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Research paper

Stability of different formulations and ion pairs of hypericin

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Abstract

Hypericin, solubilized in an instillation fluid consisting of an aqueous buffer supplemented with 1% plasma proteins, is currently used as a clinical diagnostic tool for the detection of superficial TCC (transitional cell carcinoma) tumors. However, the development of a sterile and stable hypericin stock formulation, excluding the presence of plasma constituents, would be an important factor in a more general clinical application of the method. Therefore, we investigated the stability of several heat sterilized hypericin formulations and ion pairs. Besides sodium hypericinate (in distilled water, in phosphate buffer, in polyethyleneglycol (PEG) 400), several other hypericinate salts (potassium, lysine, TRIS or hexylamine) were investigated. As to that, the physical appearance of different hypericin concentrates stored at 4 and 37 °C was investigated. Besides, after dilution into cell culture medium, the ability of hypericin remaining to accumulate in tumor cells and demonstrating photocytotoxic effects upon light irradiation was assessed. These findings suggest that PEG 400 is an excellent hypericin formulation, since it maintained the stability of the compound for at least 120 d when stored at either 4 or 37 °C. PEG 400 therefore is a suitable vehicle for the storage of hypericin prior to preparation of the bladder instillation solution.

Keywords: Bladder cancer; Formulations; Hypericin; Ion pairs; Stability

1. Introduction

Hypericin is a hydroxylated phenanthroperylenequinone that is present in a number of plants of the genus *Hypericum* [1,2]. Besides exhibiting a high fluorescence quantum yield [3,4], hypericin is a potent photosensitizer with promising photobiological activities [5–8]. Of particular interest, it was recently discovered that the compound becomes concentrated specifically in urothelial carcinoma lesions after instillation in human bladders. Consequently, the compound is currently used by urological groups as a clinical diagnostic tool for the detection of superficial TCC tumors [9–12]. Since hypericin is endowed with potent photosensitizing characteristics, the use of the compound for whole bladder wall PDT (photodynamic treatment) of superficial TCC malignant lesions has been suggested as well [13].

Deprotonation of hypericin is possible at the phenolic *bay* and *peri* groups, with pK values of 1.7 and 12.5, respectively [14–16]. The stability of the bay anion and the ease of ionization are due to the proximity of the hydroxyl groups that allows one hydrogen to be shared between two oxygen atoms, thus 1forming hydrogen bonds (Fig. 1). At physiological pH therefore, hypericin forms organic and inorganic monobasic salts [15,17,18]. Unexpectedly, these salts are practical insoluble in water and in all respects behave as lipophilic ion pairs that are closely associated. For instance, sodium hypericinate dissolve both in organic solvents producing highly fluorescent red solutions, and in phospholipid bilayers present in cellular membranes [18], resulting in fluorescent cells that can easily be visualized by endoscopy or fluorescent microscopy [9,10,19]. Conversely, in aqueous buffers hypericinate salts merely disperse as colloidal nonfluorescent high molecular weight aggregates that are no longer photodynamic active [18,20]. In the presence of albumin and plasma lipoproteins that adsorb

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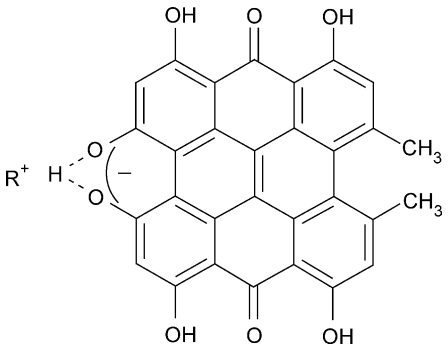

Fig. 1. Chemical structure of hypericin present as a monobasic anion in physiological conditions.

hypericin, these aggregates formed can redissociate resulting in fluorescent aqueous mixtures [18,21]. As a matter of fact, a strict correlation exists between the amount of non-aggregated hypericin and the fluorescence yield of the molecule in solution [20].

Prior to its use as a bladder diagnostic, hypericin is solubilized in an instillation fluid containing a stabilized solution of human plasma proteins (SOPP) (Red Cross, Brussels, Belgium) [9,10] to which hypericin adsorbs [18]. The amount of (lipo)proteins is known to critically determine the cellular accumulation of hypericin [7,22] and since the plasma protein preparation is not widely available, the composition of the actual instillation fluid renders a more widespread application of the diagnostic method difficult. To optimize the clinical application, an appropriate instillation fluid without plasma constituents is therefore critical. After sterilization by membrane filtration, these solutions can be kept for a few weeks at −20 °C without obvious stability problems. The availability of a sterile and stable preparation is of utmost importance for the clinical application of hypericin. A stable colloid or solution of hypericin in an aqueous buffer or biocompatible solvent without the need for plasma proteins that can be sterilized by heat would be ideal. As mentioned, a major obstacle originates from the formation of aggregates in an aqueous environment which depending on the conditions might precipitate as coarse particles. Different salts (ion pairs) of hypericin vary in some of their physical properties, including solubility in organic solvents and formation of dispersion in water. For instance, lysine hypericinate is 10× more soluble in water than sodium hypericinate [18]. Therefore it is anticipated that different ion pairs display a different tendency towards precipitation.

In this work, the stability of different hypericin formulations and ion pairs will be investigated. As to that, the physical appearance of different hypericin concentrates (300 μM) stored at 4 and 37 °C after heat sterilization was investigated for up to 120 d. Besides, after dilution into cell culture medium, the ability of hypericin remaining to accumulate in tumor cells and demonstrating photocytotoxic effects upon light irradiation was assessed.

2. Materials and methods

2.1. Synthesis of hypericin

Hypericin was synthesized from emodin anthraquinone according to Falk et al. [23]. Briefly, emodin (2.5 g), isolated from cortex Frangulae, was dissolved in 125 ml acetic acid and reduced with 5 g $SnCl_2 \cdot 2H_2O$ in 65 ml concentrated hydrochloric acid. After refluxing the mixture for 3 h at 120 °C, emodin anthrone was precipitated by cooling to room temperature. To prepare protohypericin via oxidative dimerization, 2.0 g emodin anthrone was dissolved in 44 ml pyridine/piperidine (10/1) and 4 mg of pyridine-1-oxide and 100 mg of $FeSO_4 \cdot 7H_2O$ were added. The reaction mixture was heated at 100 °C for 1 h under nitrogen in dark conditions. Protohypericin was precipitated in hexane and purified with silica column chromatography (mobile phase: ethylacetate/water with increasing amounts of acetone). A Sephadex LH-20 column (Pharmacia, Uppsala, Sweden) was used for further purification with dichloromethane, acetone and methanol as eluents. The compound was irradiated in acetone with a halogen lamp (500 W) to undergo an oxidative photocyclization reaction to hypericin ($\varepsilon_{EtOH,\ 592}$: 45.000 $M^{-1}\,cm^{-1}$). A 30 mM stock solution of hypericin (present as sodium hypericinate [24]) was made in dimethylsulfoxide (DMSO) and kept at −20 °C in the dark. The stock solution of hypericin was used afterwards to prepare the different hypericin concentrates (see further). All manipulations with the photosensitizer were performed under strictly subdued light conditions (<1 μW/cm^2).

2.2. Storage conditions and preparation of different hypericin formulations

Different hypericin concentrates (300 μM) were prepared in (a) distilled water (HyH_2O), (b) PEG 400 (20%) in distilled water (HyPEG20), (c) PEG 400 (HyPEG), (d) phosphate buffered saline (PBS) (Gibco-BRL, Paisley, Scotland) (HyPBS), (e) sodium phosphate buffer (10 mM Na^+) pH 4, pH 7 and pH 10 (HyNa pH 4, HyNa pH 7, HyNa pH 10), (f) potassium phosphate buffer (10 mM K^+) pH 7 (HyK), (g) lysine in distilled water (10 mM lysine) pH 7 (HyLys), (h) TRIS in distilled water (10 mM TRIS) pH 7 (HyTRIS) and (i) hexylamine in distilled water (10 mM hexylamine) pH 10 (HyHA) (Table 1). All concentrates were sterilized in capped glass vials by autoclaving (15 min at 121 °C) and stored at 4 or 37 °C in dark conditions.

Besides, a hypericin solution in SOPP (HySOPP) was prepared. This solution is presently used as the bladder instillation fluid for fluorescence diagnosis of bladder tumors in the clinic [9,10]. This solution was prepared by dissolving 5 mg of hypericin in 1 ml NaOH (0.1 N) and 2 ml PEG 400, followed by neutralization with 1 ml acetic acid (0.1 N). The mixture was then diluted with 33 ml of 4% SOPP and kept in the dark at room temperature for 30 min.