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A new Chlorin formulation promotes efficient photodynamic action in choriocapillaris of rabbit's eyes

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ABSTRACT

Age-related macular degeneration (AMD) as well as other choroidal diseases, demand novel therapeutic methods. Photodynamic therapy (PDT), which uses light and photosensitizer (PS) to cause specific vascular occlusion in the macula, is an interesting alternative. The only drug approved for the PDT treatment of AMD (Verteporfin) has a natural tendency to aggregate, demanding an expensive separation procedure during purification. We report a novel and affordable PS that is intrinsically protected against aggregation, the *Monomeric Chlorin at High Concentration* (*MCHC*-Chlorin), whose liposomal formulation was developed to provoke effective photodynamic action on the choroidal vasculature. Our report starts by stablishing the conditions to allow the efficient synthesis of *MCHC*-Chlorin in high yields (92%). We then tested the light stimulated occlusion of choriocapillary vessels in rabbit's eyes induced by the two MCHC-Chlorin isomers, which are directly obtained from the synthetic route. The PS formulation was infused in the rabbit's ear vein and eyes were immediately irradiated at 650 nm. Indirect ophthalmoscopy, fundus photography, fluorescein angiography and histopathological evaluations were used to evaluate levels of photo-thrombosis and collateral damage. Choriocapillary occlusion was achieved in all treated rabbits' eyes, while retina and sclera were completely preserved. There was no photochemical reaction in none of the eyes that received LASER without PS. Both MCHC-Chlorin isomers were separately tested and exhibited similar positive results with no systemic toxicity. Therefore, PDT occurred equally well in all treated eyes and none of the controls showed any effect in the ophthalmological exams. MCHC-Chlorin offers great potential and should be further studied as an alternative drug for choroidal diseases.

Increase in life expectance is causing an impressive escalation in the frequency of age-related macular degeneration (AMD), which is the most common cause of blindness in developed countries.[1] AMD is a multifactorial disease classified in two major classes: dry/atrophic and wet/exudative/neovascular. The former is more prevalent, but the latter has a poorer prognosis and has been associated with the majority of the cases of legal blindness.[2] Wet AMD is mainly caused by choroidal neovascularization (CNV), which causes a progressively loss of patient's visual acuity.

Generically speaking, the aim of the AMD treatment is to reduce vasculature in the choroid region preserving tissues in sclera and retina. The first FDA-approved treatment against this condition was Visudyne®-based Photodynamic Therapy (PDT, see below).[2,3] PDT uses photoactivable drugs (Photosensitizers) to produce toxic agents in the target tissue (Fig. 1A).[3,4]

Visudyne®, whose active principle is Verteporfin (benzoporphyrin derivative monoacid A), had tremendous success in the first five years of its launch.[3,5] It reduced blindness by half, with very small side effects. Direct-laser photocoagulation, which was the method of treatment used before Visudyne®, caused blindness by itself in a considerable fraction of the treated patients.[6] More recently, Verteporfin-based PDT has also been considered for other eye diseases, such as chronic central serous retinopathy, polypoidal vasculopathy and idiopathic retinal neovascularization.[7,8]

The method of choice to treat AMD-CNV is no longer PDT, ever since the introduction of the Vascular Endothelial Growth Factor inhibitors (anti-VEGF). This new class of drugs (made of antibody fragments) brought unquestionable long-term

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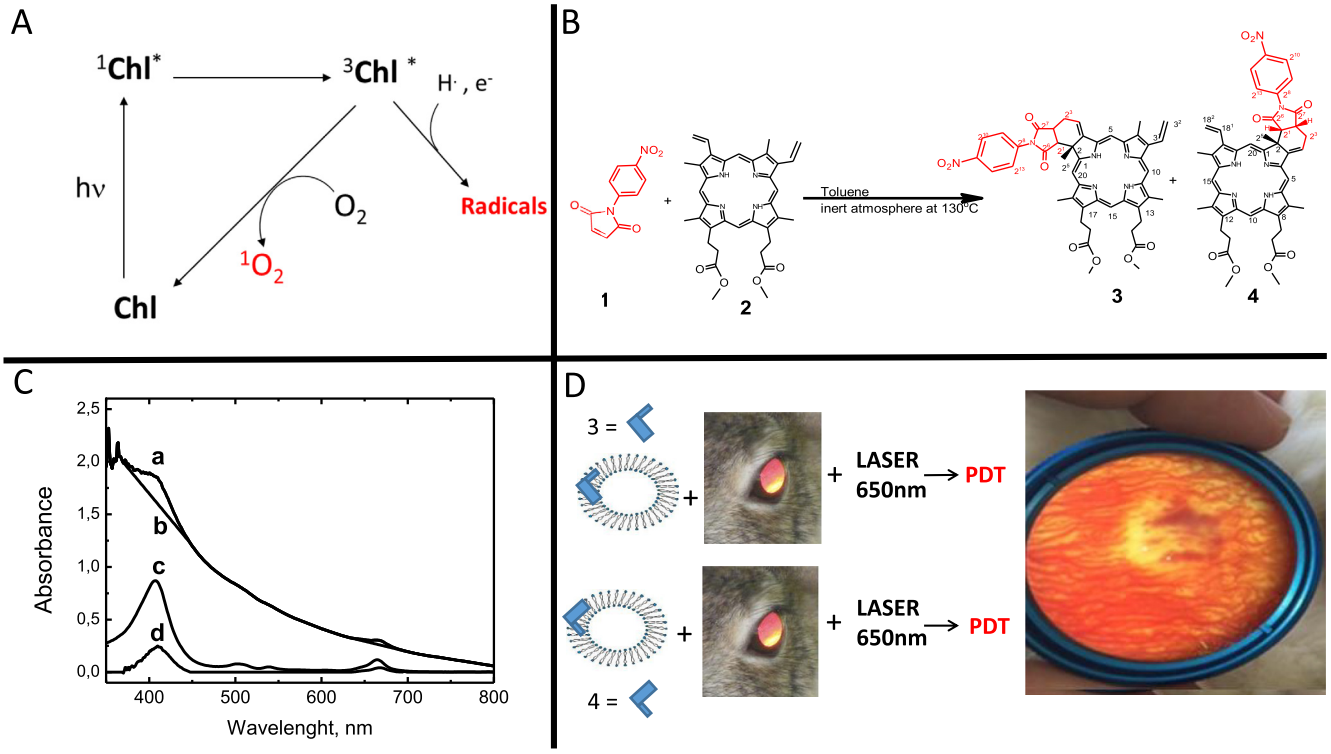

Fig. 1. (A) Photodynamic cycle of a generic photosensitizer like the Chlorin used in this work, showing light absorption, intersystem crossing to triplet, formation of radicals and singlet oxygen. These oxidizing species are short-lived and cause local tissue damage.[3,4] (B) Route for the chemical synthesis of MCHC-Chlorin. 1. p-nitrophenylmaleimide; 2. protoporphyrin dimethyl ester; 3. Chlorin (isomer A); 4. Chlorin (isomer B); (C) Absorption spectra of MCHC-Chlorin. (a) In liposome suspension (0,9% de NaCl); (b) baseline absorption of liposome suspension; (c) absorption spectra of MCHC-Chlorin in chloroform [~5 μM]; (d) baseline-subtracted absorption spectra of MCHC-Chlorin in liposome suspension. (D) Left: Experimental procedure to prepare liposome suspensions with either compound 3 or 4, followed by injection in the ear vein of rabbit's eyes and followed by irradiation with 650 nm LASER. Right: Photograph was taken using a 28D lens with the anesthetized animal immediately before retinography and fluorescein angiography, 1 day after irradiation. Picture shows the choroidal vessels and the sclera (white). We can also notice the hemorrhagic area that follows vascular occlusion. All eyes submitted to the PDT protocol showed these characteristic consequences of the photochemical reactions.

improvements in the condition of AMD patients.[9] VEGF inhibitors also brought real benefits to the pharmaceutical companies that no longer had to deal with light sources. However, not much chance for competition was given to Visudyne®, since the company that launched this new class of drugs in 2006 (Novartis) was the same that had the rights for selling Visudyne®. Nevertheless, VEGF inhibitors also cause difficulties for the AMD patients. Monthly injections of anti-VEGF drugs are extremely expensive and troublesome and there is a lot of variability in the patient responses.[10] We postulate that PDT still have an important role to play in the treatment of AMD. Besides Verteporfin, several other PS were reported to be effective in animal models of ocular neovascularization.[11–13] Interestingly, recent data suggest that Anti-VEGF drugs and PDT have synergistic effects, optimizing the overall vision of patients with AMD.[14]

The success of PDT relies very much in the efficiency of the Photosensitizer (PS).[3,4] Several features affect the performance of a PS, but aggregation is perhaps the property that most severely decreases its efficiency.[4] In fact, one of the reasons for the high cost of Visudyne® is the necessity to carry out a stereo separation of the active ingredient. Only one of the isomers formed during the synthesis of Verteporfin (Diester A) is active. The other (Dieste B), not only is inactive but also hinders the action of the first, by inducing its aggregation.[15,16]

We have recently developed the concept of l-shaped photosensitizers, which cannot interact by π-π stacking and consequently do not aggregate.[17,18] The molecule used in this work is the nitrophenyl derivative of this class of PS, which was nicknamed "Monomeric Chlorin at High Concentration" (MCHC-Chlorin), because it does not aggregate even at high concentration (Fig. 1B).[17] In the present study we exploit the potential use of MCHC-Chlorin PDT to the treatment of CNV. By using MCHC-Chlorin's liposome formulations photo-excited at 650 nm, we showed the effective targeting of choriocapillary vessels in rabbit's eyes, as well as, the integrity of neighbor tissues such as sclera and retina.

The synthesis of MCHC-Chlorin was described in our previous publications.[17,18] In here, we performed an optimization of conditions in order to maximize the yield and to facilitate purification. By varying the relative concentrations of maleimide and protoporphyrin IX, we observed that 20 equivalents in excess of maleimide to protoporphyrin IX provided the best general outcome. All of the maleimide excess was recovered during purification. We were also more careful with solvent preparation. Toluene was dried by refluxing with sodium metal, and oxygen was removed by previous degasification with ultrasound at 90 °C, and by flowing argon during the reaction. The efficient removal of water and oxygen must have helped to reduce side reactions. As a consequence, we obtained an impressive 92% yield of MCHC-Chlorin, based on the initial amount of protoporphyrin IX, which was significantly higher than the yield reported previously (67%).[17,18] It is important to comment that both A and B isomers (compounds 3 and 4 in Fig. 1B) are steric prevented from aggregating and thus photoactive. Consequently, there is practically no waste of reagent in this synthesis. In order to independently test their potency, isomers A and B were conveniently separated by thin-layer chromatography. Both isomers have the same photophysical properties. The Soret band has a maximum at 402 nm and an intense Q band at 666 nm, as described before (Fig. 1C).[17,18]

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