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How to make hypericin water-soluble

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Hypericin, isolated from *Hypericum perforatum*, is an effective photodynamic substance as demonstrated by various studies. Practical forms of applications of hypericin solutions for systemic use and introduction into body cavities are, however, lacking. We developed an aqueous solution of hypericin non-covalently bound to polyvinylpyrrolidone (PVP). PVP is a poly-*N*-vinylamide of various degrees of polymerization and forms of intermolecular crosslinks suitable for diagnostic and therapeutic applications. We used PVP (molecular weights of PVP between 10 kD and 40 kD) as a complex forming agent to prepare hypericin for photodynamic therapy and diagnostics. In pure water, hypericin forms aggregates which are non-soluble and non-fluorescent. The hypericin-PVP complex binds more than 1000 mg of hypericin in presence of 100 g PVP or less and is soluble in 1 liter of pure water. Aqueous complex solutions of hypericin-PVP display a characteristic absorption spectrum and fluorescence emission band around 600 nm wavelength. Varying concentrations of hypericin do not cause a blue- or red-shift in the absorption maximum at 595 nm. Excitation at 200 nm to 500 nm leads to emission at 590 nm; a property conducive to diagnostic investigations both *in vitro* and *in vivo*. Furthermore, hypericin-PVP exhibits high photostability in the presence of oxygen and broad band light which ensures reproducible photodynamic therapy and diagnosis. Conclusion: Hypericin forms liquid molecular chromophore complexes in water when bound to PVP thus allowing investigations in biological media.

1. Introduction

A new field of medicine has arisen over the last 25 years: the development of applications of various forms of selective photosensitization for treatment of malignant types of tissues (Dougherty and Marcus 1992; van Hillegersberg et al. 1994; Henderson and Dougherty 1992; Ochsner 1997a; Manyak et al. 1988). Two promising methods are: 1) induction of photophysical effects (e.g. light induced fluorescence) within tissues to perform photophysical diagnosis (PPD) or photodynamic diagnosis (PDD) and 2) induction of photochemical processes within tissues under the influence of light and oxygen to perform medical photodynamic therapy (PDT). Since 1992 the number of monthly presented scientific publications on PDD or PDT has been increased by a factor 3 (Medline, Current contents Life sciences) indicating the international interest in and increasing use of PDT as a successful form of tumor therapy. Moreover, several research programs have recommended PDT for treatment of non-malignant diseases such as vascular dysplasias or virus infections (Ochsner 1997a). Wide clinical use of PDT has been established mainly in the field of ophthalmology for the treatment of macular degeneration.

PDD and PDT involve interactions between a sensitizer substance administered and delivered to tissue substrates and light which must be introduced into tissue (Ochsner 1997b; Freitas and Baronzio 1991). Favorable sensitizers should be non-toxic after systemic application and should accumulate specifically within tumor tissue. Irradiation of the targeted tumor tissue using visible light induces photochemical reactions of type II and type I, both of which cause destruction of biomolecules, biomembranes and subcellular organelles (Ochsner 1997b).

Ideally, a photosensitizer should be water soluble for systemic application and introduction into body cavities. Hypericin isolated and purified from *Hypericum perforatum*, is an effective *in vitro* photodynamic substance which has been demonstrated by various studies (Diwu 1995; Van der Werf et al. 1996; Wynn and Cotton 1995; Vandenbogaerde et al. 1998; Falk 1999). Recent investigations have shown that photodynamic therapy using hypericin inhibits tumor growth successfully in various mouse tumor models via apoptosis and necrosis associated vascular damage (Falk and Mayr 1995; Kil et al. 1996; D'Hallewin et al. 2000). However, clinical applications of hypericin to investigate the photodynamic and phototherapeutic potential of hypericin are rare.

Hypericin is hydrophobic and nearly insoluble in water, oil, methylene chloride and most other nonpolar solvents but soluble in alkaline aqueous solutions, organic bases such as pyridine, and polar organic substances including acetone, ethanol, methanol, ethyl acetate, ethyl methyl ketone, and other solvents yielding red solutions with fluor-

escence emission maximums at about 600 nm and excitable in broad band regions from UV up to 590 nm (Wynn and Cotton 1995; Wirz et al. 2001).

We have developed an aqueous solution of purified hypericin non-covalently bound to polyvinylpyrrolidone (PVP) first mentioned 2000 (Kubin et al. 2000a). PVP is a poly-*N*-vinylamide with various degrees of polymerization and forms of intermolecular crosslinks suitable for diagnostic and therapeutic applications. We used PVP as a complex forming agent for the preparation of a photosensitizer for photodynamic therapy and photophysical diagnostics. In this preparation, hypericin is linked to two or more linear PVP chains with hydrogen bonds. PVP nanoclusters bearing hypericin serve as a hydrophilic carrier facilitating drug delivery (Uzdensky et al. 2003). The present work summarizes the most significant photophysical properties of water soluble hypericin-PVP complex.

2. Investigations, results and discussion

Hypericin is poorly soluble in pure water (Senthil et al. 1992). Hypericin fluorescence spectra in pure water do not show emission bands in red regions (Liebes et al. 1991) and its extinction coefficient is significantly decreased (Wirz et al. 2001). In water, hypericin exists in the form of dispersed aggregates (Liebes et al. 1991; Malkin and Mazur 1993). It has been suggested that aggregates of hypericin molecules possess a different molar extinction coefficient than monomolecularly dissolved hypericin (Falk and Maier 1994). Although hypericin forms aggregates in the presence of water, it dissolves in a monomolecular manner in polar organic solvents at concentrations of up to 10^{-3} Mol/L. In pure methanol, for example, 37 mg/L (0.07 mMol/L) of hypericin is soluble; upon addition of pyridine (1% v/v) to methanol, this increases to 320 mg/L (0.61 mMol/L) of hypericin (Wirz et al. 2002).

These properties hinder the preparation of drugs for clinical application. Due to the insolubility and non-fluorescence precipitation of hypericin in aqueous solutions, investigators have used 1% plasma protein solutions to ensure a monomolecular distribution of hypericin (D'Hallewin et al. 2000). Although this might be useful for applications into hollow organs such as the bladder, it is rather critical for intravenous administration. Because of the potential risk of transmission of infectious diseases, albumin may not be suitable for medical applications. Furthermore, plasma proteins are neither inert nor biologically inactive and could possibly be metabolized into products causing unwanted side effects. Therefore we developed a new substance combination in which hypericin is complexed to polyvinylpyrrolidone (PVP).

In the presence of PVP, hypericin forms liquid molecular chromophore complexes in water thus allowing the preparation of a dried and stable powder for diagnostic and therapeutic applications. PVP is a nontoxic polymer which is used in preparations of plasma substitutes and synthetic lacrimal fluid and is also a popular pharmaceutical adjuvant. It is favorable for medical applications because it avoids the application of tissue-irritating organic solvents which were previously used for dissolving pure hypericin.

The aqueous complex solution of hypericin-PVP displays a typical absorption spectrum. Figure 1 shows the time-course of water solubility of hypericin in the presence of PVP25 at room temperature. We refer to 'Rel. Absorbance' as the absorbance at a defined wavelength after zeroing the instrument using the matrix of the reaction mixture (identical solvent) in both optical paths of our 2-beam spectrophotometer.

Previous studies have shown that heating the solution to 70 °C does not affect stability of the complex but instantly accelerates solubility as evidenced by a significant augmentation of absorbance at the maximum of 595 nm (data not shown). Absorption at the corresponding maxima correlates with complex-concentration. These facts demonstrate the presence of an optimal chromophore solution without aggregation or microdispersive distribution.

Figure 2 shows a comparison of the absorption spectra of native hypericin in methanol and three different hypericin-PVP-complexes: PVP10, PVP25 and PVP40. The complex solutions of hypericin-PVP show typical absorption spectra. Remarkable is a red-shift of the maxima from 589 nm (non-complexed hypericin in methanol) to 595 nm (Hyp-PVP25 and Hyp-PVP40) or 596 nm (Hyp-PVP10) respectively.

Thus, our studies show that hypericin complexed to PVP is highly soluble in water and largely maintains its spectroscopic properties. The aqueous complex solution of hypericin-PVP exhibits a characteristic absorption spectrum.

Figure 3 shows a corrected 3D scan, excitation spectrum and emission spectrum of hypericin (5 μMol/L) in methanol. A thin emission band at 600 nm is visible upon excitation at wavelengths from 200 nm to 600 nm. The emission

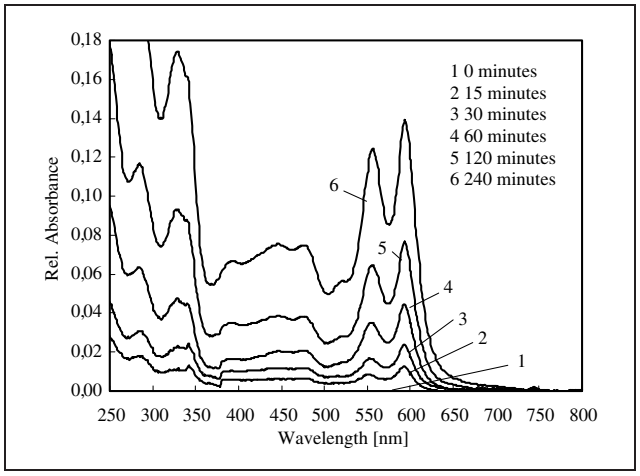

Fig. 1: Time-course of the water solubility of hypericin in presence of PVP25 at room temperature. Hypericin 100 μMol/L, PVP25 200 μMol/L in water

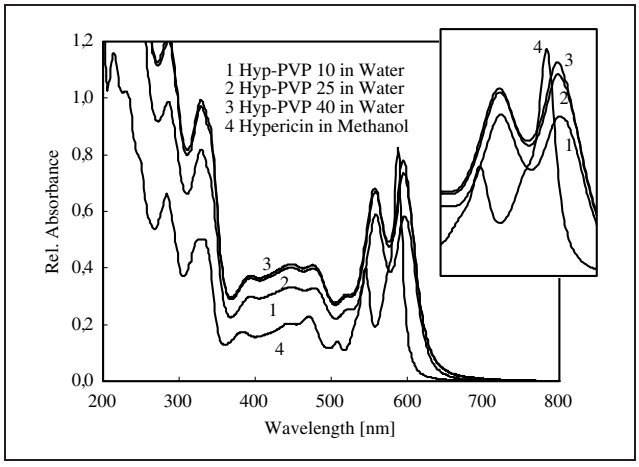

Fig. 2: Comparison of the absorption spectra of native hypericin in methanol (4) and three different hypericin-PVP-complexes: PVP10 (1), PVP25 (2) and PVP40 (3). Hypericin 20 μMol/L in methanol (4). Hypericin 50 μMol/L and PVP 100 μMol/L in water (1, 2, 3)

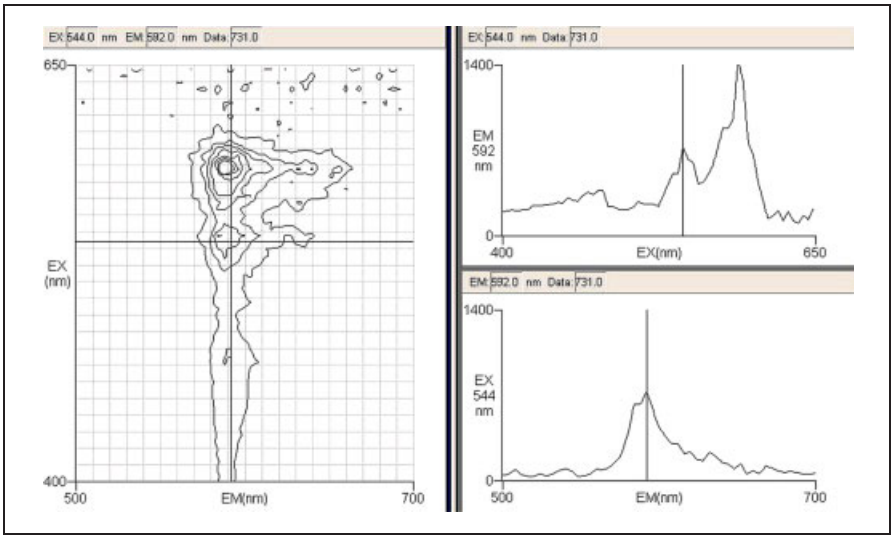

Fig. 3:
Fluorescence spectrum of hypericin (5 μMol/L) in methanol: excitation 544 nm, emission maximum 592 nm

spectrum reaches its maximum at 592 nm upon excitation at 544 nm. The excitation spectrum is comparable to the absorption spectrum of hypericin: emission measured at 600 nm was most intense at the same excitation wave length at which hypericin showed the highest degree of absorption. *Ergo*, excitation of hypericin at wave lengths from 200 to 600 nm results in a maximum emission intensity of 592 nm.

The fluorescence spectra of PVP-bound, water soluble hypericin and hypericin dissolved in methanol are similar (see Fig. 4). The 2D excitation spectra of hypericin-PVP in water shows a similar emission maximum at 596 nm which provides evidence that fluorescence properties of hypericin dissolved in water are maintained. These results are important for diagnostic applications.

Photobleaching of a photosensitizer is an unwanted photochemical effect of most sensitizers used in PDD and PDT (Moan et al. 1997; Bezdetnaya et al. 1996). Particularly porphyrins are unstable in irradiation processes making calculation and planning of therapeutic design difficult. Hypericin, however, remains a light stable sensitizer when dissolved in organic solvents or bound to PVP in water. To evaluate photostability hypericin-PVP solutions in physiological NaCl were irradiated in the presence of oxygen by a halogen lamp at a fluence rate of 125 mW/cm^2. Fig. 5 shows a comparison of hypericin-PVP25 with m-THPC-PVP25 in physiological NaCl. The relative absorbance at the particular absorbance maximum in the relevant visible region (595 nm for hypericin-PVP25 and 653 nm for m-THPC-PVP25, respectively) is shown. In accordance with Bonnet et al. (1999) photobleaching of m-THPC is observed. In contrast, hypericin-PVP25 is stable under test conditions that readily lead to photobleaching of m-THPC (presence of oxygen and broad band light). Similar results have been obtained for hypericin-PVP10 and hypericin-PVP40 (data not shown). Thus, aqueous solutions of hypericin-PVP fulfill the stringent requirement of photostability for applicable photosensitizers. These findings make hypericin-PVP a promising agent for further photobiological and clinical investigations.

Figure 6 shows the solubility of hypericin-PVP in water in a photometrically linear range. Hypericin-PVP25 was dissolved in water in increasing amounts in order to determine the limit value of solubility in aqueous solutions. The amount of hypericin-PVP was determined by UV-VIS-spectroscopy at the wavelength of maximum absorption using cuvettes with 0.1 cm pathlength. A linear correlation between concentration of hypericin-PVP and relative absorbance at wavelength of maximum absorption was observed for up to 100 mg/mL hypericin-PVP equalling 1000 mg pure hypericin in 1 L water (= 2 mMol/L). Regarding this value it is essential to note that it was not feasible to determine the maximum solubility of hypericin-PVP in aqueous solutions using this method since

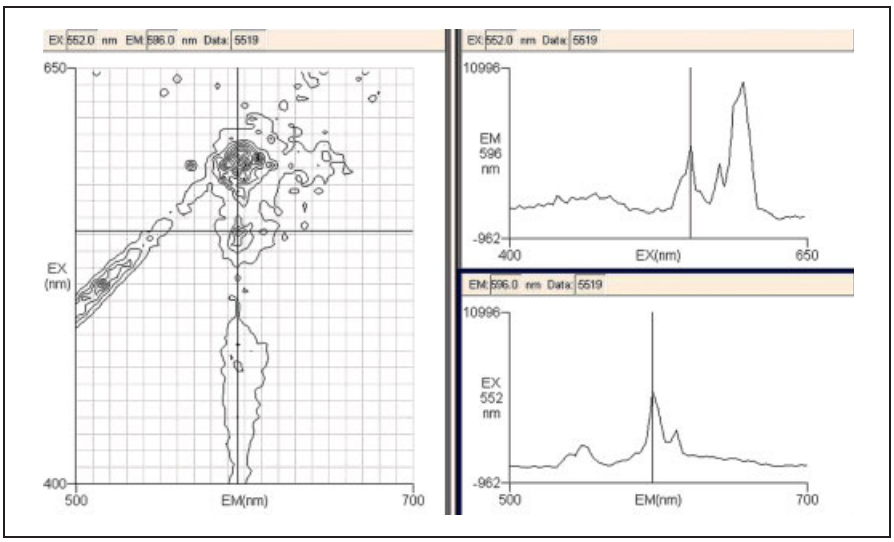

Fig. 4:
Fluorescence spectrum of hypericin-PVP in water (hypericin 25 μMol/L, PVP25 100 μMol/L): excitation 552 nm, emission maximum 596 nm

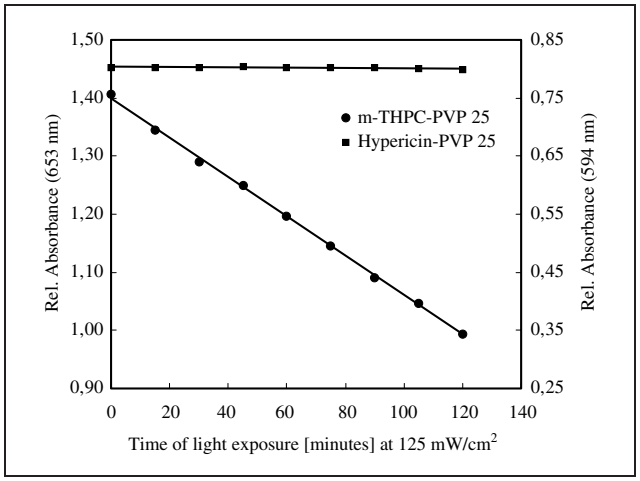

Fig. 5: Photobleaching of hypericin-PVP25 at 595 nm (1) and m-THPC-PVP25 at 653 nm (2). Both substances 50 μMol/L, PVP25 100 μMol/L in physiological NaCl solution

higher concentrations would have exceeded the limit of evaluable photometric capacity. Limitations are the molar extinction coefficient of the substance, pathlength of light and the linear working range of the instrument – according to the principle of Lambert-Beer. However, the authors observed 10 to 100-fold solubility (20–200 mMol/L hypericin) indicating that hypericin-PVP forms vitreous, semifluid liquified material showing no upper limit in solubility.

The Table shows the wavelength of maximum absorption and the corresponding molar extinction coefficient in several solvents based on the content of hypericin in the complex with PVP. Hypericin-PVP25 is almost insoluble in carbon tetrachloride and petroleum ether. It is slightly soluble in acetone and ethyl acetate and soluble in water, acetonitrile, chloroform, dimethyl sulfoxide, ethanol, methanol, methylene chloride and pyridine.

Free-form hypericin is insoluble in chloroform and methylene chloride. Complexed to PVP, hypericin is highly soluble in water, highly soluble in methylene chloride and moderately soluble in chloroform (see Table).

Fluorescence properties of hypericin-PVP depend on the concentration of hypericin in relation to PVP. We observed an increasing fluorescence at constant hypericin concentrations upon augmentation of PVP in the sample solution.

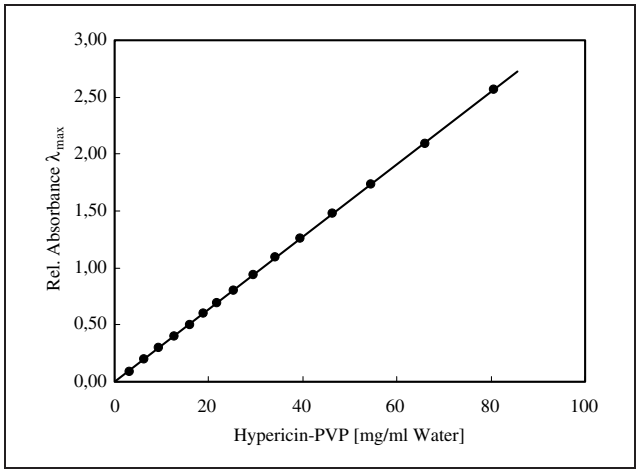

Fig. 6: Solubility of hypericin-PVP25 in water. Relative Absorbance was measured at wavelength of maximum absorption (594 nm) using cuvettes with 0.1 cm pathlength

Table: Wavelength of maximum absorption and corresponding molar extinction coefficient in several solvents based on the content of hypericin in the complex with PVP

Solvent	Molar extinction coefficient referring to the content of hypericin in hypericin-PVP		Solubility
Acetone	n.d.	596 nm	slightly soluble
Acetonitrile	39.700	594 nm	soluble
Carbon tetrachloride	n.d.	n.d.	almost insoluble
Chloroform	29.000	585 nm	soluble
Dimethyl sulfoxide	45.300	599 nm	soluble
Ethanol	35.000	597 nm	soluble
Ethyl acetate	n.d.	605 nm	slightly soluble
Methanol	30.600	595 nm	soluble
Methylene chloride	41.500	598 nm	soluble
Petroleum ether	n.d.	n.d.	almost insoluble
Pyridine	47.500	601 nm	soluble
Water	15.700	595 nm	soluble

Figure 7 shows a constant plateau at PVP concentrations above 600 μMol/L.

These results indicate a change in inter- and intra-molecular distribution of hypericin and PVP at low PVP concentration ranges. At a hypericin : PVP ratio of 1 : 60, fluorescence reached a maximum and remained constant. Figure 8 shows maximum emission of hypericin-PVP at 10 μMol/L hypericin in PVP40 (excitation at 420 nm). Lower emissions were observed using PVP10 and PVP25 whereby hypericin concentrations greater or less than 10 μMol/L led to decreased emission at 590 nm (excitation at 420 nm).

The molar ratio of PVP and hypericin is dependent on the degree of polymerization. An essential relationship demonstrates this fact: the smaller the molecular weight of PVP, the greater the molar concentration (of hypericin) has to be diluted with water to achieve a total dissolved form of chromophore complexes. This indicates that the key to solubility of hypericin in water using PVP as complexing reagent is the total chain length of PVP in the aqueous system. Regarding clinical applications, molecular weights of PVP between 10 kD and 40 kD are favored since it is assumed that molecules of these sizes can diffuse through and be physiologically excreted from cellular membranes (Römpp 1995).

Intracellular binding of hypericin-PVP-complexes was demonstrated in K562-cells. Cells were incubated with the dye complex then analysed by confocal lasermicroscopy.

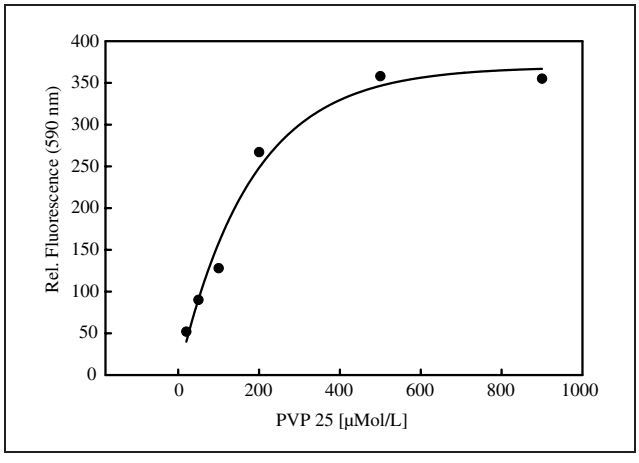

Fig. 7: Fluorescence emission at 590 nm, excitation 420 nm. Concentration of hypericin 10 μMol/L, PVP25 as indicated

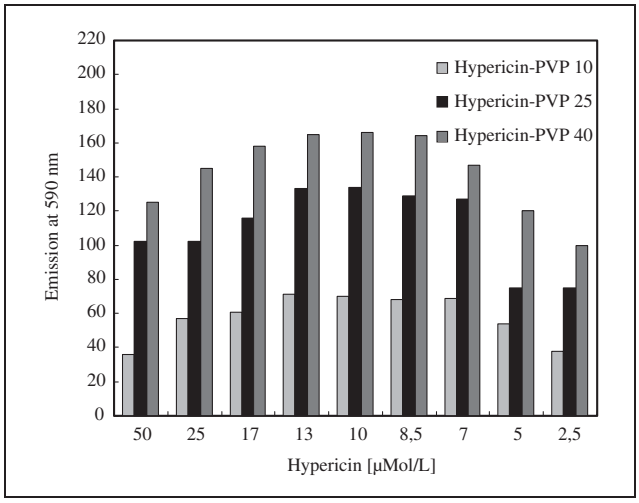

Fig. 8: Fluorescence emission at 590 nm, excitation 420 nm. Concentration of PVP 100 µMol/L, hypericin as indicated

Based on primary results, we concluded that the dye targets membrane structures within the cytoplasma and at the cell surface by way of the endoplasmatic reticulum (see Fig. 9 a, b). The dye was not detected in the nucleus.

In order to evaluate the role of PVP in the new hypericin-PVP complex we compared hypericin delivered to cells by way of the PVP-complex and the solvent DMSO which easily diffuses across cell membranes. Hypericin uptake was studied by FACS-cytometry using two different types of cells purified from human urine: leucocytes and T2-tumor positive transitional carcinoma cells (TCC-cells). Figure 10 shows the corresponding signal histograms with signal frequency (ordinate) versus signal intensity of red fluorescence F2 (abscissa). Two different incubation assays were analysed: one with hypericin-PVP40 and the other one with hypericin-DMSO. Since in both incubation assays equal concentrations of hypericin, identical cell-masses and total cell volumes were used, the peak areas were comparable and correlated with the amount of hypericin adsorbed onto the cell surface or uptaken into the cells.

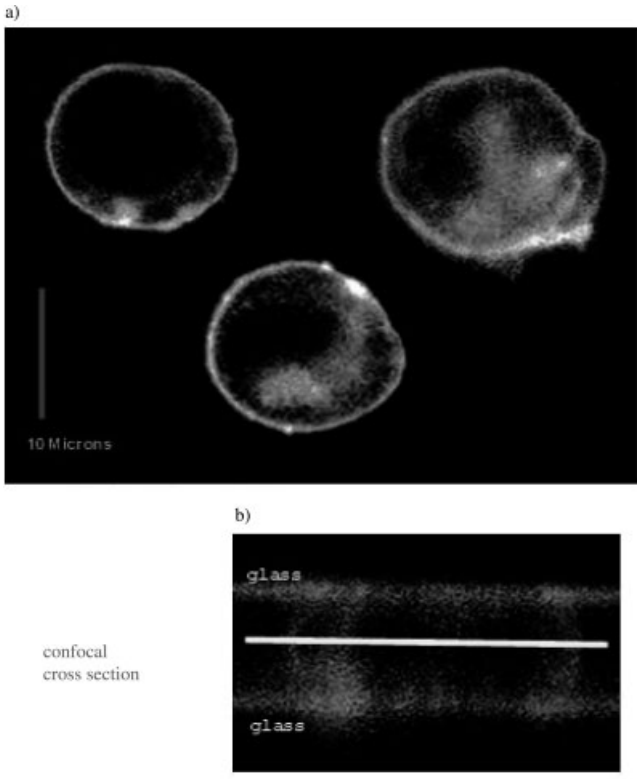

Fig. 9: a) Hypericin-PVP complex binding and cytosol transport in K562 cells. Incubation: 10 minutes in RPMI 1640 medium, 10% FCS. Excitation wavelength 514 nm, Emission 580 nm. b) Confocal cross section

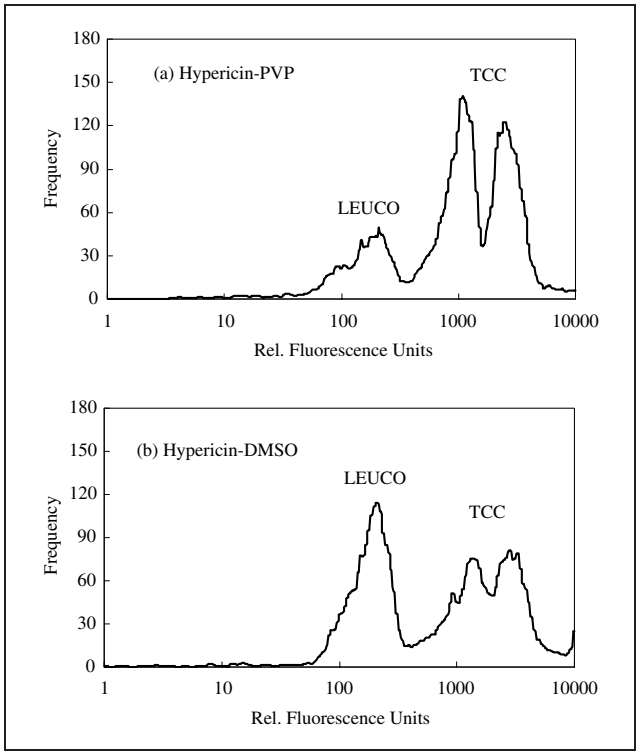

Fig. 10: Hypericin delivered to cells by (a) PVP complex compared with (b) DMSO measured by FACS-cytometry of human urinary leucocytes (LEUCO) and transitional carcinoma cells (TCC). For details see methods and materials section

Purified leukocytes showed a clear signal for red fluorescence which is located at the same position (equivalent number of relative fluorescence units) as the first peak within both histograms of urinary cells (see Fig. 10). The peaks with higher relative signal intensity represent the highly fluorescent, larger TCC-cells.

The main finding is that hypericin delivered to cells by DMSO produces fluorescence signals with almost the same frequency for leukocytes as well as for TCC-cells. No tumor-specific accumulation is detectable. In contrast, the new complex hypericin-PVP shows a more than 3-fold histogram-integral of specific fluorescence for TCC-cells under equimolar conditions indicating high tumor specificity. The ratio between the area according to TCC-cells and the area of leucocytes is 1.3 for hypericin-DMSO and 4.1 for hypericin-PVP (see Fig. 10). The nonspecific red fluorescence of leukocytes incubated with hypericin-DMSO is 3-fold higher than upon incubation with hypericin-PVP. Additionally, autofluorescence in the green spectral region is enhanced in presence of hypericin-DMSO compared to hypericin-PVP (which showed no enhancement of autofluorescence during incubation).

Summing up the results of FACS-cytometry we have clearly shown that the new complex hypericin-PVP exhibits a significantly high tumor affinity for TCC-cells whereas hypericin delivered to the cells *via* DMSO does not.

At this stage it is important to note that the fluorescence signal measured by FACS-cytometry is not specific for the

uptake of hypericin. Rather, the signal is unspecific in a topological manner. Both the dye adsorbed at the cell membrane and that taken up into the cell (and the various cell compartments) has the same impact on the FACS-signal. Nevertheless, to further the development of practicable applications of hypericin-PVP in PDT and PDD, it was essential to show that this new photosensitizer accumulates in tumor cells with a significantly high tumor specificity – regardless of its exact cellular localisation. Further studies are necessary to confirm that the cells subsequently die upon illumination.

In terms of clinical perspective, hypericin has several attributes which make it particularly attractive for investigating its clinical use: it possesses minimal dark toxicity (Thomas and Pardini 1992; Ali and Olivo 2003), is not metabolized by i.e. human organism (Kerb et al. 1996; Brockmoller et al. 1997) and is a photodynamically active molecule with marked fluorescence emission in orange/red regions. Hypericin accumulates in small superficially located tumors which can be subsequently detected by fluorescence diagnostic tools (D'Hallewin et al. 2000; Pytel and Schmeller 2002; D'Hallewin et al. 2002a; Olivo et al. 2003).

Recent investigations have shown that photodynamic therapy with hypericin successfully inhibits tumor growth in various mouse tumor models. This occurs via apoptosis and necrosis resulting in vascular damage (Hadjur et al. 1995; Chen et al. 2001, 2002a, 2002b). However, clinical applications of hypericin to investigate its photodynamic and phototherapeutic potential are rare. In small scale clinical investigations, 100–500 μg hypericin/cm^3 tumor have been injected intralesionally into basal cell carcinomas and squamous cell carcinomas and photodynamic therapy was performed successfully (Alecu et al. 1998; Kubin et al. 2000a, b).

Studies of bio-distribution using confocal laser microscopy show penetration and accumulation of hypericin in cytoplasm and endoplasmatic reticulum of human tumor cell lines (Kubin et al. 2003a, b). Localization of hypericin in mitochondria and lysosomes was reported by Ali et al. (Ali and Olivo 2002, 2003). Due to high sensitivity and specificity of hypericin at low concentrations in human hollow organs such as bladder and stomach, hypericin is a tumor selective agent (D'Hallewin et al. 2002b). Ideal properties for photodiagnostic and photodynamic applications of hypericin are light absorption at broad wavelength area, photostability, rapid clearance from normal tissue and/or slow uptake in normal cells resulting in high tumor selectivity. Hypericin complexes should be incubated for systemic applications for 0.1 to 36 h before diagnostic therapeutic procedures. Appropriate sensitizer dosage between 1 mg–5 mg per kg bodyweight would optimize the clinical effects but require further investigation (Kubin et al. 2005).

3. Experimental

3.1. Chemicals

Hypericin was obtained commercially (Planta Natural Products, Vienna, Austria), as were PVP25 (Polyvinylpyrrolidone K25 USP, Fluka, Vienna, Austria), PVP10 and PVP40 (Sigma-Aldrich, Vienna, Austria). The index number next to PVP is the average molecular weight in kD corresponding to the degree of polymerization of the vinylpyrrolidone monomers. m-Tetrahydroxyphenylchlorin (m-THPC) was obtained from Scotia Pharmaceuticals Ltd (Stirling, UK).

3.2. Synthesis of hpericin-PVP25

Hypericin (100 mg) was dissolved in 25 mL distilled ethanol using sonication for 2 min. PVP25 (10 g) and 80 mL distilled water were added to the resulting dark red solution. The mixture was heated for 5 min (70 °C) resulting in a cherry red clear solution. Fifty milliters water were added and the solution was stirred for 10 more minutes. The solvents were cautiously evaporated under reduced pressure (30 mbar) to yield a solid mass. The strongly hygroscopic solid was stored under dry and dark conditions in a dessiccator using polyphosphoric acid as a drying agent.

Hypericin-PVP10 and hypericin-PVP40 were produced using the same procedure. Solvents of hypericin-PVP10, hypericin-PVP25 and hypericin-PVP40 for spectroscopic investigations were prepared freshly. PVP was first dissolved in water after which hypericin was added. Solubility was aided by heating the mixture up to 70 °C.

3.3. Instrumentation

UV-VIS-spectra were recorded using the Hitachi U-2001 spectrophotometer. Fluorescence spectroscopy was performed on a Hitachi F-4500 fluorescence spectrometer (Hitachi High Technologies America Inc, San Jose, CA, USA).

3.4. Photostability

A 500 W broad band halogen lamp (HV halogen lamp R7S, Europart Austria, Salzburg, Austria) was used to evaluate photostability of hypericin-PVP solvents. The fluence rate was adjusted at 125 mW/cm^2 resulting in an energy intake by the sample solution of 900 J/cm^2 over the exposure time of 2 h. The sensitizer solution (hypericin 50 μMol/L, PVP 100 μMol/L in physiological NaCl solution) was stirred under irradiation in a 50 ml-beaker at room temperature. At intervals of 15 min, samples were drawn for UV-VIS spectrum analysis at 190–800 nm. Changes of absorbance were used to determine photostability and photobleaching. To compare the course of photo-modification and the method for measuring photostability, m-tetrahydroxyphenylchlorin (m-THPC, 50 μMol/L, PVP 100 μMol/L in phys. NaCl) was tested using the same modality. An aqueous solution of m-THPC was prepared by dissolving the substance in 300 μL distilled methanol using sonication for 2 min. PVP25 and physiologic NaCl were added to the resulting dark brown solution. The mixture was heated for 5 min (70 °C) yielding in a clear solution.

3.5. Solubility

In order to establish the solubility of hypericin complex bound to PVP in water, hypericin-PVP25 was dissolved in water in increasing amounts. Each addition of hypericin-PVP was followed by heating the mixture to 70 °C for 15 min and applying sonication for 2 min. The absorbance of the solution was measured at the wavelength of maximum absorption using cuvettes with 0.1 cm pathlength.

3.6. Molar extinction coefficient of hypericin-PVP

Hypericin complex bound to PVP25 was dissolved in several solvents of different properties using sonication for 5 min. The absorbance of the solution was measured at the wavelength of maximum absorption (hypericin-PVP25 in water: 595 nm, acetone: 596 nm, acetonitrile: 594 nm, chloroform: 585 nm, dimethyl sulfoxide: 599 nm, ethanol: 597 nm, ethyl acetate: 605 nm, methanol: 595 nm, methylene chloride: 598 nm, pyridine 601 nm). The molar extinction coefficient, based on the content of hypericin in the complex, was calculated.

3.7. Hypericin-PVP intracellular binding in K562 cells

K562 cells were cultivated in RPMI 1640 medium (Sigma-Aldrich, Vienna, Austria) with 10% fetal calf serum (FCS) in a Heraeus Cytoperm standard incubator (Heraeus, Hanau, Germany) under atmospheric conditions (37 °C, 5% CO_2). After 4 days, K562 cells with a cellular diameter of 12 μm were incubated with an equimolar mixture of hypericin-PVP10 and hypericin-PVP40 (total 25 μMol/L hypericin) under light protection for 10 min. Confocal microscopy was carried out at cell culture temperature (37 °C) using a thermal glass slide. 20 μL of K562 cell suspension were placed under the glass coverslip without rupturing the cells. Finally, confocal microscopy was performed using a Leitz CLSM instrument (Leica Microsysteme, Bensheim, Germany) with an excitation-wavelength of 514 nm and a high-pass filter cube allowing fluorescence emission from 580 nm to 650 nm.

3.8. FACS-cytometry of urinary sediment human leucocytes and transitional carcinoma cells

Purified human leukocytes (Coulin et al. 1997) were used as reference cells for FACS-cytometry calibration. Analysis showed a mean cell volume of 100 femtoliter using a TOA SYSMEX NE-8000 counter system. FACS-cytometry was performed using a FACS-Calibur-instrument (Beckton-Dickenson BD-Biosciences, San Jose, CA, USA) adjusted to excitation-wavelength of 488 nm, emission-wavelength of green auto-fluorescence (FL1) 510–540 nm and red hypericin-fluorescence (FL2) of 570–600 nm. After a calibration series to adjust for fluorescent noise, purified human leucocytes were incubated with hypericin as a reference to determine the signal

intensity for each counted leucocyte event (incubation: hypericin-PVP40, 200 nMol/L hypericin, 10 min under light protection). The green autofluorescence channel signal (F1) as well as the red fluorescence signals (F2) were analysed using scattergrams and histogram technique.

Finally, T2-tumor positive transitional carcinoma cells (TCC-cells) and leucocytes were prepared from human urine using a centrifugation protocol according to (Althof). Four 5 ml urine samples were centrifuged at 1,500 rpm for 10 min (Hettich Universal Centrifuge, r = 6.5 cm). After gentle resuspension with PBS-buffer the sediment was analysed histologically and with fluorescence microscopy to confirm the presence of TCC-cells and leucocytes. Two vials were filled with equal volumes of resuspended urinary sediment cells and incubated under light protection. Vial 1 was incubated with hypericin-DMSO (200 nMol/L hypericin) and vial 2 with hypericin-PVP40 (200 nMol/L hypericin). Finally, FACS-cytometry was performed as described above. The resulting FACS-scattergrams and histograms were compared to demonstrate the difference in selectivity of hypericin-PVP towards human TCC-cells versus hypericin dissolved in the solvent DMSO.

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