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Photodynamic efficacy of chlorin p6: A pH dependent study in aqueous and lipid environment

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ABSTRACT

Photodynamic efficacy of chlorin p6, a potential candidate of photodynamic therapy (PDT), has been studied at pH 5.0, 6.0 and 7.6 in aqueous and lipid environment. Increased chlorin p6 mediated photodynamic bleaching of *N*,*N*-dimethyl-4-nitrosoaniline (RNO), a measure of singlet oxygen yield, was obtained at higher pH. Rate of photodynamic bleaching of RNO was also higher at higher pH and the rate decreased with lowering in pH of irradiated solution. Photodynamic oxidation of tryptophan was also found to be higher at higher pH. Diminished oxidation of RNO was obtained with decrease in pH of irradiated solution. Both, RNO bleaching and tryptophan oxidation was significantly reduced by sodium azide, a known quencher of singlet oxygen. At lower pH, chlorin p6 mediated photodynamic malondialdehyde (MDA) and lipid hydroperoxide formation in egg lecithin liposome was higher. At higher pH chlorin p6 was found to be photodynamically more effective in aqueous environment whereas at lower pH chlorin p6 was photodynamically more effective in hydrophobic environment.

1. Introduction

Chlorins are receiving considerable interest as a potential drug for photodynamic therapy (PDT) of cancer and premalignant conditions. These molecules have strong absorption in the therapeutic window (650–900 nm), high singlet oxygen quantum yield, low dark toxicity and high tumor selectivity [1,2]. A number of investigations have been done on pH dependent photophysics of chlorin derivatives [3,4]. From spectroscopic studies it have been shown that at physiological pH chlorin p6 exists predominantly as negatively charged entity by dissociation of ionizable COOH groups (Fig. 1). With lowering of pH neutral form of the drug becomes dominant and results in aggregation [5]. Das et al. [6] has demonstrated from steady state and time resolve fluorescence studies that at physiological pH chlorin p6 is more hydrophilic and remains in aqueous region. With lowering of pH the photosensitizer becomes more and more lipophilic (hydrophobic) and possibly accumulates in lipid bilayer region. The authors attributed their results arising due to protonation and deprotonation of COOH groups of chlorin p6 with change in pH. Study has been done on pH dependent uptake of chlorin p6 in two mammalian cell lines, human colon adenocarcinoma (colo-205) and adenocarcinoma of breast (MCF-7) [7]. The results suggest that uptake of chlorin p6 by colo-205 cells were pH dependent and suggested to be due to endocytosis, while uptake of chlorin p6 by MCF-7 cells were by diffusion and therefore independent of pH [7]. These results do not necessarily suggest photodynamic efficacy of chlorin p6 at different pH. This is particularly important since, extracellular pH for a wide variety of tumors is significantly lower than the extracellular pH of normal tissue, whereas the intracellular pH of both the tissues is similar [8,9].

Photodynamic effect of a photosensitizer on biological structures is not only governed by its photophysical properties or simply uptake of the photosensitizer by cells, but also by the specificity of its interaction with important biomolecules. Localization of the photosensitizer in the tumor tissue is an important factor affecting the outcome of tumor destruction. Important biological macromolecules can have, both, hydrophobic and hydrophilic regions that may be differentially damaged by photodynamic action of chlorin p6 under different pH condition.

Present communication is intended to address how local pH affect chlorin p6 induced photodynamic damage to hydrophobic and hydrophilic biomolecules. Therefore, to avoid interference of other important biomolecules, that is present in natural system, this study has been done in model system i.e. liposome and tryptophan. Evaluation of pH dependent photodynamic efficacy of chlorine p6 in presence of other potentially interfering molecules may lead to incorrect interpretation of results. In the present study pH dependent photodynamic efficacy of chlorin p6 on hydrophobic and hydrophilic biomolecule at pH 5.0, 6.0 and 7.6 has been investigated with a view to know how chlorin p6 could damage hydrophobic and hydrophilic regions in cell under different pH condition.

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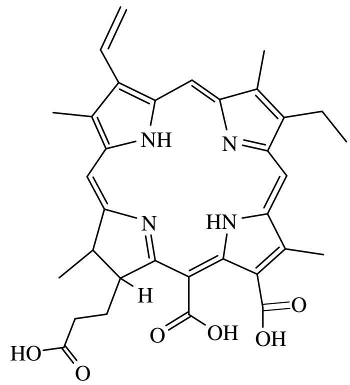

Fig. 1. Chemical structure of chlorin p6.

2. Materials and methods

2.1. Chemicals

L-Tryptophan, 2-thiobarbituric acid (TBA), imidazole, egg lecithin, xyelenol orange, butylatedhydroxy tolune (BHT) were from Sigma. *N*,*N*-dimethyl-4-nitrosoaniline (RNO) was from Fluka. Cholrin p6 was synthesized in house from spinach leaf. Method of preparation and its characterization was given elsewhere [5]. Other chemicals used were procured locally and were of the highest grade available.

2.2. Liposome preparation and tryptophan solution

Multilamellar liposome was prepared in water as described earlier [10]. Stock liposomal suspension was appropriately diluted in 25 mM Tris-base that was pre-adjusted to desired pH by Hcl. Stock tryptophan was prepared in water. Before each experiment tryptophan was diluted appropriately in 25 mM Na_2HPO_4 that was pre-adjusted to desired pH by adding 25 mM NaH_2PO_4.

2.3. Light source and irradiation procedure

Suitable aliquots of tryptophan or liposome was taken in plastic petridish (diameter 35 mm) so that depth of the liquid was not greater than 1 mm. The aliquot, in petridish, covered with lid, was then placed directly under two 20 W fluorescent daylight (TL 20 W/54, Philips, India) at a distance of 30 cm. At this point of irradiation fluence rate was 3.84 mW/cm^2 as measured by a laser power meter (Model: Lasermate; make: Coherent, USA). The irradiated solution in petridish was slowly shaken during irradiation. There was no measurable change in temperature of the irradiated liquid at the end of irradiation. Chlorin p6 of required concentration was added to tryptophan solution and liposomal suspension just prior to irradiation to visible light.

2.4. Assay of singlet oxygen yield

Chlorin p6 induced photodynamic generation of singlet oxygen was measured by the method of Kraljic and El-Mohsni [11]. Briefly, a solution containing RNO ($A_{440} \approx 0.8$), 10 mM imidazole and 2 μM chlorin p6 in 25 mM Na_2HPO_4 of stated pH was irradiated to visible light for different time period and at the end of each irradiation absorbance of irradiated and unirradiated sample was measured at 440 nm. In some cases sodium azide was added to RNO solution to attain a concentration of 10 mM just prior to irradiation.

2.5. Assay of tryptophan oxidation and lipid peroxidation

Stock tryptophan and chlorin p6 was appropriately diluted in NaH_2PO_4 solution of desired pH so that concentration of tryptophan and chlorin p6 became 4 μM and 10 μM, respectively. In some cases sodium azide from a stock solution was added to attain a concentration of 10 mM. Irradiated or unirradiated tryptophan was diluted 25 fold in 100 mM phosphate buffer pH 7.4 and tryptophan fluorescence was measured at 348 nm with excitation wavelength at 275 nm. Lipid peroxidation was assayed by measuring malondialdehyde (MDA) and lipid hydroperoxide. Malondialdehyde was measured by the thiobarbituric acid reaction following the method of Placer et al. [12] with suitable modification. Usually 1.0 ml liposome was mixed with 1.0 ml TBA reagent containing 0.53% TBA, 0.05% BHT in 7% perchloric acid and heated for 10 min. in boiling water bath. Absorbance of MDA-TBA adduct was measured at 532 nm after removing insoluble lipid with 0.7 ml 1 N NaOH and 2.0 ml chloroform. Lipid hydroperoxide was assayed by FOX2 method [13]. Briefly, 0.1 ml irradiated or unirradiated liposome added to 2.0 ml methanolic solution of BHT, xyelenol orange ammonium ferrous sulphate and H_2SO_4 so that final concentration of the reagents become 4.0 mM, 100 μM, 250 μM and 25 mM, respectively. Absorbance of the reaction mixture was measured at 560 nm after incubating the reaction mixture at room temperature for 1 h. Lipid phosphorous was measured by the method of Lelori and Cardini. [14].

3. Results and discussion

3.1. Chlorin p6 mediated photodynamic RNO bleaching

Photodynamic singlet oxygen yield of chlorin p6 at three different pH (5.0, 6.0, 7.6) was studied by measuring bleaching (decrease in absorbance) of RNO at 440 nm. With increasing time of irradiation bleaching of RNO was increasing almost linearly at all pH values used in the present study (Fig. 2). The highest bleaching of RNO was observed at pH 7.6 and the lowest bleaching was at pH 5.0 and at pH 6.0 bleaching was intermediate. Absorbance of RNO at 440 nm did not change (decrease) when RNO solution was irradiated to visible light in absence of chlorin p6. Chlorin p6 mediated photodynamic decrease in absorbance of RNO at 440 nm was suppressed by almost 90% when RNO solution was irradiated to visible light in presence of 10 mM sodium azide (Table 1). Sodium azide is a well known quencher of singlet oxygen [15]. Bleaching of RNO was thought to be a direct measure of singlet oxygen yield and was extensively used to estimate the photosensitized singlet

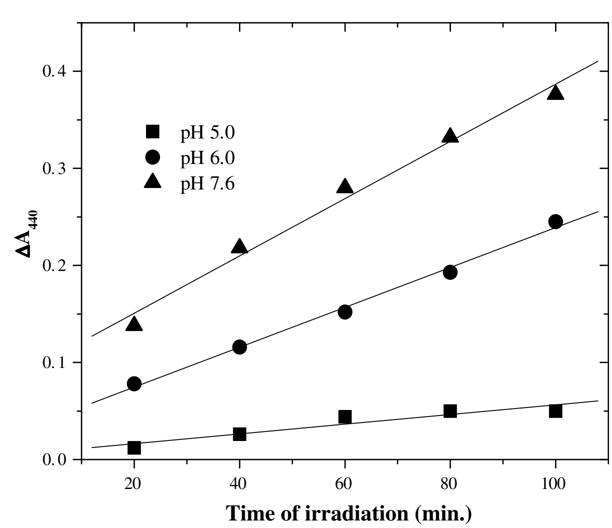

Fig. 2. Chlorin p6 mediated photodynamic RNO bleaching with time of irradiation to visible light at three different pH. Data points represent mean of three independent experiments. Standard deviations are too small to be represented.