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Comparative in vitro study of photodynamic activity of hypericin and hypericinates in MCF-7 cells

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ABSTRACT

In this work we present a comparative in vitro study of photodynamic activity between hypericin (HYP) and some hypericinates (hypericin ionic pair with lysine or *N*-methylglucamine) in human mammary adenocarcinoma cells (MCF-7). The toxicity and phototoxicity of hypericin and hypericinates were compared, as well as their cellular uptake and localization and mutagenic, genotoxic and clonogenic capacity. Our results demonstrate that different cationic moieties promote differences in the hypericinate solubility in a biological environment, and can influence the cellular localization and the phototoxicity of the photosensitizer. It was verified that hypericinates have better efficiency to generate singlet oxygen than HYP, and a lower aggregation in biological medium. In vitro assays have shown that HYP and the hypericinates are able to permeate the MCF-7 cell membrane and accumulated in organelles near the nucleus. The difference in location, however, was not determinant to the cell death mechanism, and a higher prevalence of apoptosis for all studied compounds occurred. The photodynamic studies indicated that hypericinates were more effective than HYP and were able to inhibit the formation of cellular colonies, suggesting a possible ability to prevent the recurrence of tumors. It also appears that all compounds have relative safety for mutagenicity and genotoxicity, which opens up a further safe route for application in in vivo studies.

1. Introduction

Photodynamic Therapy (PDT) is based on topical or systemic administration of a non-toxic photosensitizing dye. Photosensitizer (PS) can accumulate in diseased tissue due to the formation of complexes between the intravascular low-density lipoproteins and the compound, with a preferential accumulation occurring by neoplastic cells compared to healthy cells, due to the increased expression of lipoprotein receptors [1].

After accumulation in target cell or tissue, the photosensitizer is exposed to the vis3ible light with wavelength range of 650 nm to 850 nm, which is the range of greater light penetration into human tissue [2]. In presence of molecular oxygen or biomolecules, the photosensitizer is able to act by the mechanism type I or II for the production of reactive oxygen species (free radicals or singlet oxygen), causing cell injuries [3].

Photodynamic Therapy has important advantages compared to other anticancer therapies. In addition to being less invasive, directing the laser at the treatment region minimizes the side effects of treatment, providing less damage to patient's healthy cells. Moreover, repeated treatment doesn't usually promote cellular resistance mechanism, enabling the technique of repeating the same region necessary [4].

Several FDA approved photosensitizers are applied with excellent results against cancers, such as Photofrin® (Axcacan Pharma Inc.), Levulan® (DUSA Pharmaceuticals Inc.) and Metvixia® (PhotoCure ASA) [5]. Other compounds are under study, for example phthalocyanines, chlorins and perylene quinones derivative compounds such as hypericin (HYP).

Hypericin is a phenanthroperilene quinone present in Saint John's wort (*Hipericum perforatum*) [6]. It has the ability to generate singlet oxygen and other reactive species after irradiation with light at a wavelength range of 590–610 nm [7,8,9]. This compound is considered a potent photosensitizer, with a promising use in Photodynamic Therapy [6]. Despite its excellent photodynamic activities, hypericin is poorly soluble in physiological medium, limiting its use in medicine. In polar solvents, hypericin dissolves monomolecularly up to concentrations of 10^{-3} mol·L^{-1}, but in water or biological media it tends to form H-aggregates [10].

However, the hypericin solubility can be improved by the deprotonation of phenolic groups, which are located in bay and *peri* position,

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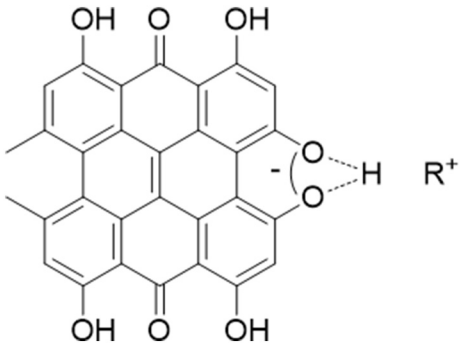

Fig. 1. Hypericinate structure.

with pKa values of 1.7 and 12.5, respectively [11,12]. Through the anion bay stability and under mild reaction conditions, it is possible to form a range of ion pairs (hypericinates) (Fig. 1) with better solubility [13,14] in biological media.

Considering this possibility, in this work we aimed to prepare some hypericinates to study the influence of the solubility in the photodynamic activity in human mammary adenocarcinoma cells (MCF-7). Through the preparation of hypericinates, our results demonstrate that this photosensitizer can present better solubility in a biological environment and maintain the good photochemical and photophysical properties, making these compounds good candidates for photodynamic application.

2. Material and Methods

2.1. Hypericin Synthesis and Hypericinates Preparation

Hypericin was synthesized according to Aigner and Falk [15]. Proto form was purified using silica column chromatography (elution with ethyl acetate-methanol 95:5, vol/vol, followed by elution of the compound with ethyl acetate-methanol 90:10, vol/vol)). After light irradiation, hypericin was obtained. Hypericinates were prepared according to Lavie et al. [13]. Briefly, 0.002 mol of hypericin was solubilized in 150 mL of acetone. Then, 5 mL of HCl solution at 7% were added, and the mixture was left still overnight, so as to occur the total precipitation of free hypericin. Precipitate was separated using centrifugation at 5000 r.p.m. during 5 min, yielding a brown colored solid. 1 Eq of free hypericin and 1 Eq of counter ions (lysine and *N*-methylglucamine) were solubilized in 20 mL of water and stirred overnight under the light. The products were lyophilized to remove water.

2.2. Absorption and Fluorescence Measurements

The emission area was obtained using Fluo 200 Time (Pico-Quanto, Germany). The measurements were performed with different concentrations (1.0, 5.0, 10, 25, 50, 100 and 200 $\mu mol \cdot L^{-1}$) of HYP, HYP-free, HYP-lys and HYP-glu in ethanol. The compounds were excited by a 574 nm diode, and emission was observed at 630 nm. Absorption measurements were carried out using Spectrophotometer UV/Vis Varian Cary 50 Bio (Varian, Australia). Fluorescence measurements were carried out using Varian Cary Eclipse spectrophotometer (Varian, Australia).

2.3. Relative Efficiency of Singlet Oxygen Production

To analyze singlet oxygen generation, 1.0 $\mu mol \cdot L^{-1}$ of hypericin or hypericinates, DPBF solution (1,3-diphenylisobenzofuran) with absorption between 0.4 and 0.6, and DMSO were placed in a cuvette and exposed to a light with wavelength of 590 nm. After switching on the LED, the absorbance values were obtained in 60 cycles of 3.6 s, when it was possible to determine the degradation rate of the DBPF. Dividing degradation rate of DPBF by absorption intensity of photosensitizer at the maximum wavelength, Re (relative efficiency) [16] of each compound is obtained. For higher values of Re it is said that the photosensitizers have a higher quantum yield of singlet oxygen compared to their standard. The Re is expressed according to the equation:

$$Re = \frac{\frac{W}{Iabs}}{\frac{Whyp}{Iabshyp}}$$

W is the DPBF degradation rate in presence of hypericinate;
W_{hyp} is the DPBF degradation rate in presence of hypericin
I_{abs} is the hypericinate light absorbing intensity at 600 nm
I_{abshyp} is the hypericin light absorption intensity at 600 nm

2.4. Evaluation of Hypericin and Hypericinates Aggregation Effect on Photodynamic Activity

To evaluate the influence of aggregation using aqueous medium, hypericin or hypericinates solution and RPMI culture medium used in MCF-7 cells culturing were added in a quartz cuvette, and an aggregation study was conducted at the same concentrations used in the mitochondrial viability assay (1: 100 v/v PS/culture medium). For each concentration, emission and absorption spectra were obtained.

2.5. Cell Culture and Treatment

MCF-7 cells were maintained in RPMI 1640 medium supplemented with 10% non-heat inactivated FBS and 1% penicillin/streptomycin. One day before the experiments, the cells were plated in 96 well microtiter plate with a density of 8.0×10^4 cells/cm^2 at 37 °C and 5% CO_2. On experiment day, the cells were treated with HYP or hypericinates with different concentrations (0.1, 1.0, 5.0, 10.0, 25.0 and 50.0 $\mu mol \cdot L^{-1}$) and incubated in individual microtiter plates for 2 h, 4 h, 8 h and 24 h, so as to evaluate toxicity. In phototoxicity assay, after incubation with 0.1 or 1.0 $\mu mol \cdot L^{-1}$ of photosensitizers, the cells were exposed to a light source.

2.6. LED Irradiation

A 590 nm LED with power density (E) of 32.0 $mW \cdot cm^{-2}$ (Biotable) provided by the Instituto de Física de São Carlos (Universidade de São Paulo), Brazil, was used to irradiate the cells. Cells were irradiated for 30 s, 1 min, 5 min and 10 min. The LED table covered the entire area of the culture dish (85.4 × 127.6 mm); this was achieved by irradiating culture dishes via LED spots right below the cell monolayer. In order to eliminate light interference, all irradiation protocols were performed in the dark.

2.7. Flow Cytometry Uptake

Cells were cultured, 12 h before the experiments, in 12 wells plates with a density of 8.0×10^4 cells/cm^2. 10 μL of treatment containing HYP or hypericinates were added. This quantity constitutes 1% DMSO in culture medium, which is the equivalent concentration of 1.0 $\mu mol \cdot L^{-1}$. The experiment's time started being counted by the time that the treatment was added to the first well. Every 5 min, the cells were removed by trypsinization, centrifuged at 1200 r.p.m. for 3 min, resuspended and washed with PBS-EDTA, followed by centrifugation 3 times. After the washing step, 50 μL of Carnoy fixer at 500 μL PBS-EDTA were added and the cells were left still for 10 min. After centrifugation, 500 μL of Carnoy were added and the cells were left still for 10 min. Finally, the fixer was removed by centrifugation, the pellet was resuspended in 200 μL of PBS-EDTA and then the cells were read on cytometer BD FACSCanto™ II.