capitis and lichen amyloidosis [31, 32].

Oral administration of HA causes hypericism, a state of skin sensitivity to visible light. This finding derives from animal studies of Mei and Luo [22]. More than 20 years later, it was demonstrated that the photosensitizing effects of HA depend on the presence of oxygen, and therefore HA was recognized as a photodynamic agent [30]. Potent photosensitizing effects in the context of PDT have been reported [11, 14, 15].

Hypocrellins are pure compounds with favorable red light absorption spectra and with high yields of singlet oxygen [6]. Initial reports also demonstrate substantial excretion from the host within 24 h [14]. With these characteristics, as well as the ability to be chemically modified, derivatives have been synthesized to optimize the photosensitizing properties. These attributes provide a strong rationale for the development and in vitro assessment of hypocrellins, with their potential for clinical use in PDT as an ultimate objective.

Materials and methods

Preparation and absorption of hypocrellin derivatives

Hypocrellin derivatives were prepared as previously described [6–8, 10]. Briefly, crude HA was prepared by acetone extraction of *Hypocrella bambusae* (*B. et Br.*) *Sacc*. Lipids were removed by counter extraction with petroleum ether. Further purification was carried out on a silica gel column, followed by 1% potassium dihydrogen phosphate–silica gel thin-layer chromatography and recrystallization from acetone. HB was prepared by quantitative potassium hydroxide dehydration of HA, followed by neutralization with HCl, chloroform extraction, and recrystallization from benzene–petroleum ether. DAHA [8] was prepared by reflux with KOH, neutralization, and chloroform extraction of HB. The product was subjected to 1% citric acid–silica gel thin-layer chromatography, using a 6: 2: 1 mixture of petroleum etherethyl acetateethanol as eluent. HB derivatives, HBBA-R2, HBDP-R1, and HBEA-R1, were prepared by amination of the phenolic hydroxyl groups of the parent compound. JL-1-1 was prepared according to the method of Liu et al. [21]. The absorption spectra of these derivatives were determined on a Hewlett Packard 8542 diode array spectrophotometer.

Determination of singlet oxygen yield

Singlet oxygen yields were determined as previously reported [6] by the 9,10-diphenylanthracene (DPA) bleaching method. Test

Table 1 Physical and chemical properties of hypocrellins of potential use in PDT. HBBA-R2, HBDP-R1, HBEA-R1 and JL-1-1 demonstrate average, or lower than average toxicity, with excellent photopotentiation (*ND* not done). For the purposes of the screening study, the LD_{50} light dose was not fixed. For the compounds tested, this dose is 0.75–1.0 J/cm^2 of 630 nm light (see Fig. 4)

Name of compound		Chemical formula	F.W.	Absorption peak in red spectral region (nm)	A_{max}/ solvent	A_{630}	Extinction coefficient ($\times 10^{-3}$) (630 nm)	$^1O_2$ yield	LD_{50} dark (μM)	LD_{50} light (μM)	Photopotentiation factor
HA	Hypocrellin A	$C_{30}H_{26}O_{10}$	546	658*	0.093/DMF	0.086	0.86	0.84	15	3–5	3–5
HB	**Hypocrellin B**	**$C_{30}H_{24}O_9$**	**528**	**658***	**0.118/DMF**	**0.100**	**1.00**	**0.74**	**20**	**1.5–2**	**10–13**
HA-Mg^{++}	HA-Mg^{++}**(Ac)**$_2$	$C_{34}H_{28}O_{12}Mg$	652	616	0.958/EtOH	0.447	4.47	0.71	> 25	> 5	–
HB-Mg^{++}	**HB-Mg^{++}**	**$C_{34}H_{26}O_{11}Mg$**	**634**	**622**	**0.604/EtOH**	**0.527**	**5.27**	**0.53**	**10**	**1**	**10**
DAHA	Dcacetylated-HA	$C_{32}H_{24}O_{10}Mg$	592	622	0.651/EtOH	0.570	5.70	0.51	> 25	> 5	–
HBAC-R1	**Cystamine-HB**	**$C_{32}H_{27}O_8NS$**	**585**	**646**	**0.417/$CHCl_3$**	**0.388**	**3.88**	**0.40**	**12.5**	**1**	**12.5**
HBAC-R2	Cystamine-HB	$C_{32}H_{27}O_8NS$	585	600	0.337/DMSO	0.244	2.44	0.31	12.5	5	2.5
HBBA-R2	**n-Butylaminated HB**	**$C_{36}H_{60}N_4O_7$**	**780**	**616***	**0.628/$CHCl_3$**	**0.619**	**6.19**	**0.32**	**> 100**	**0.2–0.6**	**167–500**
HBAM-R1	2-morpholino-ethylaminated-HB	$C_{42}H_{48}N_4O_9$	752	658				0.70	> 25	4	> 6.25
HBDD-R2	2-(*N*,*N*-diethylamino) ethylamine-HB	$C_{42}H_{52}N_4O_7$	696	646*	0.508/$CHCL_3$	0.055	0.55	0.36	> 25	7.5	> 3.3
HBDP-R1	**2-(*N*,*N*-dimethyl-amino)propylamine-HB**	**$C_{40}H_{48}N_4O_7$**	**724**	**640***	**0.463/$CHCl_3$**	**0.480**	**4.80**	**0.42**	**> 25**	**0.5–1.5**	**> 16.6–6.50**
HBEA-R1	**Ethanolamine-HB**	**$C_{34}H_{34}N_2O_9$**	**614**	**696***	**0.625/DMSO**	**0.623**	**6.23**	**0.60**	**15–25**	**0.15**	**100–167**
HBEA-R2	Ethanolamine-HB	$C_{34}H_{34}N_2O_9$	614	634*	0.162/DMSO	0.127	1.27	0.70	25	7.5	3.3
HBED-R2	Ethylenediamine-HB	$C_{38}H_{32}N_8O_6$	696	614*	1.449/DMSO	1.239	12.39	0.50	> 25	3–5	5–8.3
HBMA-IV	Methylamine-HB	$C_{30}H_{33}N_3O_6$	696	640	0.246/$CHCl_3$	0.246	2.46	0.80	8.5	1	8.5
DBHB	5,8-dibromo-HB	$C_{30}H_{23}O_9Br_2$	531	ND	ND	ND	ND	0.62	10	3	3.3
DMHB	Demethylated HB	$C_{28}H_{16}O_9$	686	648*	0.469/EtOH	0.477	4.77	0.42	> 25	3–5	> 5–8.3
JL-1-1		**$C_{30}H_{36}O_{12}$**	**578**	**594**	**0.478/$CHCl_3$**	**0.062**	**0.62**	**0.72**	**> 70**	**2–4**	**> 18.5–35**

*Significant light absorption at 630 nm

photosensitizers were illuminated at a predetermined wavelength and the kinetics of the decrease in the DPA absorption peak of 374 nm were followed as $^1O_2$ was accepted by DPA.

Cellular uptake of photosensitizers

Standard curves of fluorescence yield vs concentration were prepared for selected compounds dissolved in 3.6 ml dimethylsulfoxide (DMSO) mixed with unlabelled EMT6/Ed cells which had been removed from Petri dishes with 0.4 ml 0.5% Tween 20 in 1 *N* NaOH. This procedure resulted in equivalence of the standard curves with the actual cell uptake assays. Cells labelled for 2 h with HB or one of the four selected congeners were rinsed three times with phosphate-buffered saline to completely remove excess drug, removed from Petri plates as described above, and added to DMSO. The washing procedure was monitored spectrophotometrically. The Tween 20-NaOH solution used to remove the cells from the plates resulted in complete cytolysis and extraction of the hypocrellins from the cells. Fluorescence was quantitated with a Spex Fluoro Max fluorimeter. The illumination and detection slit widths were set at 1 nm. The optimal excitation wavelengths were determined for each compound. HB excitation was 438 nm, HBBA-Rl, 411 nm; HBEA-Rl, 352 nm; HBDP-Rl, 410 nm; and JL-1-1, 594 nm. Uptake per 10^6 cells was estimated by regression analysis of the individual uptake curves, and is outlined below. A standard, 2-h incubation period was chosen to match the empirically chosen cytotoxicity/phototoxicity preincubation. Recent intracellular uptake kinetic experiments performed by fluorescence confocal microscopy demonstrate that intracellular uptake is complete within the 2-h incubation period.

Cell line and culture conditions

EMT6/Ed mouse tumor cells have been propagated in our laboratory for several years [2]. They are maintained as monolayer cultures in Waymouth's medium containing 12.5% fetal calf serum, at 37°C in a humidified atmosphere of 95% air and 5% CO_2. They require twice weekly transfers to maintain their exponential growth. A minimum of three times per year, the cell line is passaged as solid tumors in BALB/c mice. As previously described [27], newly passaged EMT6/Ed cell lines are reestablished and propagated for at least 2 weeks in vitro prior to experimentation. This procedure maintains the malignant phenotype.

Photosensitizers and clonogenic assays

Purified hypocrellin derivatives were obtained in lyophilized form. Exponentially growing EMT6/Ed cells (25,000/ml in 2.0 ml) were seeded in 3-cm tissue culture Petri plates 1 day prior to each experiment. The photosensitizers were kept in lyophilized form until the day of experiment, at which time they were dissolved in DMSO. Stock sensitizer-DMSO solutions were diluted with Hank's balanced salt solution and added to the cells in Waymouth's medium containing 12.5% fetal calf serum for the sensitization studies. The maximum concentration of DMSO in the incubation medium was 1% (v/v), and any effects of DMSO on cloning efficiency were controlled by a series of dishes containing the appropriate concentration of DMSO, but no sensitizer. Preliminary studies had indicated that the presence of fetal calf serum did not significantly influence toxicity. EMT6/Ed cells were exposed to graded doses of the test compound for 2 h, after which time the test compound was removed by repeated washing with Hank's balanced salt solution. The cells were then illuminated as monolayers with graded doses of 630-nm light in room air (*vide infra*). The cells were trypsinized, counted, and plated at a known density in Waymouth's medium. The cultures were incubated for 6 days and subjected to a standard clonogenic assay [25].

The dark toxicity characteristic of each compound was assessed separately following a similar procedure for cell exposure to graded doses of the photosensitizer for 2 h. Precautions were taken to avoid exposure of the cells to light throughout the period that they were exposed to, or contained, photosensitizer. Survival curves represent a minimum of three independent experiments. Data were corrected for plating efficiency and the phototoxicity curves were corrected for dark toxicity for each drug concentration, where necessary.

Illumination of the cell cultures

An argon-pumped tunable dye laser system (Coherent, Innova-20, Model CR599) with Kiton Red dye (Exciton, Dayton, Ohio) was used to generate light at 630 nm. The wavelength was verified with a power meter (Laser Therapeutics) prior to experimentation. The light beam was transported by a 400-μm quartz optical fiber, coupled to a microlens and oriented 9.3 cm above the cell culture dish. The output power was 150 mW, as measured with the same power meter. The microlens provided illumination of the cell monolayer, which was rotated to provide optimal uniformity. Illuminations were performed at ambient temperature (~23°C) and the longest exposure time was 1 min. The maximum dose at the surface of the cell culture was 1 J/cm^2.

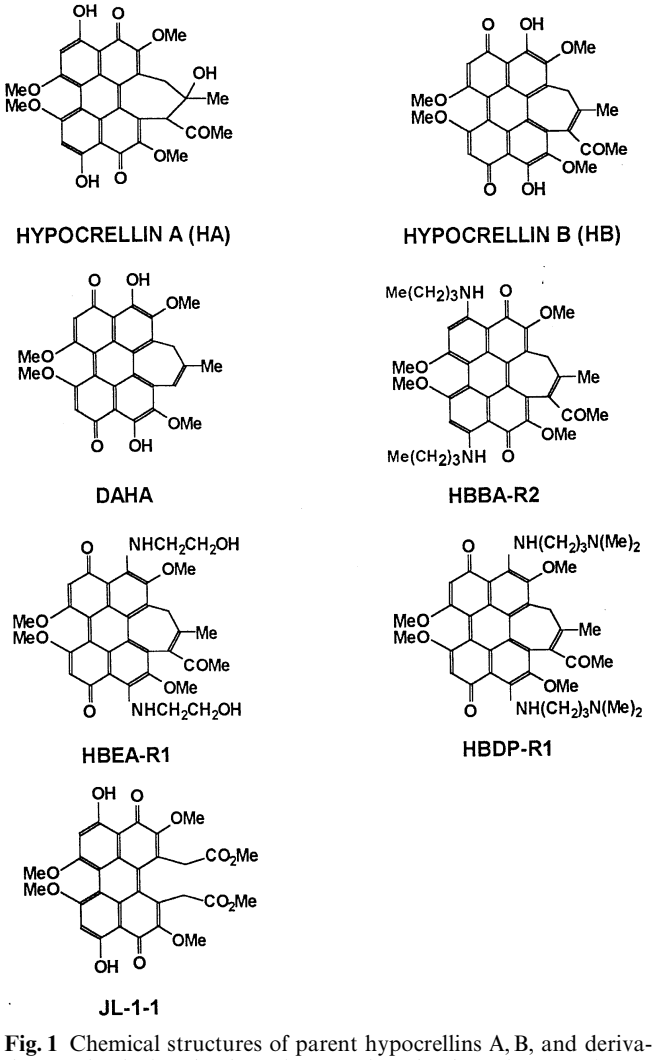

Fig. 1 Chemical structures of parent hypocrellins A, B, and derivatives, DAHA, butylaminated HB, ethanolaminated HB, 2-(*N*, *N*-dimethylamino) propylamine-HB, and JL-1-1. The phenolic hydroxyl groups of the parent compounds provide a convenient site for modification

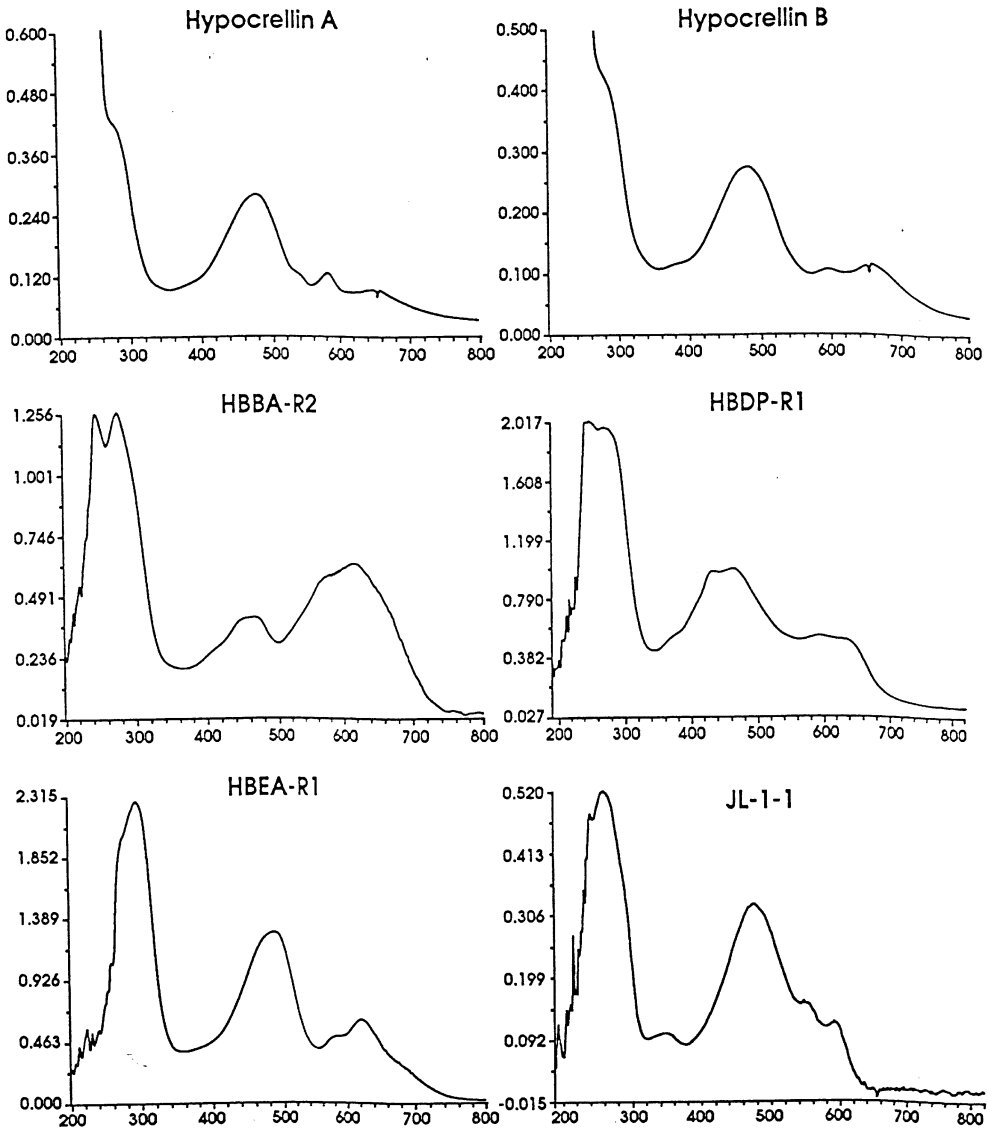

Fig. 2 Absorption spectra for HA (dimethyformamide), HB (dimethylformamide), HBBA-R2 (chloroform), HBDP-R1 (chloroform), HBEA-R1 (dimethylsulfoxide), and JL-1-1 (chloroform) (*ordinate*: absorbance, *abscissa* wavelength, nm)

Results

The physical and chemical properties of hypocrellin compounds synthesized and screened for toxicity in vitro are listed in Table 1. Compounds with exceptionally favorable characteristics of dark cytotoxicity and photopotentiation are indicated in boldface.

The average molecular mass of the hypocrellins and their derivatives is approximately 640 Da (range 528–780 Da). For perspective, this contrasts with a large molecular mass of approximately 1,130–4,520 Da for the mainly ester- and ether-linked oligomers of hematoporphyrin (two to eight porphyrin units) thought to comprise the bulk of Photofrin. The chemical structures of selected hypocrellins are shown in Fig. 1.

The hypocrellins used in this study each have absorption bands in the red spectral region. Compounds with significant absorption around 630 nm are highlighted with asterisks in the 'Absorbance peak' column of Table 1. Absorption spectra of the parent compounds, HA and HB, and of four efficient congeners that are photosensitizers, are illustrated in Fig. 2. It is interesting to note that the singlet oxygen yield (Table 1) is not strongly correlated with photopotentiation.

The sensitizer uptake studies were performed under incubation conditions identical to those used for clonogenic assays. The standard curves and cellular uptake data are shown in Fig. 3a and b, respectively. The ranges of photosensitizer concentrations required to exert 50% toxicity in the dark or light were estimated from survival curves for each photosensitizer. These values could be used to estimate the molar quantity of sensitizer absorbed, per 10^6 cells, required to affect 50% lethality. This was accomplished by

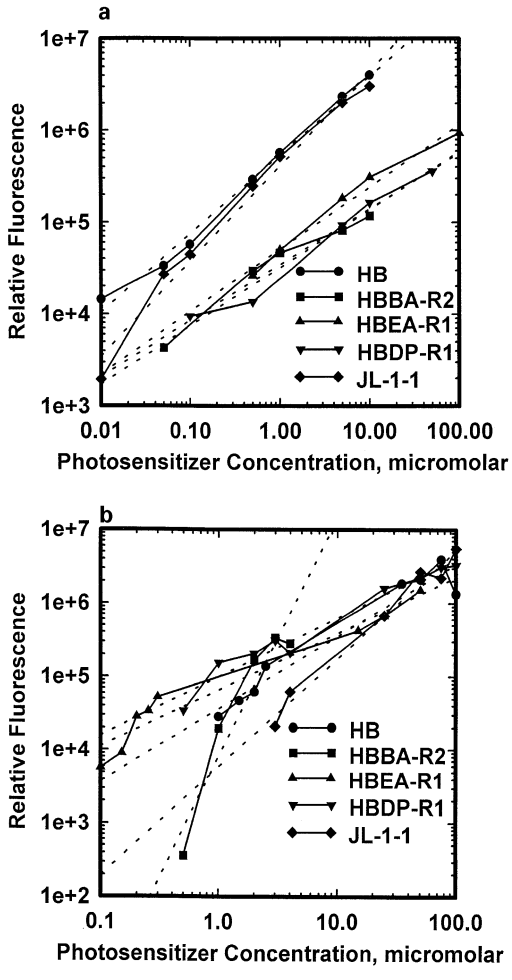

Fig. 3a, b Cellular uptake of selected photosensitizers (**a**) and standard concentration curves (**b**). Uptake by EMT6/Ed cells, adjusted to 10^6 cells (*ordinate* relative fluorescence at the appropriate detection wavelength (see text), *abscissa* concentration of photosensitizer, μM)

reading the relative fluorescence value per 10^6 cells, for the appropriate photosensitizer concentration LD_{50} value from Fig. 3b, and determining the corresponding molar concentration of photosensitizer from the standard curve (Fig. 3a). This value was corrected for the difference between cell volume and extraction volume. For HB, the LD_{50} value per 10^6 cells was 8 nmol, HBEA-R1, 120 nmol; and JL-1-1, 32 nmol. The administration of 630-nm light markedly reduced intracellular photosensitizer quantities required to achieve 50% cell killing. For light doses up to 1 J/cm^2, 160 pmol/10^6 cells of HB, or a 50-fold reduction in bound drug was required. HBEA-R1 was effective at 1 nmol/10^6 cells (120-fold less), HBDP-R1 at 11 nmol/10^6 cells, and JL-1-1 at 0.32 nmol/10^6 cells (a 100-fold photopotentiation of 50% lethality).

The properties of an ideal photosensitizer include low dark cytotoxicity, and excellent photopotentiation of cellular damage. Among the compounds tested, the concentration range for LD_{50} in the clonogenic assay ranged from approximately 10 μM to > 100 μM (mean, approx. 25 μM) . In the presence of 630-nm light, the LD_{50} photosensitizing dose of hypocrellins was reduced (0.15 to > 6 μM; mean, approx. 3 μM), in some cases substantially. HBBA-R2 demonstrated up to 500-fold photopotentiation in vitro, while HBEA-R1, HBDP-R1 and JL-1-1 were characterized by 167-, 50-, and 35-fold photopotentiation factors, respectively. The photopotentiation values based on clonogenic assays generally varied proportionately with drug uptake, for those compounds for which both values were determined.

Survival curves of the in vitro dark toxicity of four hypocrellin derivatives are displayed in Fig. 4a. Error bars represent the standard deviations of five replicate culture plates from three independent experiments for each compound. Each derivative had a characteristic cytotoxicity. HBBA-R2 and JL-1-1 did not evoke 50% lethality within the concentration range tested (≤ 80 μM). HBDP-R1 produced 50% cell death at concentrations in excess of 40 μM. Further studies are required to determine the precise value. HBEA-R1 was effective at about the 20-μM level. The present screening analysis for the properties of cytotoxicity and phototoxicity, represents a first step in the selection of promising hypocrellin congeners for further development. More rigorous determination of the LD_{50} values awaits completion of studies in progress (systemic toxicity and tissue distribution in vivo, and intracellular localization and uptake kinetics) in order to make informed choices of the most appropriate hypocrellin congener(s) to develop further. Graded doses of HBEA-R1 and HBBA-R2 have been administered to Balb/c mice, to a maximum of 31 and 39 mg/kg body weight (50 μM), respectively.

The excellent photopotentiation characteristics of HBBA-R2 were due to extremely low dark cytotoxicity on the one hand, and excellent sensitization, on the other (Fig. 4a, b). HBEA-R1 was another excellent photosensitizer, with a reciprocal drug-light dose response in the 0.15–0.3-μM, 0.25–1.00-J/cm^2 ranges (Fig. 4a, c). Data presented in Fig. 4d indicate that the LD_{50} for phototoxicity of HBDP-R1 lay in the 0.5–2.5-μM range, in the presence of 0.75 J/cm^2 630-nm light. Finally, the toxicities of JL-1-1 are represented in Fig. 4e. Again, there was a reciprocal relationship between drug and light dose, with excellent phototoxicity in the 2.0–4.0-μM drug concentration range.

Discussion

The basic structures of the parent hypocrellins render them amenable to site-specific modification [5]. A major advantage of hypocrellins as photosensitizers for PDT rests in their ability to be synthesized in pure, monomeric form. This feature significantly facilitates

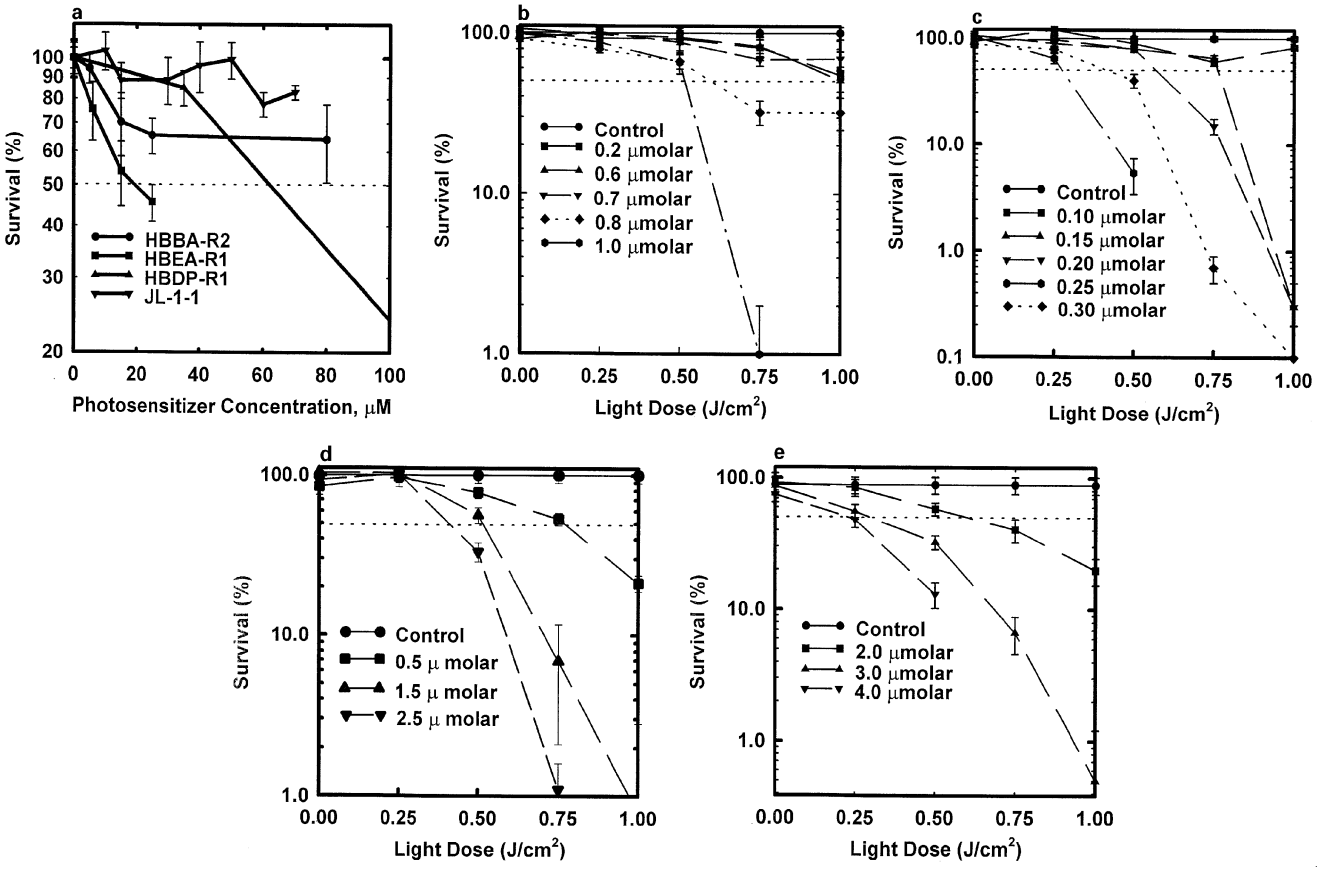

Fig. 4a–e Survival curves of cytotoxicity (**a**); (*ordinate* percent survival, *abscissa* photosensitizer concentration, μ*M*) and phototoxicity (**b–e**); (*ordinate* percent survival, *abscissa* light dose, J/cm^2) of hypocrellin sensitizers, determined by clonogenic assays of EMT6/Ed cells in monolayer culture. **b** HBBA-R2, **c** HBEA-R1, **d** HBDP-R1, **e** JL-1-1. Error bars represent standard deviations of five replicate culture plates

studies on phototoxic mechanisms in vitro; however a major advantage is foreseen in the simplicity with which pharmacokinetic studies may be executed. The low molecular mass should facilitate rapid distribution in tissues. The octanol–water partition coefficient is amenable to site-specific alteration, a feature which will expedite synthesis of a series of derivatives of graded hydrophobicity. For example, addition of the terminal hydroxyls and the quaternary nitrogen atoms in the sidechains of HBEA-R1 and HBDP-R1, respectively, may promote water solubility. This property will affect association with plasma proteins and lipoproteins, and therefore, the tumor and normal tissue distribution and kinetics of uptake and clearance. Some degree of selective tumor uptake might be achieved by modification of the pKa of the sensitizer, since the interstitial milieu of some tumors is more acidic than that of normal tissues [16, 24].

Butylamino substitution of the phenolic hydroxyls, yielding HBBA-R1, raised the extinction coefficient (ε) over sixfold compared with HB, with a concomitant enhancement of phototoxicity. This substitution resulted in a fivefold reduction in cytotoxicity, compared with HB. Ethanolamine substitution resulting in HBEA-R1 augmented ε to a similar degree, with attendant heightened phototoxicity. Additionally, 2-*N*,*N*-(dimethylamino) propylamination of the the phenolic hydroxyl of HB to yield HBDP-R1, resulted in a fivefold boost in ε, with heightened phototoxic activity. While these three modifications actually reduced singlet oxygen yield to varying degrees, enhanced photoxicity may relate to altered intracellular sensitizer distribution, with advantageous targeting of the phototoxic species.

Of more than 25 hypocrellin derivatives screened to date, all have shown acceptable levels of cytotoxicity in vitro. At least seven of the compounds photopotentiate by a factor of ten, with four possessing exceptional photopotentiating ability. Direct molar comparison of the hypocrellin concentrations required to effect a given degree of cytotoxicity or photopotentiation, with those for P-II is impractical, since the molecular mass of the photosensitizing component(s) of photofrin-II (P-II) is not clearly defined. Considering the low molecular masses characteristic of the hypocrellin derivatives, and their predominance as monomeric forms, we did not

deem it necessary to adhere to the tradition of longer drug preincubation times characteristic of some porphyrins. Preincubation kinetic studies confirm that varying the preincubation time for periods up to 24 h has no significant effect on the drug concentration required to exert 50% cytotoxicity or photopotentiation of cell kill. Recent fluorescence confocal microscopy studies indicate that for most compounds examined, uptake is complete within the 2-h incubation period. Kinetic studies are essential to avoid premature rejection of potential photosensitizing compounds.

It is our goal to improve and optimize the physicochemical properties (and ideally, cytotoxicity and photopotentiation) of promising hypocrellin derivatives, through successive in vitro clonogenic screening assays. This initial approach has been useful and efficient in estimating sensitizer concentration ranges required to effect cytotoxicity and photopotentiation. For this reason, the drug and light dose responses for the four compounds presented here are not comprehensive, but will be made when specific sensitizers are selected for further preclinical evaluation. We are performing concurrent in vivo studies in rodents to assess putative systemic toxicity and pharmacokinetic properties of selected compounds. Preliminary analysis of HBEA-R1 and HBBA-R2 indicates that these two compounds retain significant potency in vitro at 688 nm. Further work is required to determine whether the photopotentiation factor determined for LD_{50} values in vitro is upheld in tumor growth delay/TCD_{50} studies in vivo.

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