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In vitro transport and uptake of protohypericin and hypericin in the Caco-2 model

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Abstract

The intestinal absorption characteristics of protohypericin, a protonaphthodianthrone present in *Hypericum* extract, were studied and compared with those of hypericin. The Caco-2 model was used as a model of the intestinal mucosa to assess transepithelial transport and cell uptake. The experimental work was performed in specific light conditions that prevented both the photoconversion of protohypericin into hypericin and the photosensitization of the cells. Following application of the individual compounds (80–200 μM) to the apical side of the monolayers, the appearance in the basolateral compartment was found to be very low ($< 0.5\%/5$ h), but was comparable for both compounds. A lag-time of 2–3 h was observed, suggesting gradual saturation of binding sites on the membrane or inside the cells. Uptake experiments of protohypericin and hypericin by Caco-2 cells revealed a very significant cellular accumulation (4–8%); uptake was characterised by saturation after 3 h. The findings of this study suggest that protohypericin has comparable absorption characteristics as hypericin and may contribute to the beneficial effect of *Hypericum* extract after oral dosing. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Hypericin; Protohypericin; Caco-2; Transport; Uptake; *Hypericum*

1. Introduction

Nowadays, *Hypericum* plant extracts are commonly being used for the treatment of mild to moderate depression in traditional herbal medicine. *Hypericum* extract is the most widely prescribed antidepressant in Germany and is frequently used in the USA as a popular over-the-counter preparation (Raffa, 1998). Of importance, a meta-analysis of randomised clinical trials confirmed that *Hypericum* extract is significantly superior to placebo and as effective as standard antidepressants (Linde et al., 1996). The extract contains a complex variety of constituents, belonging to several phytochemical classes, and numerous investigators have reported on the

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pharmacological activity of the total extract and its constituents (e.g. Butterweck et al., 1997; Nahrstedt and Butterweck, 1997).

Especially the hypericins (naphthodianthrones, Fig. 1), but more recently also hyperforin, are considered the constituents of *Hypericum* extract responsible for its antidepressant effect (Wagner and Bladt, 1994; Chatterjee et al., 1998). So far, a number of studies have been undertaken to evaluate the pharmacokinetics and the gastrointestinal transepithelial transport characteristics of hypericins. Using an in vitro system of the gastrointestinal mucosa (Caco-2 monolayer), 3–5% of total hypericin was reported to cross the monolayer after a 5-h incubation period (Sattler et al., 1997). A pharmacokinetic study revealed that, following oral intake of the standardised *H. perforatum* extract in healthy human volunteers, the systemic availability of hypericin and pseudohypericin was limited and amounted to 14 and 21%, respectively (Kerb et al., 1996).

Some protonaphthodianthrones (protohypericin, protopseudohypericin, Fig. 1) are also present in *Hypericum* extracts. These structures represent partially cyclic precursors of the naphthodianthrones which, upon irradiation with visible light, convert efficiently into their naphthodianthrone analogues (Falk and Schmitzberger, 1992). The ratio of protonaphthodianthrones/naphthodianthrones in the fresh plant and in the extract may vary considerably between 1:5 and 5:1, probably depending on the extent of photoconversion that took place during the preparation of the extract (Falk and Schmitzberger, 1992; Krämer and Wiartalla, 1992). Therefore, the protonaphthodianthrones constitute a variable but significant fraction of the *Hypericum* extract.

So far, no studies have been performed on the absorption and transport characteristics of the protonaphthodianthrones, presumably because of both a lack of pure compounds and the technical complications encountered working with photoconvertible structures. In the present study, protohypericin was synthesised and its transport and uptake compared with that of hypericin using an in vitro model of the gastrointestinal mucosa (Caco-2). The experimental work was performed in specific light conditions that prevented both the photoconversion of protohypericin into hypericin and the photosensitization of the cells.

2. Materials and methods

2.1. Materials

Protohypericin and hypericin were prepared as described previously (Falk et al., 1993). After synthesis, the crude protohypericin was purified by silica column chromatography (ethyl acetate/water 100:2.5), followed by elution of the compound (ethyl acetate/acetone/water, 80:20:2.5) under specific light conditions (see further). Protohypericin was then further purified with Sephadex LH20 column chromatography (dichloromethane/methanol/acetone, 55:30:15). In the case of hypericin synthesis, protohypericin was light irradiated before the second purification step to convert protohypericin to hypericin by a photocyclisation reaction. Hypericin was then further purified by Sephadex LH20 column chromatography. The synthesis yield for protohypericin was 20% (purity > 98%, content of hypericin: 1.01%, as determined by HPLC), and in the case of hypericin 28% (purity > 99%).

Both compounds were characterised by ^{1}H NMR (Gemini 200 MHz, Varian), LSI mass spectrometry (Kratos Concept IH) and UV/Vis spectrophotometry (Hewlett-Packard, CA). The data were comparable with literature data (Falk et al., 1993). The stock solutions of protohypericin and

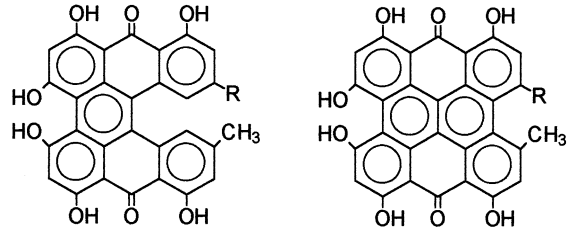

Fig. 1. Structure of protohypericin (R = CH_3), protopseudohypericin (R = CH_2OH) (protonaphthodianthrones), hypericin (R = CH_3) and pseudohypericin (R = CH_2OH) (naphthodianthrones).

hypericin were made in DMSO. They were diluted to the desired final concentrations (80 or 200 μM) in transport medium. The concentration of DMSO in the final solutions was 0.5%, a concentration which had been shown not to affect the integrity of Caco-2 monolayers.

All chemicals used for culturing the cells were purchased from Gibco (Life Technologies, Merelbeke, Belgium). Bovine serum albumin (BSA) and D-(+)-glucose were purchased from Sigma (St Louis, MO). Sodium fluorescein and sodium hydroxide were obtained from UCB (Leuven, Belgium). DMSO was obtained from Janssen Chimica (Geel, Belgium). Cell culture medium consisted of DMEM supplemented with 10% foetal calf serum (FCS), 1% non-essential amino acids solution and 100 IU/ml penicillin–streptomycin. Transport medium consisted of 500 ml Hanks' Balanced Salt Solution (HBSS) containing 25 mM glucose, 10 mM Hepes and 10% foetal calf serum (FCS); FCS was included in the transport medium to maintain the solubility of hypericin and protohypericin.

2.2. Methods

2.2.1. Light conditions

In order to avoid photoconversion or photosensitization, all manipulations with protohypericin and hypericin were performed under the illumination by a KL 1500 electronic cold light source (Schott Glaswerke, Wiesbaden, Germany) equipped with a 645 nm sharp cut-off glass filter (Melles Griot, Zevenaar, The Netherlands).

2.2.2. Caco-2 cell culture

Caco-2 cells were kindly provided by Dr Y. Schneider (UCL, Belgium). Cells were grown in 75 cm^2 culture flasks in an atmosphere of 5% CO_2 and 90% relative humidity at 37°C. Cells were passaged every 3–4 days (at 70–80% confluence) at a split ratio of 1:7.

For transport studies, Caco-2 cells were plated at a density of 40 000 cells/cm^2 on Transwell membrane inserts (3 μm pore diameter, 12 mm diameter, Caring Costar Corporation, Cambridge, MA). For uptake experiments, cells were seeded directly in flat bottom 96-well tissue culture plates (Corning Costar Corporation, Cambridge, USA). Confluence was reached within 3–4 days after seeding and the monolayers were used for the experiments between days 18 and 24 post-seeding. Cell passages between 114 and 127 were used in the experiments.

2.2.3. Transport experiments

Transport of protohypericin and hypericin across the Caco-2 cell monolayers was studied using monolayers 18–24 days post-seeding. Before the experiments, the monolayers were rinsed with transport medium and preincubated for 30 min. After the preincubation, transepithelial electrical resistance (TEER) of the monolayers was measured at 37°C using a Millicel ERS apparatus (Millipore) to check cell monolayer integrity. Only monolayers displaying TEER values above 300 $\Omega \cdot cm^2$ were used in the experiments. Transport was initiated by adding 1.5 ml of transport medium to the basolateral side and 0.5 ml of a solution of the compound (80 or 200 μM) in transport medium to the apical side. At predetermined time points, samples (20 μl) were taken from the basolateral side and replaced by the same amount of transport medium. At the end of the transport experiment (5 h), samples (20 μl) were also taken from the apical side. All monolayers were checked for integrity after the experiment by measuring TEER values as well as transport of the paracellular leakage marker sodium fluorescein (by measuring the UV-absorption of the basolateral solution at 490 nm, 1 h after adding a solution of 1 mg/ml sodium fluorescein to the apical side).

2.2.4. Uptake experiments

Uptake of protohypericin and hypericin was carried out using Caco-2 monolayers seeded in flat bottom 96-well tissue culture plates. Before the experiments, the monolayers were rinsed twice with transport medium (37°C) and preincubated for 30 min. The cells were then incubated with the compounds (80 or 200 μM) dissolved in transport medium. After incubation, the cells were washed three times with PBS containing 2% BSA and were consequently extracted with 200 μl DMSO.

2.3. Analysis of the samples

In the case of the transport experiments, 20-μl samples of the basolateral and apical medium were diluted with 200 μl DMSO, while in the case of the uptake experiments, cells were incubated for 1 h with 200 μl DMSO to extract the compound. Before fluorescence measurements, all samples containing protohypericin were placed on a plastic diffuser sheet above a set of seven L18W/30 fluorescence lamps (color temperature 3000 K, Osram, Germany) for 10 min. At the surface of the diffuser the uniform fluence rate was 4.5 mW/cm^2, as measured with an IL 1400A photometer (International Light, MA). The content of hypericin of the samples was analysed by a Microplate Fluorescence Reader (FL600, Bio-tek, Winooski, USA) with excitation and emission wavelengths set at 485 and 645 nm, respectively.

The protein content of the monolayers was determined using the method of Lowry et al. (1951) with bovine serum albumin as standard.

3. Results and discussion

The present study was undertaken to compare the in vitro transport and uptake of protohypericin and hypericin using an in vitro cell culture system of the intestinal mucosa (Caco-2 cell monolayers). For that purpose, protohypericin was synthesised and purified under filtered light conditions (>645 nm). These light conditions did not convert protohypericin to hypericin, as was shown in preliminary work exposing diluted solutions of protohypericin to the filtered light used. Consequently, the prepared protohypericin contained only small amounts of hypericin (1.01%). In addition, the filtered light did not photo-activate hypericin (see further).

Before analysing medium and cells for their content of protohypericin by fluorescence measurements, protohypericin was first converted to hypericin by light irradiation. This was necessary since protohypericin is a virtually non-fluorescent compound, while hypericin features a high fluorescence quantum yield (Lavie et al., 1995), enabling the quantification of minute amounts using a fluorescent reader. HPLC analysis of the light irradiated DMSO samples indicated 100% conversion of protohypericin to hypericin in these conditions.

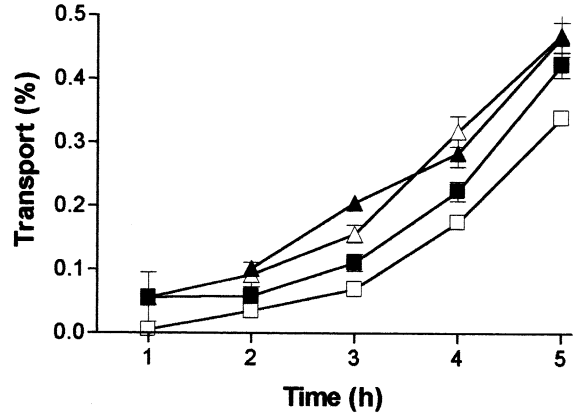

Fig. 2. Transport of protohypericin and hypericin across Caco-2 monolayers at 37°C, expressed as a percentage of the initial amount added to the apical compartment, as a function of time (mean ± S.D.; $n=3$): ■, hypericin 200 μM; ▲, hypericin 80 μM; □, protohypericin 200 μM; △, protohypericin 80 μM.

The results of the transport of 80 and 200 μM protohypericin and hypericin solutions from the apical side to the basolateral side of the cells' monolayer are shown in Fig. 2. Transport of both compounds across Caco-2 monolayers was very low at any concentration tested ($<0.5\%$ over 5-h incubation period), and was characterised by a disproportional increase in transport with concentration. As can be observed, there was a lag time of 2–3 h for the transport of both compounds across the monolayer; no significant differences in transport results could be observed for protohypericin and hypericin

The amount recovered in the apical compartment after the 5-h incubation amounted to ± 85% of the initial amount incubated for both compounds. In addition, preliminary experiments had shown that both protohypericin and hypericin in the inserts without cells did not show any adsorption. We therefore decided to determine the intracellular accumulation of both compounds in another set of experiments.

The results for the uptake of protohypericin and hypericin by the Caco-2 cells are illustrated in Fig. 3. Uptake of both protohypericin and hypericin by the cells was found to be saturable. The percentage of the amount of hypericin or protohypericin accumulated in the cells with respect to the original amount incubated amounted to 4–8%. Since the two experiments were conducted separately, the results shown in Figs. 2 and 3 cannot be directly correlated. However, it appears that there is a significant and comparable amount of protohypericin and hypericin accumulating in Caco-2 cells.

The present data on the transport of hypericin across the Caco-2 monolayer are inconsistent with the results obtained in a recent study where substantial transport of the compound (apical concentration: 200 μM) was noticed (Sattler et al., 1997). This discrepancy could be explained in terms of the different incubation conditions that were employed in both studies: in the present study, 10% FCS was included in the transport medium to maintain the solubility of hypericin and protohypericin, while in the study of Sattler et al. cyclodextrin was used to solubilise hypericin. However, we recently reported that cultured cells were able to concentrate intracellularly 200- to 300-fold the compound present in 10% FCS containing medium (Vandenbogaerde et al., 1998)

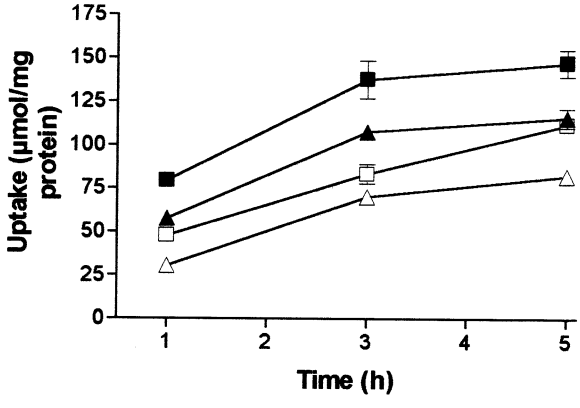

Fig. 3. Accumulation of protohypericin and hypericin in Caco-2 cells at 37°C, expressed as the amount (μmol) per mg of protein as a function of time (mean ± S.D.; $n = 5$): ■, hypericin 200 μM; ▲, hypericin 80 μM; □, protohypericin 200 μM; △, protohypericin 80 μM.

and also in this study a large amount of hypericin was recovered in the Caco-2 cells, illustrating that proteins present in FCS (e.g. albumin) are not a limiting factor for the delivery of hypericin to the cells and, likely, cannot account for the low transport efficiency observed in the present study.

Conversely, it should be stressed that hypericin is an extremely potent photosensitizer, as numerous investigations have documented (for an overview, see Ugwu et al., 1998). Therefore it is absolutely imperative to conduct the experimental work with hypericin in conditions that prevent the photo-activation of the compound. Importantly, it has been reported that even the slightest exposure of cells incubated with hypericin to laboratory illumination resulted in marked photocytotoxicity of cultured cells, especially when concentrations in the micromolar range are used (Vandenbogaerde et al., 1997). Therefore it is believed that exposure of cells treated with hypericin to normal laboratory light may lead to the photosensitization of the cells resulting in an artificially increased transport of the compound across the photosensitised Caco-2 cells monolayer. In the present study this artefact was avoided by the use of filtered light with a wavelength above 645 nm. As this red light does not overlap with the absorption spectrum of hypericin, it allows a normal visual inspection and manipulation of cells and equipment without photo-activating the compound. Preliminary experiments had indeed shown that exposure to normal light during sampling and TEER measurements resulted in a significantly higher decrease of TEER values as compared to exposure to filtered light (data not shown).

From the findings of this study, we can suggest that protohypericin has comparable absorption characteristics to hypericin, and may therefore contribute to the beneficial effect of *Hypericum* extract after oral dosing.

Recently, it was found that pure hypericin and pseudohypericin exert in vivo an antidepressant activity in the forced swimming test solely when solubilised by procyanidins present in *Hypericum* extract (Butterweck et al., 1998). This finding implies that other constituents of the extract dramatically influence the absorption of the hyper-

icins (and protohypericins) by the gastrointestinal tract. A study using human volunteers showed that, after oral intake of *Hypericum* extract, the systemic availability of hypericin and pseudohypericin was 14 and 21%, respectively (Kerb et al., 1996). Future work investigating the effect of specific fractions present in *Hypericum* extract on the in vitro transport across the Caco-2 monolayer of the pure compounds, will explore this hypothesis.

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