Towards Characteristics of Photodynamic Drugs Specifically Aimed at Microvascular Diseases

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Abstract: Photodynamic therapy (PDT) is a versatile methodology to treat various diseases but the drugs (photosensitizers) specifically aimed at individual character of diseases are urgently needed. Among those, the perilenoquinoid photosensitizers were thought of low PDT effectiveness to solid tumors due to low absorption on the "photodynamic window" but may be specially suitable for PDT to some microvascular diseases, such as age-related macular degeneration (AMD) and port wine stains (PWS), as well as other superficial diseases. Two strategies were discussed to convert the photosensitizers into clinically acceptable drugs in consideration of the drug-delivery and biological activity.

Keywords: Photodynamic therapy (PDT), Photosensitizers, Solid tumor, Microvascular disease, Target-related characteristic drug, Drug delivery, Biological activity, Drug preparation.

1. INTRODUCTION

Photodynamic therapy (PDT) has been a routine methodology to treat some tumors clinically besides operation, chemotherapy and radiation therapy. PDT is a much safer clinical treatment than chemotherapy or radiation therapy for selectively light-activating the drugs adsorbed on diseased targets previously while the drugs are inactive without irradiation. In photochemical basis, PDT effects depend on photo-generated chemically reactive species including free radicals and reactive oxygen species (ROS). Absorbing a photon, a photosensitizer molecule in ground state is promoted to the singlet excited state and further convert into the triplet excited state *via* the intersystem crossing. A triplet-excited photosensitizer in the lifetimes of microseconds to milliseconds may accept an electron from a biological molecule or donate an electron to an oxygen molecule to form the radical or ROS. Alternatively, it may transfer the excitation energy to a ground-state oxygen molecule to form singlet oxygen ($^1O_2$), the most important species for PDT effect [1]. In principle, the photogeneration of $^1O_2$ is a catalytic reaction, i.e., $^1O_2$ generated continuously without consumption of photosensitizer molecules, which is the molecular basis for PDT to be efficient. The chemical species are so reactive that they are ready to react with most of biological molecules. The high reactivity resulting in cell death or apoptosis is the molecular mechanism for PDT effect to treating various diseases [2], furthermore, the high reactivity means that the species having a short free diffusion distance in biological environment, no more than 100 nm [3], which is significant to restrict PDT effect exactly on the diseased targets illuminated selectively. Indeed, at least in principle, PDT is suitable for clinical treatment to almost all kinds of tumors. In the new century, PDT has got great success in treating some commonly occurred microvascular diseases, named as MVD for simplicity, such as age-related macular degeneration (AMD) and port wine stains (PWS). It should be indicated that the vascular endothelial growth factor A (VEGF-A) neutralization has been validated clinically, but it is only effective for one-third of the patients and may also negatively affect retinal function [4]. Further, formation of new vasculatures or the vasculature endothelial cells is not necessarily restricted to the cases of tumors or AMD but also involved in normal physiological vascular repairs [5], besides, "VEGF" strategy may prevent formation of new vasculatures but not remove those already exist. In contrary, PDT to AMD or PWS is selectively blocking and removing the proliferated microvasculatures but not hurting retina or normal tissue, which is obviously a better choice, at least in principle. At present, PDT drugs specially aimed to MVD are urgently needed. For clinical treatment to MVD, PDT targets are the microvasculatures, which are different from solid tumors in dimension, composition and microenvironment, therefore, the drugs should be specially aimed to the characteristics of diseased targets.

2. A PDT DRUG SHOULD BE SPECIFIC BUT NOT UNIVERSAL

In contrast to traditional operation, radiation and chemistry therapies, PDT is too superficial to treat large-sized solid tumors effectively due to not only limited penetration of light to tissues but also deficient supply of oxygen [6]. Clinically, PWS and AMD are classified as common-illness category and occur due to abnormal generation of microvasculatures in high density. PWS is vascular birthmark consisting of superficial and deep dilated capillaries in epidermis producing reddish to purplish discoloration with an

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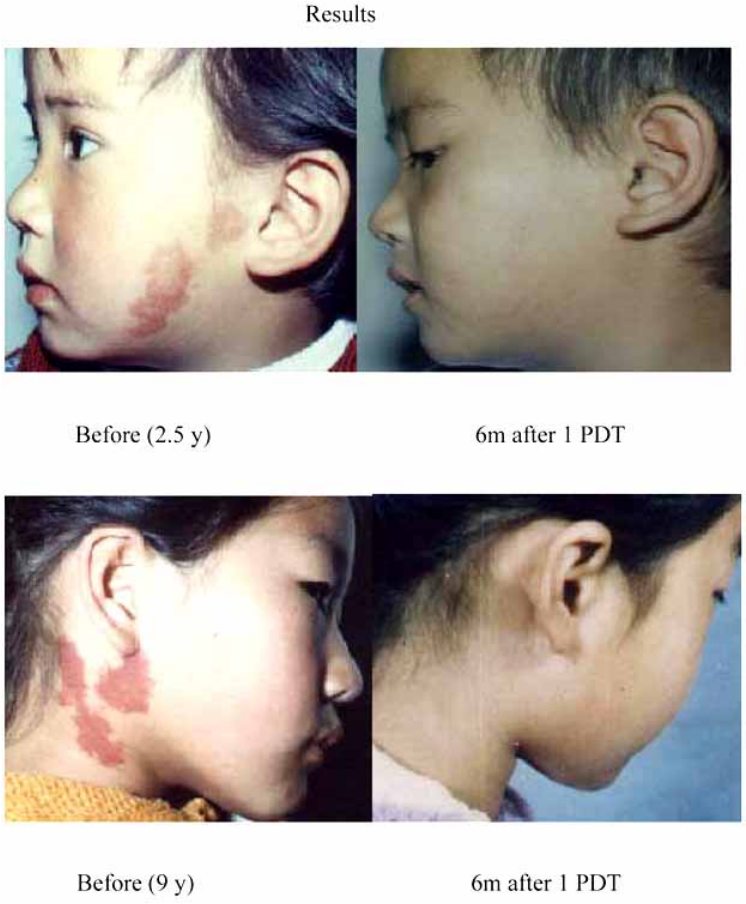


Fig. (1). Two PWS cases and PDT results after one-time treatment.

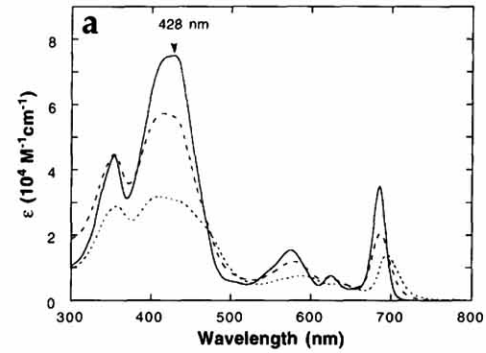
incidence about 3 to 5 among 1000 people [7]. In the past two decades, the laser department in Chinese PLA General Hospital achieved the non-scaring PWS treatment by the use of PDT and has successfully treated thousands of the cases, as selectively shown in Fig. (**1**). In contrast to the great success of the PDT methodology, clinically available drugs (hematoporphyrin-like photosensitizers) possess so low PDT activity that have to be used in a heavier dose (about 10mg/kg), higher laser intensity (100mW/cm, 532 nm) longer treating time (40 min).

AMD occur in a similar incidence with PWS and affects 30–50 million people in the world to result in permanent loss of the sight [8]. Commonly, PDT to MVD targets the vasculature endothelial cells to block and finally remove the abnormal microvasculatures. Besides what mentioned above, another significant difference between the targets for MVD and solid tumors is the supply of oxygen which is fundamental factor for PDT. Therefore, MVD is a suitable case for PDT treatment for the abundant oxygen supply. As mentioned above, "phototherapeutic window" (600 nm to 900 nm) was proposed for effective PDT to solid tumors, on the other hand, AMD and PWS belong to superficial diseases appeared on shallow surface no deeper than 1 mm, therefore, the red light penetrating into the tissue deeper is not only unnecessary but also harmful to the normal tissues. Besides, for PDT to solid tumors, the time intervals between the drug delivery and the illumination are usually taken hours or days to wait for a superior distribution of the drugs in tumor tissues [9, 10]. In contrast, the PDT irradiation is done almost immediately after the intravenous injection while much longer waiting leads to drug diffusion out of the vesculatures and results in a hurt to normal tissue [11].

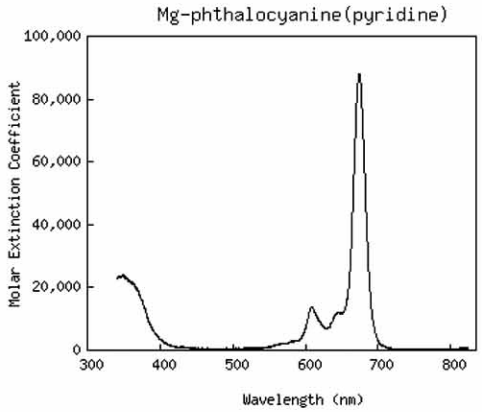
"Suiting the medicine to the illness" is a common knowledge but has not achieved for PDT yet. Generally, the molecular mechanism for PDT to various diseases is common, i.e., photogenerated free radicals and/or ROS reacting to biological molecules, cells and tissues, while, specifically, the difference of the diseased targets in composition, size and microenvironment requires a drug to be specific rather than universal. To obtain the maximum PDT effect, the drug (a photosensitizer) is required to have an absorption spectrum matching to the illuminating light. For several decades, the photosensitizers with strong absorption on the "phototherapeutic window" had been pursued for effective PDT to solid tumors because red light can penetrate into tissues deeper [12]. Currently, clinically available PDT drugs and the photosensitizers under investigation are mainly the porphyrin-, phthalocyanine- and perilenoquinone-like pigments. The first two groups possess PDT-interested absorption on a wavelength range from 600 nm to 700 nm (see Fig. (**1**)) [13-16] while the main peak at around 400 nm is less important for it may be directly absorbed by some biological molecules and lead to some nonspecific photochemistry reactions [17]. Perilenoquinoid pigments are a group of photosensitizers

different from porphyrins and phthalocyanines. Among these, hypocrellins and elsinochromes are most important for they are abundant in the natural fungus sacs of *hypocrella bambusae* and have been synthesized chemically or biologically [18, 19]. Hypocrellins and elsinochromes possess the super-high PDT activity, low dark-toxicity and fast metabolizing period [20-22], however, the absorption mainly on the range from 450 nm to 600nm (see Fig. (**2**)) is a drawback for PDT to solid tumors. For more than a decade, great efforts have been paid to improve the red absorption of hypocrellins [23-27]. However, even for the well-modified hypocrellin derivatives, the extinction coefficients of red light are about 10 times smaller than the phthalocyanines, which implies that hypocrellins are not competitive for PDT treatment to solid tumors.

Hypocrellins or the perilenoquinoid photosensitizers absorb the light mainly on a range from 450 nm to 600 nm which penetrating into tissues less than 1 mm is too superficial to give an effective PDT to solid tumors but just coincides with the depth of the diseased targets for PWS and AMD. Apparently, this family of photosensitizers is especially suitable for PDT to MVD or other shallow-surface



UV-vis spectra of BPDMA (Benzoporphyrill derivative monoacid) in methanol (__) , in PBS (__) and in PBS containing 20% methanol (...).



UV-vis spectra of Magnesium phthalocyanine (MgPc) dissolved in pyridine

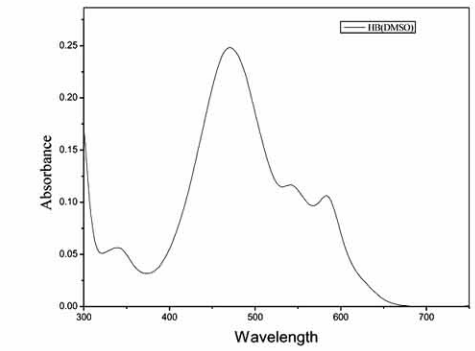


Fig. (2). UV-vis spectra of Benzoporphyrill derivative monoacid (a porphyrin derivative) (top), Magnesium phthalocyanine (middle) and HB (bottom).

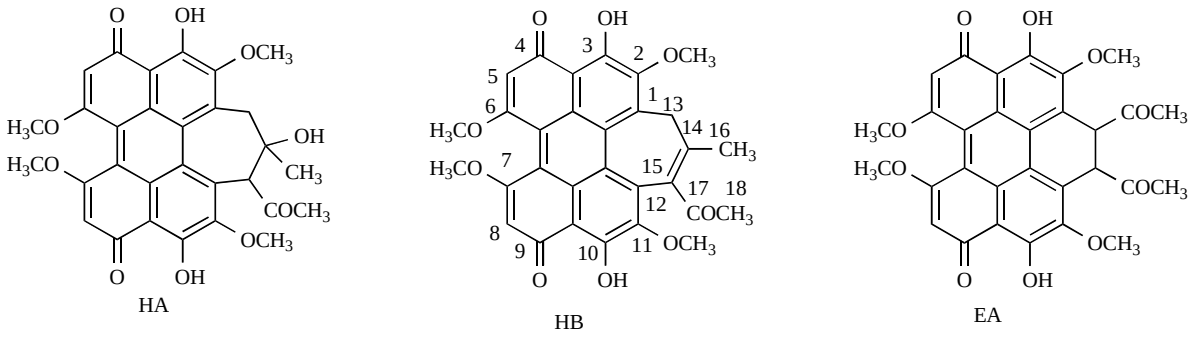


Fig. (3). The chemical structures of HA, HB and EA.

illness to restrict PDT effect precisely to the diseased targets. It is generally believed that green or orange light is more harmful to retina than red light, however, whether the retinal pigments are irreversibly bleached depends on not only the light frequency but also the intensity. In fact, the inherent high photosensitization activity, suggested by the singlet oxygen yields from 0.8 to 0.98 [28, 29], implies that less drug or light dosage may be used to minimize the side-effects. However, the safe and PDT-effective intensities for green or orange light (550nm to 580 nm) have to be quantitatively determined.

At present, the only drug approved for clinical PDT to AMD is benzoporphyrin derivative monoacid ring A (BPD-MA) possessing PDT-usable absorption peak at around 690 nm [30,31]. Clinically, it was indicated that the irradiation time for BPD-MA-PDT to AMD should not be longer than 80 seconds, which implies that the drug can only be used before it diffuses out of the microvasculatures. In fact, the time-limited illumination would unavoidably lead to a serious waste of the PDT effect. It was also noticed that a clinical use of BPD-MA to treat PWS caused permanent facial scar due to much longer irradiating time, suggesting an unwanted injure to dermal tissue (an unpublished result).

3. THE PERILENOQUINOID PHOTOSENSITIZERS AND THEIR BIOLOGICAL PDT EFFECTS

Among perilenoquinoid photosensitizers, hypocrellin A (HA) and B (HB) were intensively studied because they are the main components in the natural fungus. Further, HB is medicinally important for it can be prepared in much higher purity (≥99%) than HA which can be quantitatively transformed into HB [32]. In the perilenoquinoid family, Elsinochrome A (EA) is an important member for it not only possesses the highest singlet oxygen yield of 0.98, with 0.82 for HA or 0.76 for HB as a reference, but also has been synthesized artificially [28, 19]. Evaluated by the quantum yields of $^1O_2$ which is most important species for PDT, the perilenoquinoid pigments possess much higher photosensitivity than most of the photosensitizers [33,34]. The chemical structures for HA, HB and EA are shown in Fig. (**3**).

For PDT treatment to microvascular diseases, drugs have to be delivered to the targets, vasculature endothelial cells, *via* blood circulation. The diseased regions are more susceptible to PDT effect for the microvasculatures in abnormal high-density absorb much more photosensitizer molecules. After getting into the blood circulation *via* intravenous injection, drug molecules may be bounded to the vasculature endothelial cells, proteins or suspended freely in blood, and finally reaching an equilibrium distribution. The equilibrium shifts toward the diseased region under illumination to compensate the photosensitizer consumption, therefore, studies in molecular binding of photosensitizers to the drug-carrier biomolecules and transportation of drugs to the targets are necessary to get some clinical indications. In the molecule-level studies by using phosphate buffered saline (PBS, pH 7.4) to mimic blood plasma, human serum albumin (HSA) and polysaccharides to the drug-carriers and liposome to lipid membranes of the cells, it was demonstrated that the environment-sensitive fluorescence of HB could not only recognize its binding to a protein, polysaccharide or liposome [35-37] but also distinguish a specific from an unspecific binding [38-40]. These provide not only an insight into the molecular behaviors of photosensitizers in biological environments but also a possible method to monitor molecular transportation of the drugs.

PDT activity of HA or HB to vasculature endothelial cells were determined with hematoporphyrin derivative (HpD) as a reference, as shown in Fig. (**4**) [41]. It was confirmed that hypocrellins were far more PDT active than HpD.

Some conclusions were derived basing on experimental results on model animals for HB-PDT to MVD as shown in Fig. (**5**) [42-44]. Firstly, HB-PDT is highly effective, suggested by that the PDT effect for 1mg/Kg of HB irradiated for 5 min was equivalent to that of 10mg/Kg of hematoporphyrin monomethyl ether (HMME) for 40 min. Secondly, the dermal or retinal tissue was almost not injured even by the use of green (532 nm) laser due to far lower light intensity. Thirdly, suitable clinical dosage of HB was suggested to be less than 0.5 mg/Kg. It was reported that liposomal HA irradiated by the yellow laser (568 nm) was effective in occluding the choriocapillaris of pigmented rabbits [45]. These results suggest the potential of hypocrellins for PDT treatment to MVD.

Low dark-toxicity of hypocrellins is expectable for *Hypocrella bambusae*, a natural fungus, has long been used as an eatable food and folk medicine. Indeed, hypocrellins in escalated dose had no demonstrable systemic toxicity to rodents [46]. It was also observed that the rodents did not show observable poisoning reaction even under a dose of 100 times more than PDT dosage (an unpublished result). The

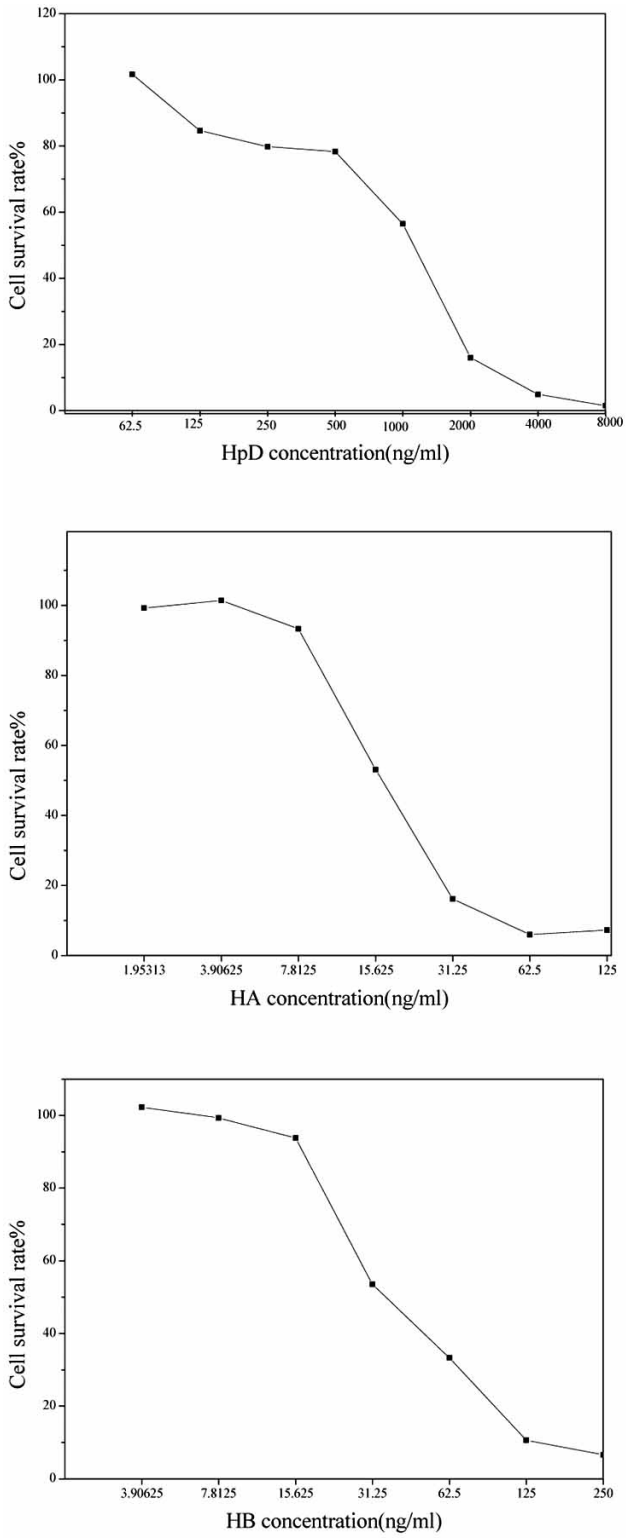


Fig. (4). Plots of the death percentage of vasculature endothelial cells to concentration of HpD (top), HA (middle) and HB (bottom) irradiated by copper laser (510.6 nm). The half-death concentrations are 1135, 17.87 and 41.91 ng/ml respectively.

metabolic period of hypocrellins in living animals was about 24 hours [47], far shorter than most of the photosensitizers, which is very important on clinical purpose for it greatly shortens the confinement of patients to a darkened room after PDT treatments.

4. TOWARDS CLINICALLY APPLICABLE DRUGS

For PDT to MVD, the drug-delivery requires a photosensitizer to be hydrophilic for safe transportation in blood circulation, while the biological PDT activity requires it to be lipophilic for cell uptake. HA, HB and EA are all liposoluble organic compounds to have high affinity to the target cell membranes, which benefits to PDT activity to the biological targets but resulting in the molecular aggregation may block vascular networks. The hydrophilic drug is safety for drug-delivery but lose the PDT activity *in vivo* due to the poor cell-uptake. To solve the problem, liposomal drug is a common strategy [48, 49], such as liposomal BPD-MA [50]. Liposome composed of amphiphilic phosphatides and cholesterols holds lipophilic drugs in the lipid bilayer but leaves the polar groups on out-surface. Thermodynamically, liposome of part-fluidic property is an unstable construction and ready to release the drugs in blood circulation, which is desirable for PDT to MVD but a problem for drug-storage. Therefore, a liposomal drug is usually dried to a solid. Besides, some water-soluble nanoparticles of hypocrellin-proteins or -polysaccharides were prepared with a similar PDT activity to the liposomal [51, 52]. The solidified nanoparticles are thermodynamically stable but too firm to release drugs quickly in blood, therefore, these preparations may be suitable for PDT to tumors but not to MVD. The oil-emulsion of hypocrellins has a narrow size distribution (40-60 nm) and higher PDT activity to the cells, but the storage-term is no more than 6 months [53]. All of the preparations have a good bio-compatibility but large amount of the photo-inactive biological materials unavoidably reduces the effective concentration of drugs. In fact, the drug concentrations in the preparations are no more than 1mg/ml, which is a crucial problem for volume-limited intravenous injection.

For more than a decade, several tens of hypocrellin derivatives have been synthesized to improve the water solubility, mainly by introducing one or two hydrophilic groups to HA or HB. Among these, sulfonated, di-glycoconjugated, dimercaptoacetic-acid- and cyclodexin-substituted hypocrellins as well as the metal chelates are completely water-soluble [54-57], but they lost PDT activity due to poor cell-uptake [58]. Some amine-, amino- or mercapto-substituted derivatives [59-61] did remain some of the PDT activity, but the water-solubility is too low to make an aqueous solution in clinically acceptable concentration. Previously, many publications announced obtaining the amphiphilic derivatives of hypocrellins, but the "amphiphilicity" has only qualitative significance. Taking consideration of the drug-delivery and PDT activity for medicinal purpose, it is focused to finding a compromise between hydrophilicity and lipophilicity, i.e., a quantitative instead of a qualitative criterion of the water-solubility which is just enough for making the aqueous solution in clinically acceptable concentration and arithmetically equals to the quotient of the personal dosage, depending on PDT activity, to reasonable volume for intravenous injection. In fact, the quantitative concept is not limited to hypocrellins only but possesses a common significance for lipo-soluble drugs. In fact, the low PDT activity of HMME is mainly ascribed to the unnecessarily high water-solubility.

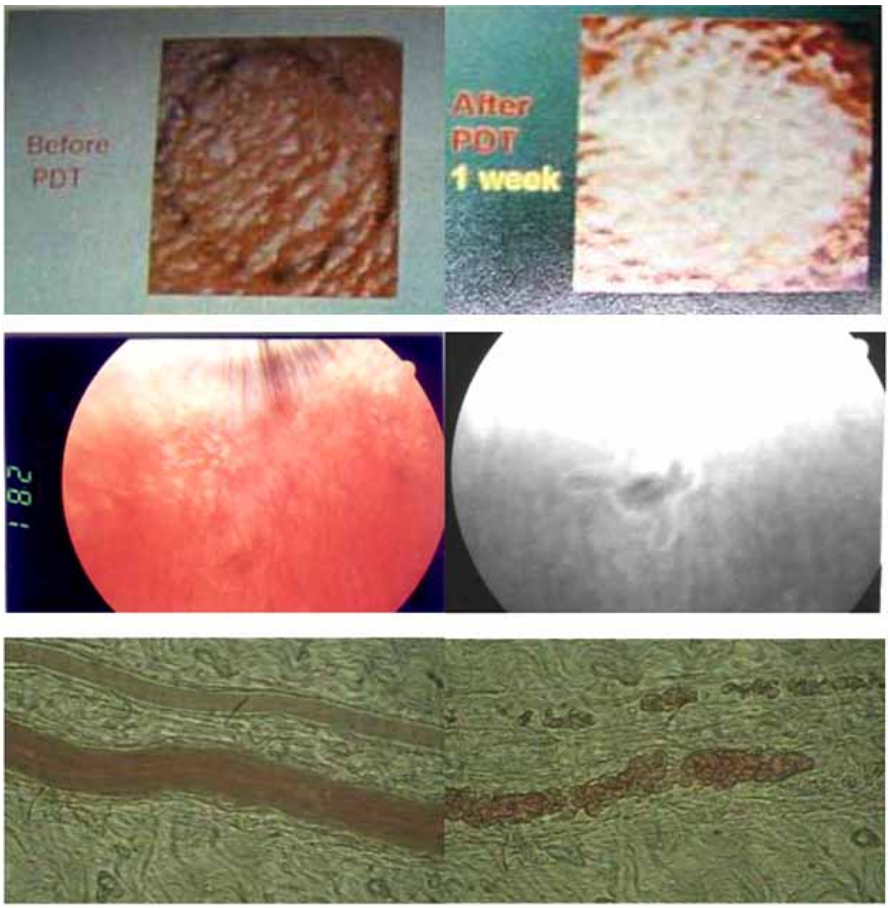


Fig. (5). PDT effects of HB (0.5mg/Kg) to cockscomb (top), eyeball of pigmented rabbit (middle) and choriocapillaris (artery and vein) of mouse (bottom), irradiated by copper or 532 nm laser.

As mentioned above, sulfonated hypocrellins are too hydrophilic to be PDT active to the target cells due to a strong inorganic acid attached. In comparison, taurine subtituted HB (THB) did retain some of the PDT activity for HB (about 10%) [62] because the 2-carbon-atom aliphatic chain in the substituting group somewhat reduce the molecular polarity. Following this consideration, 2-amino-propanesulfonic-acid-HB (APSHB) and 2-amino-butanesulfonic-acid-HB (ABSHB), with the aliphatic chain of 3 and 4 carbon atoms respectively, were synthesized [63]. The chemical structures are shown in Fig. (**6**).

As expected, with increase in the carbon-atom number, the maximum solubility for THB, APSHB and ABSHB forms a progressively decreased sequence, 9.2 to 7.5 to 4.2 mg/ml respectively, while the PDT activity a progressively increased sequence, as shown in Fig. (**7**).

Dependence of the water-solubility to the carbon atom number clearly depicts the significance of the quantitative or optimized amphiphilicity in Fig. (**8**) [64]. Based on PDT activity of the derivatives relative to HB and reasonable volume for one-time intravenous injection (less than 50 ml), it was estimated that the minimum acceptable solubility is 3.1

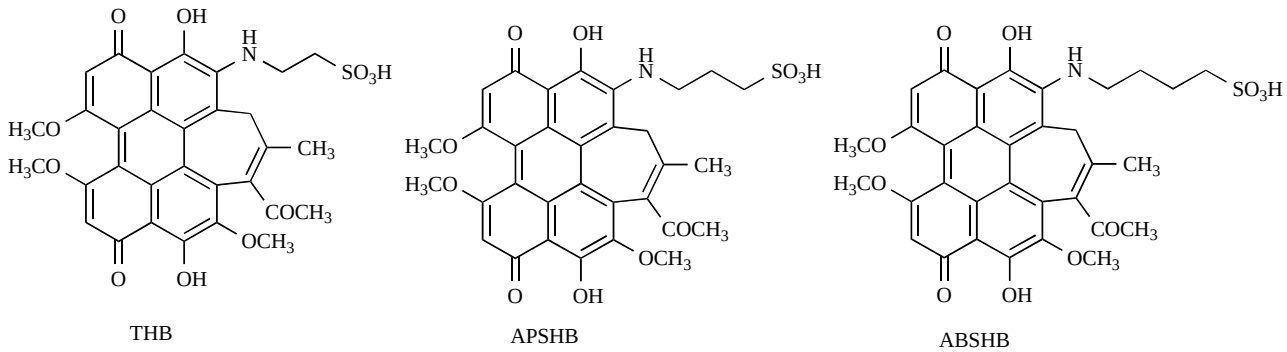


Fig. (6). The chemical structures of THB, APSHB and ABSHB.

mg/ml which is just located between 4 and 5 carbon atoms, i.e., ABSHB possesses the optimized amphiphilicity.

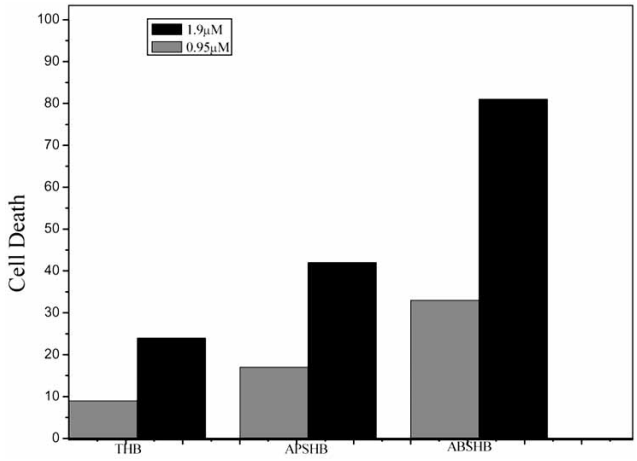


Fig. (7). Percentage deaths of A549 cells (5×10^4 cell/well) irradiated by a 532 nm laser of 20 mW/cm^2 for 1000 s in the presence of THB, APSHB and ABSHB.

Based on the PDT activity to A549 cells (data not shown), ABSHB remains only one third of the activity for HB, which is ascribed to lower $^1O_2$ yield (0.23) than HB (0.76). Hypocrellins may be structurally modified by introducing a substituting group to the site 5, 8, 2, 11, 13 and 17. Among these, the site 5, 8 or 2 substituted derivatives commonly have lower $^1O_2$ quantum yields, no more than 0.30 with 0.76 for HB as a reference [65-67], while the derivatives substituted at the site 13, 14 or 17 on the 7-member-ring possesses a similar or even higher $^1O_2$ yields than HB [68-70]. Singlet oxygen is generated *via* an energy transfer from the excited triplet state of a photosensitizer to an oxygen molecule in ground state, therefore, lower photosensitization activity for the derivatives substituted at site 5, 8 or 2 is ascribed to lower yields of the triplet state due to disturbing the perilene-ring structure, which was also suggested by a higher $^1O_2$ yield for the cysteine-substituted derivative at site 5 or 8 to form a new 6-member-ring which stabilizes the perilene-ring structure [71]. Furthermore, the $^1O_2$ yield for the derivatives substituted at site 2 by aliphatic amines is much higher than that substituted by a hydrophilic group [59], suggesting the effect of the substituting group on the intersystem crossing efficiency.

Based on analysis of the structure-dependent photosensitization activity, Schiff-base derivatives of HB substituted by amino-sulfonic-acids at site 17, 17-amino-propanesulfonic-acid-HB (NSHB) and 17-butanesulfonic-acid-HB (butSHB), were synthesized [72, 73]. The water-solubility for NSHB or butSHB is very similar to that for APSHB or ABSHB respectively, while the $^1O_2$ yield was significantly promoted, 0.72 and 0.94 for NSHB and butSHB respectively with 0.76 for HB as a reference. Biological experiments proved that NSHB and butSHB could remain 71% and 82% of the PDT activity for HB respectively, as shown in Fig. (**9**), which is a synthetical result of both the photosensitivity and the cell-affinity. For the plot of cell death to photosensitizer concentration usually takes an "S" shape, the concentration was adjusted to produce a cell-death in the linear region so that the relative activity was reasonable. Although PDT activity of the derivatives is somewhat lower than their parent HB, it is clinically acceptable for inherent PDT activity of HB is much higher than most of the photosensitizers. In fact, the biological PDT activity for butSHB (82%) or NSHB (71%) is higher than or equal to the liposomal HB (70%, unpublished result). Most importantly, the derivatives may be directly used for intravenous injection without need of further drug preparation.

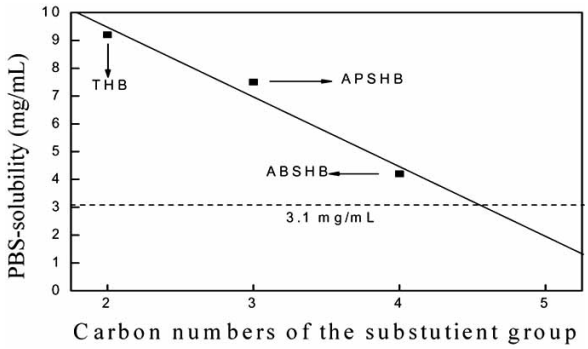


Fig. (8). Plot of water-solubility of derivatives to the carbon atom number in the substituting groups.

5. PROSPECT OF PDT DRUGS SPECIALLY AIMED TO MICROVASCULAR DISEASES

To get clinical indications for PDT treatment to MVD, some more investigations are necessary. After intravenous injection, the drugs (photosensitizer molecules) may be bounded to drug-carrier protein, such as HSA, or vasculature endothelial cells or suspended freely in blood, and finally to reach an equilibrium distribution which will continuously shift to compensate drug consumption on the diseased region under irradiation. Therefore, the binding (loading) and releasing (unloading) kinetics of drug molecules to/from drug-carrier biomolecules have to be investigated in molecular details, more profoundly, the time-dependent transportation of drug molecules to the targeted cells not only in molecular level but also on the model animals is key important for selecting a time to start PDT irradiation.

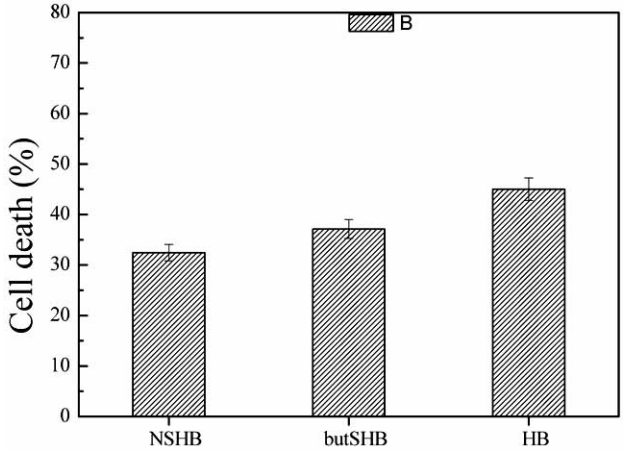


Fig. (9). Percent deaths of A549 cells irradiated with a 532 nm laser of 20 mW/cm^2 for 1000 s in the presence of NSHB, butSHB or HB (0.12 μM).

The photo-generated ROS is responsible for the PDT effect while may also oxidize the photosensitizers into photo-inactive products, named photo-bleaching. Photo-bleaching leads to waste of both ROS and photosensitizer, which is generally thought of a disadvantage. Specially, For PDT to MVD, photo-bleaching possesses a great importance for restricting PDT effect precisely onto the diseased targets - the abnormally proliferated microvasulatures. Obviously, PDT effect may be exactly restricted to the microvasulatures if photo-bleaching is kinetically faster than diffusing of drugs out of the vasculatures, which is especially important for PDT to the vital organs, such as eyes. In the consideration, the rates for photo-bleaching and the photosensitizer diffusing are important parameters for PDT to MVD, which has to be investigated not only *in vitro* but also *in vivo*.

MVD is most suitable illness for PDT because the targets are the oxygen-abundant microvasculature networks, therefore, PDT has a great potential to treat the commonly-occurred diseases. Besides, "antiangiogenic therapy" was also a strategy to treat tumors by inhibiting formation of new vasculatures providing nutrients for tumor growth [74−76]. However, angiogenesis is not only necessary for fast growth of tumors but also for physiological vascular repairs occurred commonly for an adult person. That is, tumors are not necessarily the only targets for "antiangiogenic therapy". Alternatively, it may be expectable that vasculature-targeting PDT is a more reasonable strategy because the laser irradiation is focused to the vascular networks to provide nutrients for tumors.

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ABBREVIATIONS

PDT = Photodynamic therapy

AMD = Age-related macular degeneration

PWS = Port wine stains

MVD = Microvascular diseases

ROS = Reactive oxygen species

BPD-MA = Benzoporphyrill derivative monoacid

HA = Hypocrellin A

HB = Hypocrellin B

EA = Elsinochrome A

PBS = Phosphate buffered saline

HSA = Human serum albumin

HpD = Hematoporphyrin derivative

HMME = Hematoporphyrin monomethyl ether

THB = Taurine subtituted HB

APSHB = 2-Amino-propanesulfonic-acid subtituted HB

ABSHB = 2-Amino-butanesulfonic-acid substituted HB

NSHB = 17-Amino-propanesulfonic-acid substituted HB

butSHB = 17- Amino-butanesulfonic-acid substituted HB

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