In vitro accumulation and permeation of hypericin and lipophilic analogues in 2-D and 3-D cellular systems

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Abstract. Previous studies have shown that hypericin is an excellent diagnostic tool for the fluorescence detection of carcinoma *in situ* in the human bladder. The present work was performed to get a better insight into the mechanism of cellular uptake of hypericin (HYP) using RT-112 human papillary TCC cells of the bladder. Using lipophilic hypericin acid amide derivatives like hypericin acid hexylamide (AM6), hypericin acid octylamide (AM8) and hypericin acid dodecylamide (AM12), the effect of increased lipophilicity on the binding to serum proteins was investigated, as well as the cellular accumulation and permeation, both in 2-D and 3-D cell conditions. Density-gradient ultracentrifugation of the compounds pre-incubated with fetal bovine serum (FBS) showed that HYP and to a lesser extent AM6 predominantly bind to LDL, whereas AM8 and especially AM12 preferably associate with HDL. The cellular accumulation of the compounds did not significantly differ when LDL or AcLDL was supplemented to medium, and with all compounds the highest uptake could be observed in case of medium without supplements. Using medium without supplements it was further observed that the compounds with the highest lipophilicity accumulated substantially less in RT-112 cells. We further found a significant difference in the intracellular concentration of AM8 and AM12 when LDL or FBS was supplemented to MEM, but not in case of HYP and AM6. Of particular interest, AM8 and especially AM12 showed enhanced intraspheroidal permeation in the presence of FBS. It is believed that the relative stronger binding to HDL reduces the intracellular accumulation, as seen in the 2-D conditions, and therefore increases the probability of paracellular transport in a 3-D multicellular system by passive diffusion. In conclusion the data suggest that the amount of free hypericin or its lipophilic congener determines the extent of intracellular accumulation. This concentration is both determined and limited by binding to different lipoproteins present in the medium, and by the formation of stable homoaggregates. The findings further highlight AM8 and AM12 as compounds better tailored for paracellular transport than HYP itself and therefore as potentially very interesting diagnostic tools for TCC lesions in the bladder.

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Abbreviations: PDT, photodynamic therapy; ROS, reactive oxygen species; TCC, transitional cell carcinoma; CIS, carcinoma *in situ*; LDL, low density lipoproteins; Ac LDL, acetylated low density lipoproteins; PBS, phosphate-buffered saline; FBS, fetal bovine serum; MEM, minimum essential medium

Key words: hypericin, photodynamic diagnosis, photodynamic therapy, transitional cell carcinoma, bladder cancer, spheroids, intracellular uptake, paracellular transport

Introduction

The majority of bladder tumors exhibit a papillary configuration which, by projecting toward the lumen, is easily detectable by endoscopy. Although these tumors usually show one or more recurrences after initial therapy, they rarely progress to muscle invasive disease (1). In contrast, carcinoma *in situ* (CIS) is a flat lesion that shows a highly malignant potential, thus requiring early detection to be effective, irrespective of the therapeutic treatment chosen. Since CIS are flat lesions with no extensions present into the bladder lumen, the diagnosis of CIS by white light cystoscopy, sonography or other radiological methods is difficult and subjective (2).

Hypericin (HYP), a compound belonging to the class of phenanthroperylene 7-14 diones (Fig. 1) is found in more than 300 species of the genus *Hypericum*, with *Hypericum perforatum* (St. John's wort) as the most representative species (3). Previous studies have shown that this compound can be used as a diagnostic tool for the fluorescence detection of CIS in the human bladder. The sensitivity and specificity were found to be 94 and 95%, respectively, proving that the compound is far better than other diagnostic tools used for the same purpose (2,4,5). Since hypericin is a powerful

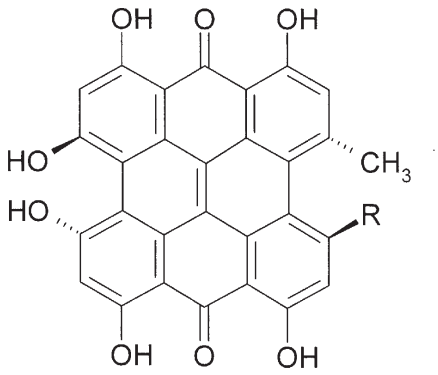

Figure 1. Chemical structure of hypericin (R=CH_3), hypericin hexylamide (R=$CONH(CH_2)_5CH_3$), hypericin acid octylamide (R=$CONH(CH_2)_7CH_3$) and hypericin dodecylamide (R=$CONH(CH_2)_{11}CH_3$).

photosensitizer, this situation offers the opportunity not only to detect, but also to treat tumor sites photodynamically (6).

There are numerous studies suggesting that modifications in the expression of the transmembrane glycoproteins cadherins and integrins, that mediate cell-cell and cell-extracellular matrix adhesion, may play a crucial role in the development and progression of bladder cancer (7,8). Cancer cells lacking homophilic Ca^{2+} dependent glycoproteins (cadherins) can escape from their origin, invade and metastasize distant organs (9). Due to the lack of cohesiveness between cancer cells, paracellular transport of material is possible, a phenomenon unlikely to occur with normal urothelial cells that line the inner wall of the bladder. As a matter of fact, a recent study using multicellular spheroids establishes a strong correlation between limited hypericin permeation into the spheroid and E-cadherin expression (10). Another aspect of the uptake of hypericin in transitional cell carcinoma (TCC) lesions, relates to the way the compound is finally taken up intracellularly. Possible mechanisms include endocytosis, pinocytosis and passive diffusion (11). Endocytosis is highlighted as the most important method of intracellular accumulation of the lipophilic photosensitizer benzoporphyrin derivative, both *in vitro* and *in vivo* (12). Lipophilic compounds bind to low density lipoproteins (LDL) and consequently are internalised by LDL receptor-mediated endocytosis. Although HYP is amphiphilic, it is known to bind to LDL particles (13) and a similar endocytotic mechanism has been suggested (11).

The present work was performed to obtain a better insight into the mechanism of cellular uptake of HYP using RT-112 human papillary TCC cells of the bladder, and to investigate the effect of increased lipophilicity on the binding to serum proteins, and on the cellular accumulation and permeation of hypericin analogues in 2-D and 3-D cell conditions, respectively. By attaching the different hydrocarbon chains in the bay region of hypericin acid, derivatives could be synthesised keeping the hexahydroxy-phenantroperylenedione skeleton intact (Fig. 1).

Materials and methods

Chemicals. HYP was synthesized according to a previously published method (14). Hypericin acid hexylamide (AM6), hypericin acid octylamide (AM8) and hypericin acid dodecylamide (AM12) were produced according to ref. 15. LDL and acetylated LDL (AcLDL) were purchased from Molecular Probes (Willow Creek, OR, USA).

Cell line. 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 in minimum essential medium (MEM) with Earle's salts containing 2 mM L-glutamine (Gibco-BRL, Paisley, Scotland, UK), under 5% CO_2 at 37˚C. The medium was supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) non-essential amino acids (Gibco-BRL), 1% (v/v) antibiotic/antimycotic solution (Gibco-BRL) and tylosine (60 μg/ml) (Eli Lilly, Brusseles, Belgium).

Intracellular accumulation. RT-112 cells were seeded onto 6-well tissue culture plates (Costar, Cambridge, MA, USA) at concentration of $9x10^5$ cells/well and incubated for 24 h at 37˚C. Subsequently, the medium was replaced with fresh MEM supplemented (or not) with LDL (10 μg protein/ml), acetylated LDL (AcLDL) (10 μg protein/ml) or FBS (10%), and hypericin or derivatives (1 μM). After an incubation for 2 h at 37˚C, the cells were washed twice with phosphate-buffered saline (PBS) (Gibco-BRL) and harvested by treatment with trypsin solution (Gibco-BRL). The cell suspension was then pelleted by centrifugation (5 min, 600 g) and the compounds were extracted twice with 0.5 ml methanol/ethyl acetate (50/50, v/v). The residue was dissolved in 250 μl dimethylsulfoxide, of which 200 μl was transferred to a 96-well tissue culture plate (Costar, Cambridge, MA, USA). Fluorescence measurements were performed using a microplate fluorescence/absorbance reader (FL600, Biotek, Winooski, VT, USA). Concentration of the photosensitizers was calculated from calibration curves. The excitation and emission filters were 590/20 and 645/40 nm respectively. The number of cells per condition was determined in a separate experiment, using a particle counter (Coulter Electronic, Luton, UK). The results were calculated assuming a mean volume of 3 μl per 10^6 cells (16). All experiments were performed in triplicate. The difference in the cellular uptakes was statistically evaluated using One-way ANOVA with Tukey-Kramer post test.

3-D cell culture. The 96-well tissue culture plates (Costar) were layered with 1.5% agarose (Sigma, Steinheim, Germany) in MEM. Spheroids were initiated by inoculation of $5x10^3$ RT-112 cells in 200 μl of culture medium supplemented with 1% sodium pyruvate (100 μM) (Gibco-BRL). After 7 days of incubation, spheroids reached a size of 400-500 μm in diameter and were used in the experiments. During growth, medium was replaced once.

3-D permeation. Spheroids were incubated in MEM supplemented with 10% FBS with the individual compounds (10 μM) in 6-well plates (Costar) for 2 h. After incubation they were washed thoroughly with PBS, collected, embedded in Tissue Tek medium (Miles, Elkhart, IN, USA) and frozen in liquid nitrogen. Frozen samples were cut in a cryostat microtome (5-μm thickness). Centrally cut cryostat sections were

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. Images were acquired using a light-sensitive charge-coupled device digital camera (AxioCam HR, Carl Zeiss). KS imaging software system (Carl Zeiss Vision, Hallbergmoos, Germany) was used to measure the average fluorescence in concentric layers of 9.5-μm thickness from the rim to the center of the spheroid. Fluorescence intensities were determined as the mean of 4 spheroids. Corrections were made for autofluorescence and differences in intrinsic fluorescence of the compounds. After curve fitting using a non-linear regression model (Prism, San Diego, CA, USA) F_{50} and F_{100} values were determined. These values correspond to the relative fluorescence at a distance of 50 and 100 μm from the rim, respectively. Statistical analysis of the F_{50} and F_{100} values was done by one-way ANOVA with Tukey-Kramer post test.

Serum protein binding. Density-gradient ultracentrifugation was carried out as previously described (17). FBS samples were incubated with the compounds (10 and 30 μM) for 2 h at 37˚C under dark conditions. Proteins were separated by density-gradient ultracentrifugation using a KBr/NaCl gradient. The step gradient was formed by careful layering KBr/NaCl solutions of different densities (1.063, 1.019, 1.006 g/ml) on top of serum incubated with the compounds. Serum solution was previously brought to a density of 1.21 g/ml. The samples were centrifuged for 20 h (100 000 x g, 4˚C) using SW 40 Ti rotor in Beckman Optima LE-80K ultracentrifuge. Immediately following centrifugation, 0.5 ml aliquots were removed sequentially from the bottom of the tube by means of a peristaltic pump. Fluorescence intensity of the samples was determined using fluorescence/absorbance microplate reader as described above. All the experiments and the majority of the synthetic reactions were done in strictly subdued light conditions.

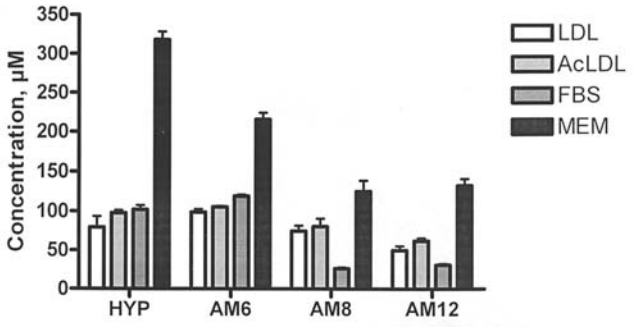

Figure 2. Intracellular concentrations in monolayer cell culture (RT-112 cells) of hypericin (HYP), hypericin acid hexylamide (AM6), hypericin acid octylamide (AM8) and hypericin acid dodecylamide (AM12) after 2-h incubation in minimal essential medium (MEM), MEM supplemented with 10 μg/ml low density lipoproteins (LDL), MEM supplemented with 10 μg/ml acetylated low density lipoproteins (AcLDL) or with 10% fetal bovine serum (FBS). Data represent mean ± SD (n=3).

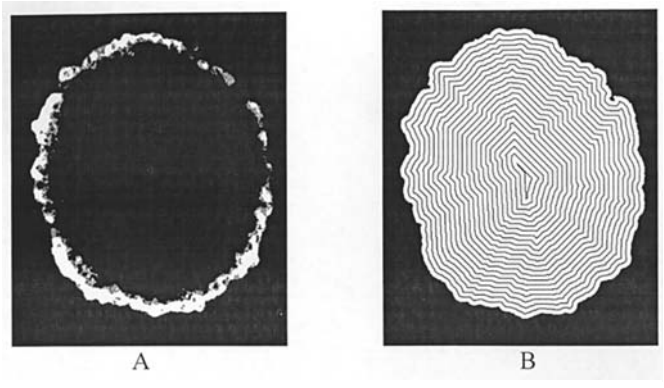

Figure 3. (A) Fluorescence photomicrograph of centrally cut cryostat cross section of a RT-112 spheroid after 2-h incubation with 10 μM hypericin in MEM supplemented with 10% FBS. (B) Quantification of fluorescence was performed by KS imaging software system in concentric layers of 9.5-μm thickness from the rim to the centre of the spheroid.

Results

Intracellular accumulation of hypericin analogues. To investigate the effect of increased lipophilicity on the cellular accumulation of the different hypericin analogues, we first compared the accumulation of the dyes in monolayers of the human TCC cell line RT-112.

Fig. 2 shows the average cellular accumulation of HYP and its analogues in a monolayer culture of RT-112 cells. The concentrations were calculated by the external standard method. Calibration curves showed good linearity (R^2>0.999) in the range of concentrations measured. The cellular accumulation of the compounds did not significantly differ when LDL or AcLDL was supplemented to MEM (P>0.05, in all cases), and with all compounds the highest uptake was observed in the case of MEM without supplements (P<0.01, when compared with the LDL results). Using MEM without supplements it was further observed that the compounds with the highest lipophilicity accumulated substantially less in RT-112 cells (P<0.01, for each of the compounds compared with the others, except for AM8 vs AM12: P>0.05). We further found a significant difference in the intracellular concentration when LDL supplemented and FBS supplemented MEM were compared (p<0.01), in the case of AM8 and AM12 but not in the case of HYP and AM6 (P>0.05).

Intraspheroidal fluorescence distribution. Cell-cell adhesion proteins have a pronounced influence on the distribution of hypericin in the 3-D structure of multicellular spheroids (10). To check the influence of the structural changes and subsequent physical characteristics of the synthesised hypericin acid derivatives on the distribution in 3-D cellular organization, we incubated multicellular spheroids in the presence of hypericin and its analogues. KS imaging software system (Carl Zeiss Vision) was used to measure the average fluorescence in concentric layers of 9.5-μm thickness from the rim to the centre (Fig. 3). After measuring the relative fluorescence, the data were fitted by a non-linear regression model. Fitted curves are presented in Fig. 4. Table I shows relative fluorescence intensities at the depth of 50 and 100 μm from the rim of the spheroid. These F_{50} and F_{100} values are decreasing in the following order: AM12 > AM8 > HYP, AM6. The differences between all individual F_{50} or F_{100} values are very significant (p<0.01), except in case of HYP and AM6

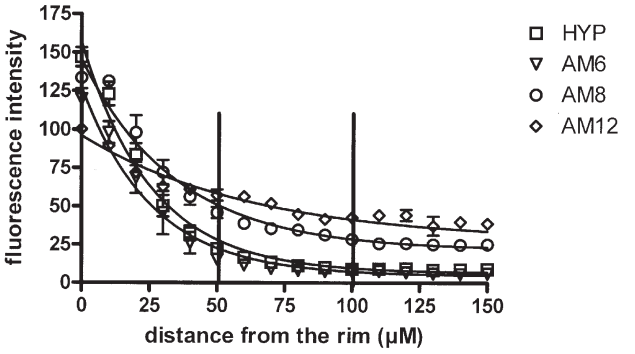

Figure 4. Intraspheroidal distribution (3-D culture system, RT-112 cells) of hypericin (HYP), hypericin acid hexylamide (AM6), hypericin acid octylamide (AM8) and hypericin acid dodecylamide (AM12) after 2-h incubation in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Data represent mean ± SD (n=6-8). Vertical lines represent the F_{50} and F_{100} values (see Table I).

Table I. Relative fluorescence intensity at radial distance of 50 μm (F_{50}) or 100 μm (F_{100}) from the rim.[a]

Compound	AM12	AM8	AM6	HYP
F_{50} ± SD	57.2±8.9	45.9±10.3	15.4±6.8	22.2±8.4
F_{100} ± SD	42.7±2.1	28.6±3.9	7.4±1.9	9.1±1.7

[a]Spheroids from RT-112 cells were incubated with hypericin or derivatives (10 μM) for 2 h in MEM supplemented with 10% FBS. Frozen sections were analyzed with fluorescence microscopy and imaging software. Data represent mean ± SD (n=4).

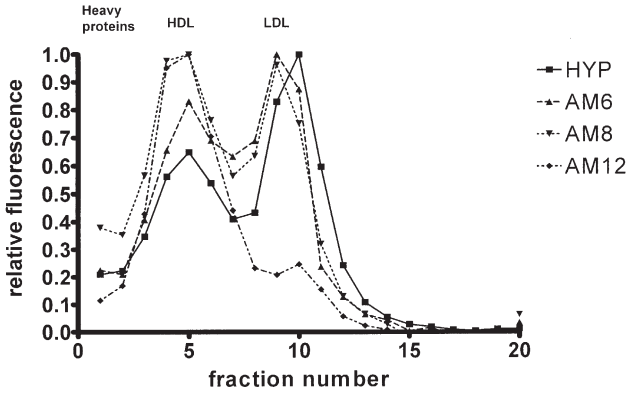

Figure 5. Fluorescence profile of hypericin (HYP), hypericin acid hexylamide (AM6), hypericin acid octylamide (AM8) and hypericin acid dodecylamide (AM12) bound to FBS *in vitro*, separated by density-gradient centrifugation.

($p>0.05$ for F_{50} and F_{100}) and AM8 vs. AM12 ($p>0.05$ for F_{50}).

Serum protein binding. The binding of hypericin and analogues to the different serum constituents of FBS was assessed by

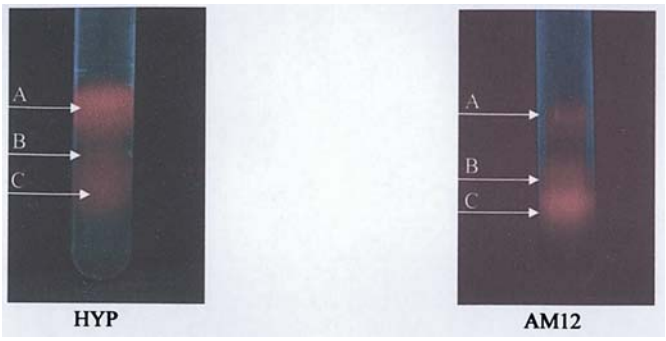

Figure 6. The binding of hypericin acid dodecylamide (AM12) and hypericin (HYP) to fetal bovine serum constituents. The position of the various lipoproteins in the gradient was determined by comparison with literature. A corresponds to low density lipoprotein (LDL), whereas B and C correspond to high density lipoproteins (HDL).

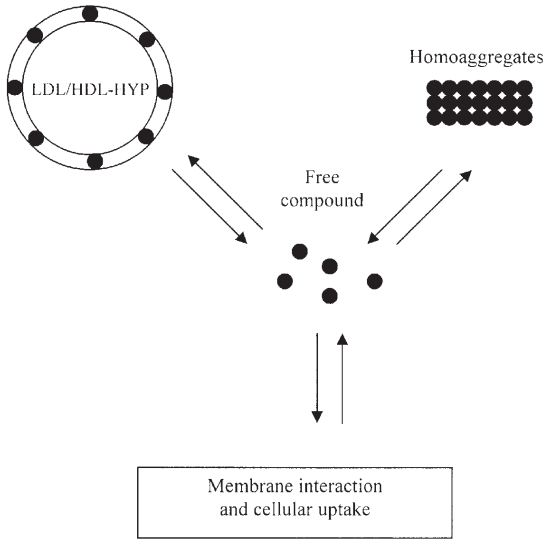

Figure 7. Schematic representation of the proposed equilibria in the medium. Free compound can interact with the LDL or HDL particle (if present), or with other free molecules to form homoaggregated stacks, or it can interact with the cellular membrane and then be taken up intracellularly.

density-gradient ultracentrifugation. The position of the various lipoproteins in the gradient was confirmed by comparison with literature data (13,17). Fluorescence profiles are shown graphically in Fig. 5. It can be seen that at a 30 μM concentration hypericin and to a lesser extent AM6 predominantly bind to LDL, whereas AM8 and especially AM12 preferably associate with high density lipoproteins (HDL). Fig. 6 shows the tubes under UV light (λ=365 nm) after density-gradient ultracentrifugation of FBS incubated with HYP and AM12. A shift in the binding profile can easily be seen. Similar results were obtained using the compounds at a lower concentration (10 μM) (not shown).

Discussion

After intravesical instillation, hypericin shows high specificity and sensitivity towards superficial TCC of the bladder (2,4,5). This feature makes it an excellent tool for diagnosis of flat CIS lesions which otherwise are difficult to detect. The

selective uptake of hypericin has been explained in terms of an enhanced migration of the compound through malignant tissue with a decreased expression of E-cadherin (10). On the other hand, hypericin binds to LDL and LDL-receptor mediated internalization has been proposed to favor a selective uptake of hydrophobic antitumoral drugs in malignant cells overexpressing LDL receptors (12). To test this latter hypothesis, RT-112 TCC cells were incubated with hypericin in serum-free medium or medium supplemented with LDL, AcLDL or FBS. Acetylation of the lysine residues of the apoprotein of LDL abolishes the recognition by LDL receptors, leaving the overall structure and content of the particle intact (18). Irrespective of the use of LDL or AcLDL, similar intracellular concentrations of hypericin were observed, strongly indicating that the intracellular accumulation of hypericin is not mediated by LDL and its receptor. Similar results were obtained with the hypericin analogues. The present findings therefore identify LDL as a passive carrier for hypericin.

As investigated, 10% FBS contains somewhat identical low concentrations of LDL as used in the LDL-conditions (data not shown). Although hypericin displays affinity also for albumin, LDL and HDL (19,20), our results show that in the conditions used, hypericin becomes associated in particular with LDL. It can be argued therefore that 10% FBS or LDL-conditions are comparable both in terms of LDL content and hypericin binding, resulting in a similar cellular uptake of hypericin.

As seen in previous studies with hypericin (11) and some other lipophilic photosensitizers (21), the highest cellular uptake was observed when the incubation took place in MEM medium without LDL or FBS. Such results again support passive diffusion or pinocytosis as the major internalisation route, which requires no specific receptors. As a matter of fact, the presence of LDL or other hypericin binders hampers cellular uptake, due to competition between cellular interaction and binding to LDL.

Overall similar results were obtained with hypericin acid hexylamide, a compound that does not differ substantially from hypericin in terms of hydrophilic/lipophilic properties. Indeed both compounds co-elute on a C-18 reversed phase HPLC column (15), and likely the rise in hydrophilicity by introducing an amide moiety in the hypericin analogues is neutralized by coupling the molecule with a lipophilic C6-alkyl chain. Introducing longer chains (octyl, dodecyl), however, increases the elution time on RP-HPLC, an indication that the derivatives feature a more lipophilic character (15). All these compounds exhibited decreased intracellular uptake when LDL was supplemented to the medium compared with medium only. These results demonstrate once more that LDL particles are passive carriers competing with the cellular membranes for hypericin and their analogues. As compared to LDL conditions, the presence of FBS unexpectedly reduced the intracellular concentrations of the AM8 and AM12 amides. An explanation relies probably on the increased relative affinity of the compounds for HDL, and in binding stronger to this lipoprotein, on a reduced free medium concentration. Since the non-polar lipid core of lipoproteins consists mainly of triacylglycerol and cholesteryl esters which are surrounded by a single surface layer of phospholipid and cholesterol molecules, the main difference between LDL and HDL particles lies in their apolipoproteins embedded in the surface layer (22). Of interest, it was shown previously that HYP binds to LDL at the boundary between the lipidic and protein part of the particle (23). Our results therefore suggest an increased affinity of AM8 and especially AM12 for HDL by means of an interaction between the alkyl chain and lipophilic parts present in the HDL apolipoprotein.

It is generally accepted that the more lipophilic a compound is, the more it shows an affinity for cellular membranes and the more readily the compound is taken up intracellularly. For instance, the extent of cellular uptake of desmopressin conjugates lipidized with fatty acids of different chain length was found to correlate positively with their lipophilicity (24). Hence, in the absence of competing binders like LDL or HDL, one would expect AM8 and AM12 to be among the best intracellular-accumulating hypericin analogues, whereas the opposite was true. Although hard evidence is presently missing, this discrepancy can be explained in terms of aggregate formation. In the absence of binding to (lipo)proteins hypericin forms homoassociates in aqueous solutions exhibiting a stacking pattern similar to the one observed for the crystalline structure (25). The stable association of hypericin molecules that become dispersed in the medium therefore represents another competing factor that limits the amount of non-associated hypericin molecules that can be taken up by the cells. From the results it is anticipated that the alkyl chain present in the amide analogues further stabilizes the aggregates formed, and consequently limits the amount of free molecule in solution.

Spheroids show important characteristics of the small avascular tumors such as CIS of the bladder as well as sparingly vascularized tumors (26). Within the spheroidal structure, gradients of cellular oxygen levels (27), proliferation (28), pH (29), nutrients (30) and of applied compounds (31) can be observed. Therefore, spheroids can be useful in studying some aspects involved in PDT such as the role of oxygen and drug penetration, which can not be achieved with the monolayer cell culture systems. Since spheroid experiments are accomplished *in vitro*, this model facilitates studies of tumor cell-specific phenomena in isolation from complex host-dependent factors.

Of particular interest, AM8 but especially AM12 showed enhanced intraspheroidal permeation in the presence of FBS. It is believed that the relative stronger binding to HDL reduces the intracellular accumulation, as seen in the 2-D conditions, and therefore increases the probability of para-cellular transport in a 3-D multicellular system by passive diffusion. Conversely, HYP and AM6 show affinity for LDL and it is anticipated that this binding is weaker as compared to the binding of AM8 and especially AM12 to HDL. Hence by an improved intracellular accumulation, HYP and AM6 accumulated especially in the outer layers of the spheroids. The quantitative details of the binding of the compounds to LDL or HDL will be further studied in the near future but are beyond the scope of the present report.

In conclusion, the data suggest that the amount of free hypericin or its derived analogue determines the extent of intracellular accumulation. This concentration is determined and limited by both binding to different lipoproteins present

in the medium, and by the formation of stable homoaggregates. As a result, a complex picture of different equilibria emerges (Fig. 7). The findings further highlight AM8 and AM12 as compounds better tailored for paracellular transport than HYP itself and therefore as potentially very interesting diagnostic tools for TCC lesions in the bladder.

Acknowledgments

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