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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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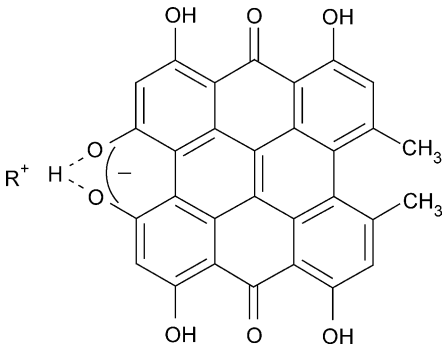

Fig. 1. Chemical structure of hypericin present as a monobasic anion in physiological conditions.

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.

Table 1
Different formulations of hypericin

	Hypericin formulations
HyH_2O	300 μM hypericin in distilled water
HyPEG20	300 μM hypericin in PEG 400 (20%) in distilled water
HyPEG	300 μM hypericin in PEG 400
HyPBS	300 μM hypericin in PBS
HyNa pH 4	300 μM hypericin in sodium phosphate buffer (10 mM Na^+) pH 4
HyNa pH 7	300 μM hypericin in sodium phosphate buffer (10 mM Na^+) pH 7
HyNa pH 10	300 μM hypericin in sodium phosphate buffer (10 mM Na^+) pH 10
HyK	300 μM hypericin in potassium phosphate buffer (10 mM K^+) pH 7
HyLys	300 μM hypericin in lysine in distilled water (10 mM lysine)
HyTRIS	300 μM hypericin in TRIS in distilled water (10 μM TRIS)
HyHA	300 μM hypericin in hexylamine in distilled water (10 mM hexylamine)
HySOPP	75 μM hypericin in SOPP (1%) in PBS

After sterilization by membrane filtration, the solution was further diluted with PBS to obtain a 75 μM hypericin solution in 1% SOPP. This solution is aliquoted for single use, kept at −20 °C in the dark and diluted in normal saline prior to use for each experiment after thawing.

2.3. Physical appearance and irreversibility of hypericin aggregation

The different concentrates stored at 4 or 37 °C were visually examined for physical change for up to 120 d. After this period, the concentrates were diluted a 300-fold in PEG 400 resulting in a final 1 μM hypericin concentration, and shaken for 2 or 24 h at 37 °C. After centrifugation, the fluorescence of the supernatant was measured by a microplate fluorescence reader (FL600, Bio-Tek Instruments, Winooski, VT, USA) with excitation and emission filters of 590/20 nm and 645/40 nm, respectively. HySOPP was diluted in normal saline (75 times) and examined in the same way.

2.4. Cell culture

RT-112, a human moderately differentiated non-invasive papillary TCC cell line was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cells were cultured as a monolayer in MEM (minimum essential medium) with Earle's salts containing 2 mM L-glutamine under 5% CO_2 at 37 °C. The medium was supplemented with 10% (v/v) fetal calf serum (FCS), 1% (v/v) non-essential amino acids, 1% (v/v) antibiotic/antimycotic solution (Gibco-BRL, Paisley, Scotland) and tylosin (60 μg/ml) (Eli Lilly, Brussels, Belgium).

2.5. Photocytotoxicity assay

RT-112 cells were seeded onto 96-well tissue culture plates at 5×10^3 cells per well and incubated for 24 h at 37 °C. The medium was then replaced with medium containing 10 μM hypericin prepared from the hypericin concentrates stored at 4 or 37 °C for various time periods up to 120 d. HySOPP on the contrary was diluted in normal saline (7.5 times) prior to application on RT-112 cells. After incubation under dark conditions at 37 °C for 2 h, the cells were washed twice with PBS and irradiated (in the presence of PBS) for 5 min using a 1 mW/cm^2 laser light. For irradiation, light emitted by a Rhodamine 6G dye laser (375B, Spectra Physics, Mountain View, CA, USA) pumped by a 4W Argon laser (Spectra Physics) was coupled into a fiber optic microlens (Rare Earth Medical, West Yarmouth, MA, USA) to obtain an ultra-uniform intensity distribution. The laser was tuned at 595 nm and the fluence rate at the surface of the tissue culture plate was measured with an IL 1400 radiometer (International Light, Newburyport, MA, USA).

After light irradiation, cells were further cultured for 2 days and the surviving fraction was determined with a MTT antiproliferation assay, which is based on the ability of mitochondrial enzymes to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma, Steinheim, Germany) into purple formazan crystals. For this purpose, the medium was replaced by a 1 mg/ml MTT solution in fresh medium, followed by incubation at 37 °C for 4 h. The MTT solution was then removed and replaced with 200 μl DMSO. The concentration of formazan per well was determined by measuring its absorbance at 550 nm using a microplate reader (FL600, Biotek, Winooski, VT, USA). The surviving fractions of the cells were calculated from three replicates. The controls consisted of cells that were irradiated in the absence of the photosensitizer.

2.6. Cellular accumulation

RT-112 cells were seeded onto Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL, USA) at 6×10^4 cells per well, and incubated for 24 h at 37 °C. The medium was then replaced with medium containing 1 μM hypericin prepared from the hypericin concentrates stored at 4 or 37 °C for 120 d or with 1 μM HySOPP diluted in normal saline. After incubation under dark conditions at 37 °C for 2 h, the cells were washed twice with PBS and examined by fluorescence microscopy (Axioskop 2 plus fluorescence microscope, Carl Zeiss, Göttingen, Germany) using a 535/25 nm band-pass excitation filter and a 590 nm long-pass emission filter. Fluorescence images were acquired using a light-sensitive charge-coupled device digital camera (AxioCam HR, Carl Zeiss). Rapid observation and electronic image storage avoided significant photobleaching of the hypericin-induced fluorescence in the cells.

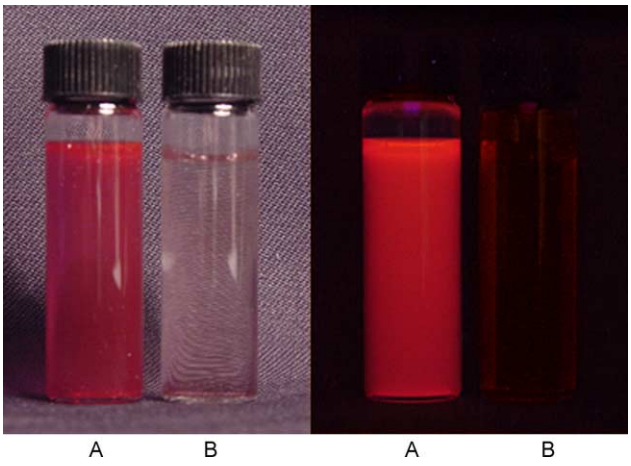

Fig. 2. The fluorescence of hypericin in different formulations. The left picture shows hypericin under normal room lighting in PEG 400 (A) and distilled water (B). The right picture shows the same vials observed under UV light ($\lambda = 365$ nm). Only in case pure PEG 400 was used as a solvent, fluorescence could be detected.

2.7. Statistical analysis

One-way ANOVA analysis with Tukey–Kramer post test using Instat software (GraphPad, San Diego, CA, USA) was performed to determine the significance of differences between the means. Significance was accepted at $P < 0.05$.

3. Results

3.1. Physical appearance of hypericin formulations and irreversibility of hypericin aggregation

The physical appearance of different formulations of sodium hypericinate (present in distilled water, 20% PEG 400 in distilled water, PEG 400, or phosphate buffer pH 4, pH 7 ($\pm$saline), or pH 10), or other hypericinate salts (in situ formed from sodium hypericinate by excess (10 mM) of K^+, lysine, TRIS or hexylamine present in distilled water) stored at 4 and 37 °C as concentrates (300 μM) was investigated for up to 120 d after heat sterilization. Only in case pure PEG 400 was used as a solvent, hypericin was completely dissolved exhibiting maximal fluorescence under UV irradiation. In all other cases, no fluorescence could be detected implying an aggregation of the molecule (Fig. 2).

Under normal light conditions, essentially the following intermediate or end situations could be distinguished: (A) the mixture looked clear without any visible particles present, (B) a fine precipitate was formed that could easily be resuspended by gentle shaking resulting into a cloudy appearance of the mixture, or (C) a coarse precipitate was formed which was difficult or impossible to resuspend. Table 2 gives an overview of all the results obtained, with day 0 representing the physical appearance of the mixture immediately after sterilization. When prepared in pure PEG 400, sodium hypericinate remained in solution at both temperatures, whereas in PBS the compound precipitated as coarse particles already during the autoclaving procedure. Depending on the storage time and temperature, the rest of the preparations showed variable results. No precipitation was visible when hypericin (sodium salt) was stored in distilled water up to 42 d at 37 °C, or up to 60 d at 4 °C. In TRIS buffer, the hypericinate solution remained clear for up to 2 weeks, after which a precipitate started to form both at 4 and 37 °C. In some cases the low temperature turned out to be beneficial for stability (e.g. HyH_2O and HyPEG20), while in other cases the higher temperature was favorable (e.g. HyNa pH 7, HyNa pH 10 and HyK).

The degree of fluorescence in a vehicle indicates the extent of hypericin present in its dissolved or deaggregated state [21]. To assess the level of irreversible aggregation after a 120 d storage at 4 and 37 °C, all concentrates were diluted a 300-fold in PEG 400 (dissolving hypericin excellently), and after centrifugation, the fluorescence of the supernatants was measured 2 h (Fig. 3) or 24 h after dilution. Fluorescence present in a freshly made hypericin

Table 2
Physical appearance of different formulations of sodium hypericinate or other hypericinate salts as a function of storage time and storage temperature (4 °C/37 °C)

	Day 0	Day 8	Day 14	Day 21	Day 35	Day 42	Day 60	Day 120
HyH_2O	A/A	A/A	A/A	A/A	A/A	A/A	A/B	B/B
HyPEG20	A/C	A/C	A/C	A/C	B/C	B/C	B/C	B/C
HyPEG	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A
HyPBS	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C
HyNa pH 4	B/B	B/B	B/B	B/B	B/B	B/B	B/B	B/B
HyNa pH 7	B/A	B/A	B/A	B/A	B/B	B/B	B/C	B/B
HyNa pH 10	B/A	B/A	B/A	B/A	B/A	B/A	B/A	B/A
HyK	B/A	B/A	B/B	B/B	B/B	B/B	B/C	B/B
HyLys	B/B	B/B	B/B	B/C	B/C	B/C	B/C	B/C
HyTRIS	A/A	A/A	A/A	B/C	B/C	B/C	B/C	B/C
HyHA	C/B	C/B	C/C	C/C	C/C	C/C	C/C	C/C

A, the mixture looked clear without any visible particles present; B, a fine precipitate was formed that could easily be resuspended by gentle shaking resulting into a cloudy appearance of the mixture; C, a coarse precipitate was formed which was difficult to resuspend.

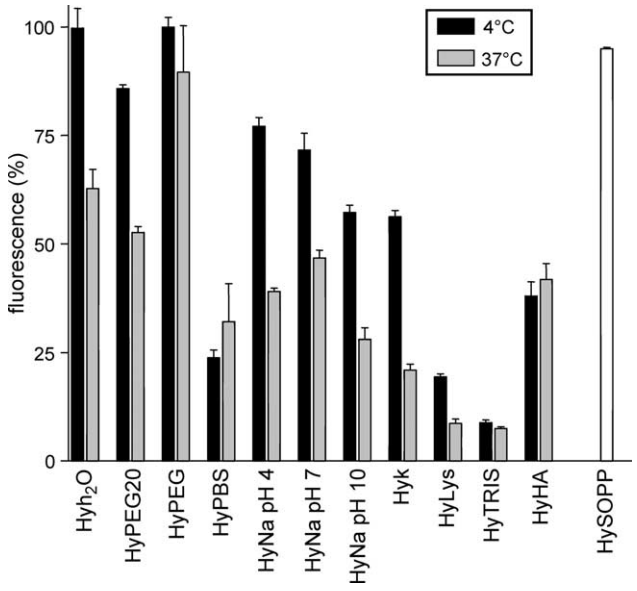

Fig. 3. Fluorescence of hypericin present in the different formulations after 120 d storage. Hypericin preparations were 300 times diluted in PEG 400 and the dilutions were kept at 37 °C for 2 h before quantifying the fluorescence with a microplate fluorescence reader. Fluorescence present in a freshly made hypericin preparation of PEG 400 was considered as 100%. The fluorescence in different preparations was evaluated as a function of the storage temperature (4 °C and 37 °C). Each value represents the mean (±SD) ($n=3$).

preparation of PEG 400 was considered as 100%. While HyH_2O, when stored at 4 °C, exhibited a similar fluorescence as found in case of HyPEG, the rest of the samples showed somewhat lower to significantly lower fluorescence levels after storage at this temperature (Fig. 3). With the exception of HyPBS and HyHA, the rest of the preparations also showed less fluorescence when stored at 37 °C. For instance, when stored at 4 °C, HyNa pH 4 and HyNa pH 10 exhibited 77.1 and 57.2% fluorescence, respectively, but their fluorescence levels declined by a factor two when stored at 37 °C. When the fluorescence recovered after dilution was analyzed 24 h later, HyLys showed an increase in fluorescence with a factor 1.3 and 2.7 compared to the 2 h condition for the storage at 4 and 37 °C, respectively. In all other cases, the fluorescence recovered 24 h after dilution was not significantly different from the 2 h condition. The fluorescence present in HySOPP stored at −20 °C was also investigated after dilution in normal saline to obtain a 1 μM hypericin solution. Similar fluorescence was found as in case of diluted HyPEG.

3.2. Cytotoxicity assay

The ability of hypericin remaining in the different concentrates to induce photocytotoxic effects was assessed on RT-112 tumor cells as a function of storage time and temperature by diluting the concentrates to a final 10 μM hypericin concentration in cell culture medium. When used immediately after preparation, but before heat sterilization, all hypericin formulations exhibited a 100% photokilling of RT-112 tumor cells. However, immediately after autoclaving, HyPBS, HyLys, HyTRIS and HyHA lost more than 75% of their photo-activity, while all other hypericin preparations remained 100% photocytotoxic.

The sterilized concentrates were then kept at 4 or 37 °C and their photocytotoxicity efficacy was evaluated as a function of time over a 120 d period (Fig. 4A and B). The results show that HyH_2O, HyPEG, HyNa pH 4 and HyNa pH 7 maintained their photo-activity at 4 °C even after 4 months of storage (Fig. 4A). Other hypericin formulations preserved their photocytotoxic content for a limited period of time, after which they gradually lost their photo-activity. For instance, after 42 d at 4 °C, HyK, HyNa pH 10 and HyPEG20 lost about 7, 45 and 70% of their photo-activity, respectively. Storing the samples at 37 °C, the results show that only HyPEG was still 100% photo-active after a 120 d period, whereas the rest of the formulations lost at least 75% of their photocytotoxic content (Fig. 4B). Indeed, HyH_2O, HyNa pH 4 and HyNa pH 7, which remained stable at 4 °C, exhibited a rapid decrease in their photo-activity under these higher temperature conditions as a function of time. For instance, after 42 d at 4 °C, about 58.7, 79.8 and 62% of their photocytotoxic effect was lost for, respectively, HyH_2O, HyNa pH 4 and HyNa pH 10. The photocytotoxicity pattern of HyPEG20, HyK and HyNa pH 10 was similar to that of HyNa pH 4.

HySOPP sterilized by membrane filtration and stored at −20 °C, remained completely active.

3.3. Cellular accumulation

The ability of hypericin remaining in the different concentrates to accumulate in RT-112 tumor cells after 120 d storage at 4 or 37 °C was studied using fluorescence microscopy. As to that, the concentrates were diluted to a final 1 μM hypericin concentration in cell culture medium (or normal saline in case of HySOPP) with which the cells were incubated for 2 h. Fig. 5 shows microphotographs of RT-112 cells after incubation with the diluted concentrates of HyH_2O, HyPEG and HyNa pH 10. The results show that when stored at 4 °C, HyH_2O, HyPEG, HyNa pH 4 and HyNa pH 7 exhibited at least 4 times higher fluorescence than the rest of the preparations. With the exception of HyPEG, the results also show that there was minimal fluorescence that could be detected in the cells when the preparations were stored at 37 °C. Fluorescence in RT-112 tumor cells, after incubation with HySOPP, exhibited a similar level as obtained after incubation with HyPEG.

4. Discussion

The technique using hypericin as a fluorescent diagnostic is highly sensitive and specific in detecting superficial bladder tumors in patients [9–11]. For that purpose,

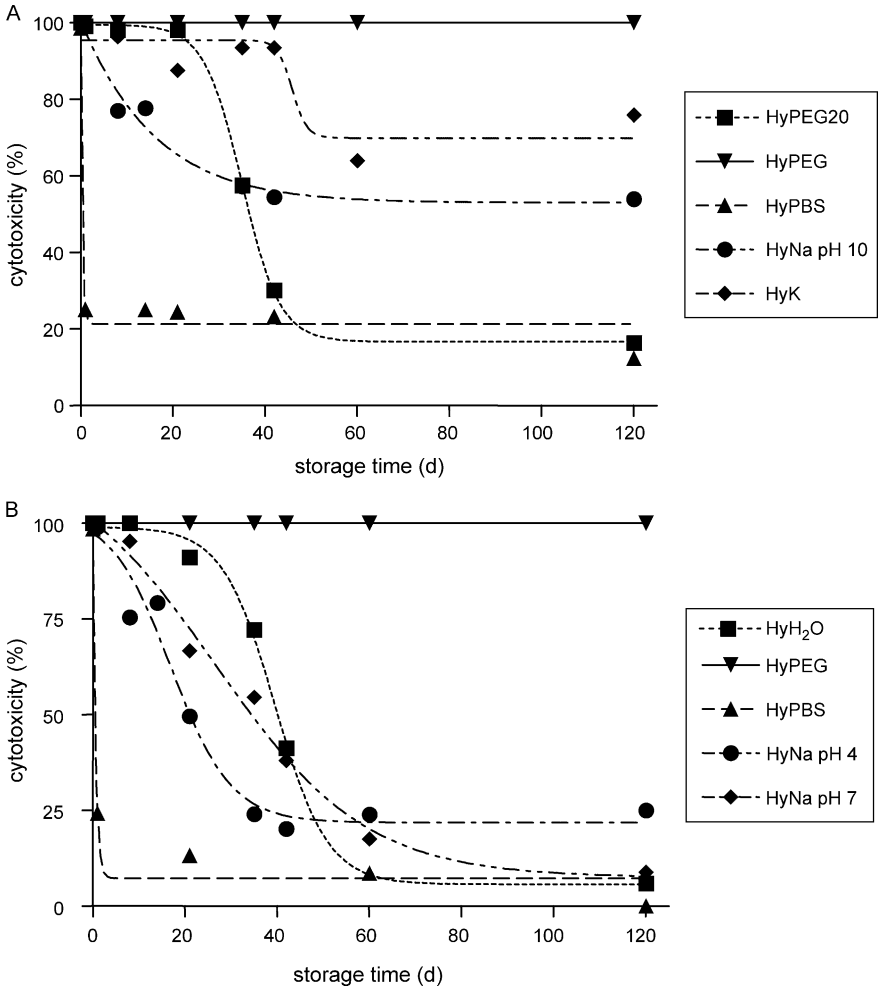

Fig. 4. Photocytotoxicity of the different hypericin preparations on RT-112 cells. Cells were incubated for 2 h with different hypericin preparation after a 30-fold dilution in medium and irradiated with 1 mW/cm^2 for 5 min at 595 nm. The photocytotoxic effect of the different preparations was then evaluated as a function of their storage time and temperature at 4 °C (A) and 37 °C (B). Data represent the mean of at least three measurements. In all cases, the coefficient of variation was <5%. For reasons of clarity not all data are shown. However, when stored at 4 °C, HyH_2O, HyNa pH 4 and HyNa pH 7 resembled HyPEG, while HyLys, HyTRIS and HyHA behaved similar as HyPBS with 75% loss of their photocytotoxicity after sterilization. When stored at 37 °C, the latter concentrates resembled HyPBS, while HyPEG20, HyNa pH 10 and HyK showed a pattern similar to the one of HyNa pH 4.

hypericin is solubilized in the presence of plasma proteins and as a sterile solution stored at −20 °C prior to bladder instillation. The objective of the present work was to find a vehicle that for the stability of the compound does not rely on the presence of plasma proteins and that after heat sterilization is suitable for the storage of hypericin for at least a few months. It is believed that such a vehicle further optimizes the clinical application for detection of superficial TCC tumors and will set off a more widespread use of the method.

As can be concluded from the fluorescent properties of hypericin in the different concentrates, only in case pure PEG 400 was used as a vehicle, the compound was present in its monomeric state, while in all other aqueous systems non-fluorescent aggregates were formed that were not visible to the human eye, or that appeared physically as a fine or coarse precipitate. Of interest, some formulations kept their initial post-sterilization physical state throughout the 120 d period, while others progressed from a non-visible to a fine, or from a fine to a coarse precipitate.

The physical form of a molecule present in a vehicle can be expected to influence substantially the kinetics of its cellular uptake and its subsequent biological activity. To induce a photocytotoxic response, hypericin should be present as a monomer, but not as an aggregate [18,20]. Since in the presence of albumin, plasma lipoproteins or cellular membranes hypericin aggregates are assumed to readily redissociate [18,21], it was anticipated that upon dilution in medium and exposure to cultured cells, all aqueous concentrates stored would result in a somewhat equal cellular uptake of monomerized hypericin. Since most aqueous vehicles lost increasingly their photo-active content during storage, especially when kept at the highest storage temperature, the photocytotoxicity data clearly show that this was not the case. It therefore can be assumed that newly formed aggregates undergo a stabilization

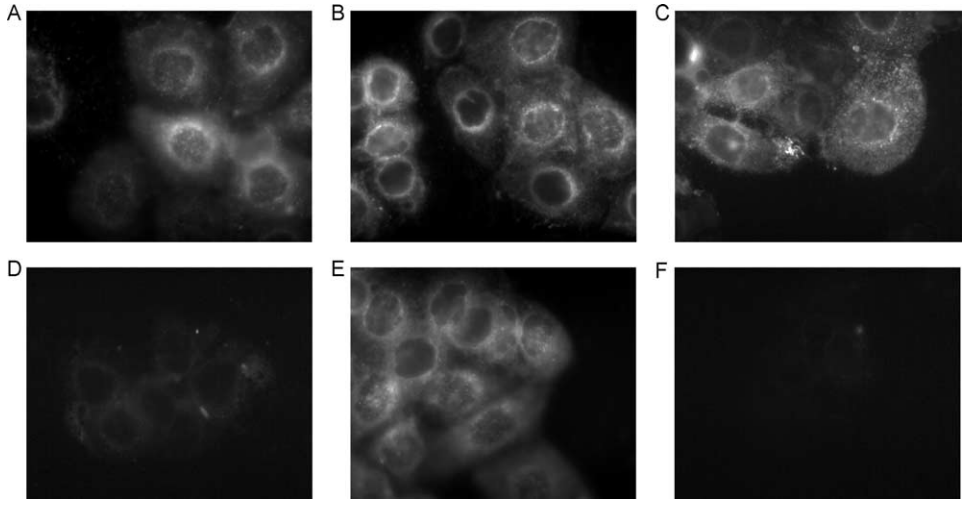

Fig. 5. Fluorescence photomicrographs of the cellular accumulation of hypericin in RT-112 cells. Cells were incubated with different hypericin preparations, stored for 120 d, after a 300-fold dilution in medium. The pictures represent the fluorescence of HyH_2O (A, D), HyPEG (B, E) and HyNa pH 10 (C, F) stored at 4 °C and 37 °C, respectively. Microphotograph A, B and E was taken with a less sensitive gain, resulting in a decrease of the fluorescence by a factor 4.

process as a function of storage time, a phenomenon likely accompanied by an increase in size and resulting in a enhanced resistance against monomerization, and that the kinetics of this progression heavily depends on the conditions (ionic strength, pH, size of counter ion) present. Significantly, a few formulations lost most their activity already during a short heat treatment. Conversely, featuring both hydroxyls and carbonyls, hypericin has been implicated in redox reactions in dark conditions [25,26], and it cannot not be excluded that within the tightly packed aggregates also chemical intermolecular modifications occur leading to inactive constituents or an irreversible aggregation state.

In general, a straightforward correlation between the visible appearance of the concentrates and their photocytotoxic effects was not found. This principle is well demonstrated by the samples that contained hypericin dispersed in water. For instance, when kept at 4 °C this concentrate exhibited during the whole storage period full photo-activity, and the change in physical appearance in the 60–120 d period did not seem to induce any effect. Conversely, in case the storage temperature was 37 °C, no fine precipitate could be observed in the 0–42 d period, even though meanwhile the concentrate lost gradually more than 50% of its photocytotoxic content. A similar situation can be observed with the HyNa pH 4 concentrate that invariably shows a fine precipitate, seemingly independent from its photocytotoxic content as monitored during storage at 4 or 37 °C. However, in other circumstances some relationship between the two parameters could be found, e.g. in case of HyPEG20 a rapid decline in photocytotoxicity is noticed in the 21–42 d period, coinciding with a change in the physical appearance, while on the other hand also all concentrates that showed a coarse precipitate (C status) consistently revealed low photo-activity.

Polyethyleneglycol 400 is known to be an excellent solvent for hypericin and therefore was used to investigate the extent of hypericin monomers that after the 120 d storage period still could be liberated from the aqueous concentrates. The results showed that some concentrates stored at 4 °C (e.g. HyH_2O, HyNa pH 4, HyNa pH 7), exhibited a high fluorescence, while others (e.g. HyLys, HyTRIS) showed a very low fluorescence when diluted in PEG 400. Since after the storage period these concentrates revealed a high and a low photocytotoxicity, respectively, a relationship between the reversibility of the aggregation and the photocytotoxic data seems to exist. This is further corroborated by the observation that, as compared to the concentrates stored at 4 °C, almost all concentrates that were kept at 37 °C released less fluorescence, and in general were endowed with less photo-activity. On the contrary, HyPEG20 concentrate stored for 120 d at 4 °C resulted in a high fluorescence when diluted in PEG 400, but exhibited a very poor photo-activity on the cells. This discrepancy may be explained by the different conditions in which the experiments took place. Indeed, PEG 400 does not resemble the lipoproteins present in medium supplemented with serum or the phospholipids present in cellular membranes, and in case of photocytotoxicity experiments only these components are present to monomerize hypericin dispersed in the aqueous concentrates. It was anticipated from these results that their potency to do so differed from that one of PEG 400.

Accordingly, the amount of hypericin-related fluorescence was investigated that was present in RT-112 cells after incubating them with the different concentrates stored for 120 d. As it turned out, all concentrates that remained 100% photo-active, exhibited at least a 4 times higher fluorescence as compared with the less photo-active preparations. It therefore was concluded that a high

fluorescence present in RT-112 cells, corresponding with a high cellular uptake of monomerized hypericin, resulted in a high cytotoxicity efficacy after light activation. The data further demonstrate that the method assessing the fluorescence present in concentrates diluted in PEG 400 cannot be used to predict the reversibility of the hypericin aggregation in the presence of cells.

In conclusion, the present work shows that the formulation of hypericin concentrates as well as the storage temperature can have a substantial impact on the photoactivity and cellular accumulation of the compound. All aqueous concentrates, even when kept at 4 °C, finally produced aggregates that only incompletely were able to release their hypericin content when applied to cells. It is anticipated that the same is true when solutions are made from the aqueous concentrates that are instilled in order to visualize malignant lesions in the bladder wall. In contrast when hypericin was dissolved in PEG 400, the compound did not form aggregates and was stable at either 4 or 37 °C. All experiments performed, showed similar results as the one obtained with the currently used clinical solution containing plasma proteins. Besides, LC-MS analysis of these hypericin formulations in PEG 400 revealed no deterioration products after 120 d storage. PEG 400 is therefore a suitable vehicle for the storage of hypericin. For instillation purposes, the concentrate can be diluted a 20-fold in an appropriate medium prior to application, decreasing PEG 400 concentration to a biocompatible level.

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