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Synthesis, in vitro cellular uptake and photo-induced antiproliferative effects of lipophilic hypericin acid derivatives

Ivo Crnolatac,[a] Ann Huygens,[a] Arthur van Aerschot,[b] Roger Busson,[b] Jef Rozenski[b] and Peter A. M. de Witte[a,*]

[a]*Laboratorium voor Farmaceutische Biologie en Fytofarmacologie, Faculteit Farmaceutische Wetenschappen, Katholieke Universiteit Leuven, Van Evenstraat 4, 3000 Leuven, Belgium*
[b]*Laboratorium voor Medicinale Chemie, Faculteit Farmaceutische Wetenschappen, Rega Instituut, Katholieke Universiteit Leuven, Minderbroedersstraat 10, 3000 Leuven, Belgium*

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Abstract—Hypericin, a naturally occurring hydroxylated phenanthroperylene dione, is used as a powerful photosensitizer for photodynamic therapy as well as a diagnostic tool for the fluorescence detection of flat neoplastic lesions in the bladder of patients. Both applications are based on the tumouritropic characteristics of the compound. To get more insight into some of the physicochemical properties of hypericin affecting its tumouritropic characteristics, we set out to synthesize a series of more lipophilic hypericins. For this purpose, a synthetic pathway to hypericin acid amides with hydrocarbon chains of different lengths stably attached by an amide bond at position C10 was explored. Hypericin acid proved inert in amide forming reactions, whereas the precursor protohypericin acid showed higher reactivity and resulted in the desired amide derivatives, which afterwards can be easily converted into their phenanthroperylene dione form. Hexyl-, octyl-, decyl- and dodecylamides of hypericin acid were successfully synthesized in this way. In vitro cellular uptake and photo-induced antiproliferative effects of the compounds were evaluated, using the human moderately differentiated non-invasive papillary transitional carcinoma RT-112 cell line. Whereas the more lipophilic amides were taken up limitedly, the hexylamide accumulated approx. as well as hypericin itself. From the antiproliferative data it can further be concluded that not only the cellular uptake, but also the light-induced activity, is affected by the introduced structural changes.

1. Introduction

Hypericin (**1**) is a secondary metabolite belonging to the chemical class of phenanthroperylene 7-14 diones. The compound is found in plants of the genus *Hypericum*,[1,2] with *Hypericum perforatum* (St. John's wort) as the widely known one. The commonly used synthesis procedure for **1** starts from the natural compound emodin (**2**), usually obtained as a glycoside by extraction of the bark of the plant *Frangula alnus*.[3–5] The coupling of two emodins yields protohypericin (**3**) which upon light irradiation is converted into **1** (Scheme 1).

As the compound is a potent photosensitizer[6] with tumouritropic characteristics both after local bladder instillation and systemic application, hypericin is presently under investigation as a diagnostic tool for the fluorescence detection of flat neoplastic lesions in the bladder of patients[7] and as a promising agent for photodynamic therapy (PDT).[8–13] PDT is a relatively new cancer treatment, which consists of the application of a photosensitizer that selectively accumulates in the tumour tissue. Upon irradiation with light of a specific wavelength, the photosensitizer goes into an electronically excited state. Following an intersystem crossing into the triplet state, the photosensitizer reacts with oxygen producing singlet oxygen species that cause tumour cell death.[14,15]

A basic understanding of the tumouritropic principle of hypericin would support the development of new and even more selective compounds. Of importance, previous results have shown that, as compared to normal

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* Corresponding author. Tel.: +32 (0) 16 323 432; fax: +32 (0) 16 323 460; e-mail: peter.dewitte@pharm.kuleuven.ac.be

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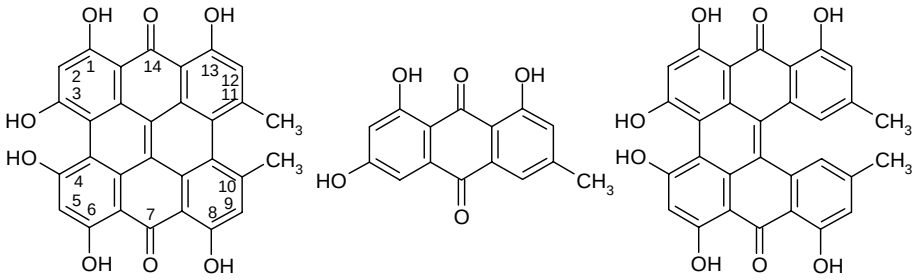


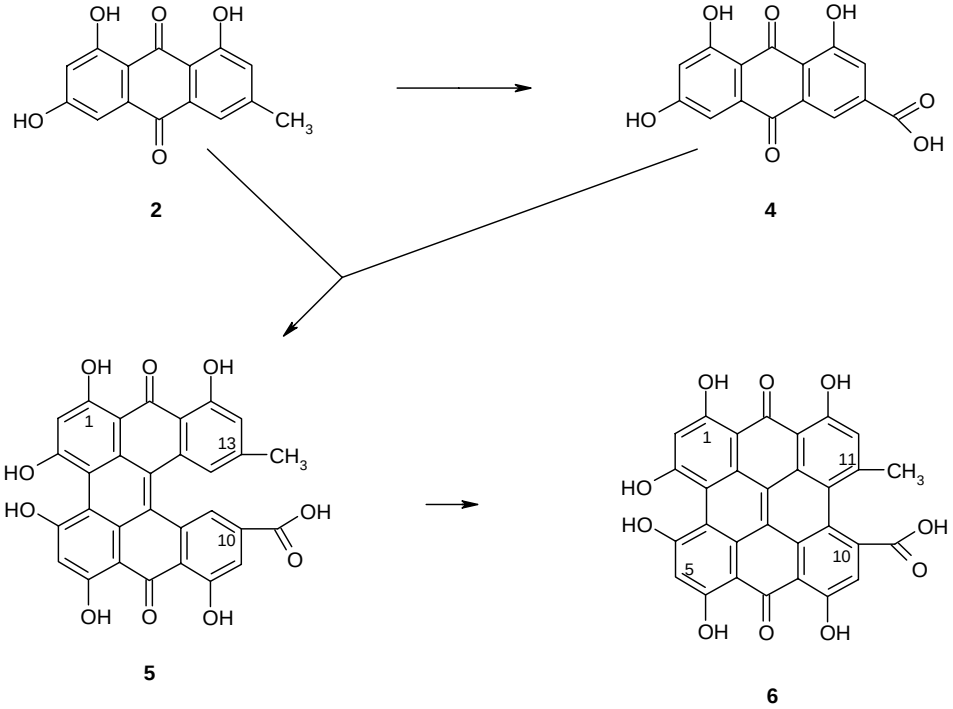
Scheme 1.

cells, isolated malignant cells intrinsically do not take up more hypericin.[16] Besides, hypericin is not metabolized and an increased conversion to other (more) fluorescent compounds particularly in malignant cells should be excluded.[17] The in vivo tumouritropic behaviour of hypericin therefore must be related to the specifically modified 3D-histoarchitecture of tumour tissue, and likely, the combination of different biological (i.e., tumour-dependent) and physical (i.e., compound-dependent) aspects finally results in the overall phenomenon of accumulation of hypericin in tumour tissue.

To get more insight into the physicochemical properties of hypericin affecting its tumouritropic characteristics, we set out to synthesize a series of lipophilic hypericin derivatives and to investigate their in vitro cellular uptake and their dark and photo-dependent cytotoxicity. To preserve the intact hexahydroxy-phenanthroperylene dione skeleton of hypericin, believed to be responsible for its highly fluorescent features, we prepared analogues with hydrocarbon chains of different lengths stably attached by an amide bond at position C11(C10). Retention times on a reversed-phase octadecyl high performance liquid chromatography (RP-C18 HPLC) column were used as a relative measure of the lipophilicity of the compounds.

2. Results

Synthesis of **6** was done according to the previously published method[18] by coupling **2** with emodic acid (**4**) which was synthesized starting from **2**. Similar as in the preparation of **1**, the intermediate protohypericin acid (1,3,4,6,8,15-hexahydroxy-13-methyl-7,16-dioxo-7,16-dihydro-dibenzo[*a*,*o*]perylene-10-carboxylic acid) (**5**) was obtained and converted into the final product by light irradiation (Scheme 2).



Scheme 2.

Compound **5** was used as a precursor for hypericin acid amides. After protection of the phenolic groups, the obtained product was treated with oxalyl chloride. Following the substitution of chlorine with an amine, with concomitant deprotection of phenols, the compounds were irradiated to convert them into the desired hypericin analogues. Four different amide derivatives were synthesized in this way, namely: hexahydroxy-11-methyl-7,14-dioxo-7*H*,14*H*-phenanthro[1,10,9,8-opqra]perylene-10-carboxylic acid hexylamide (**9**, $C_{36}H_{27}NO_9$); 1,3,4,6,8,13-hexahydroxy-11-methyl-7,14-dioxo-7*H*,14*H*-phenanthro[1,10,9,8-opqra]perylene-10-carboxylic acid octylamide (**10**, $C_{38}H_{31}NO_9$); 1,3,4,6,8,13-hexahydroxy-11-methyl-7,14-dioxo-7*H*,14*H*-phenanthro[1,10,9,8-opqra]-perylene-10-carboxylic acid decylamide (**11**, $C_{40}H_{35}NO_9$) and 1,3,4,6,8,13-hexahydroxy-11-methyl-7,14-dioxo-7*H*,14*H*-phenanthro[1,10,9,8-opqra]perylene-10-carboxylic acid dodecylamide (**12**, $C_{42}H_{39}NO_9$) (Scheme 3).

The chromatographic retention times of the compounds on a RP-C18 column served as a measure of their relative lipophilicities. Using the chromatographic method described in the experimental part, the retention times were: 6.0 min, 8.7 min, 14.5 min and 24.9 min for **9**, **10**, **11** and **12**, respectively. For reasons of comparison, the free acid **6** eluted after 2.9 min and **1** after 6.0 min.

The results of the experimental work assessing the cellular uptake of hypericin and its derivatives (**11** not included) are displayed in Figure 1 ($n = 3$).

All compounds showed light-induced antiproliferative effect (Fig. 2). The calculated IC_{50} values for **1**, **9**, **10** and **12** were 0.48 μM ± 0.09 (mean ± SD), 1.76 μM ±

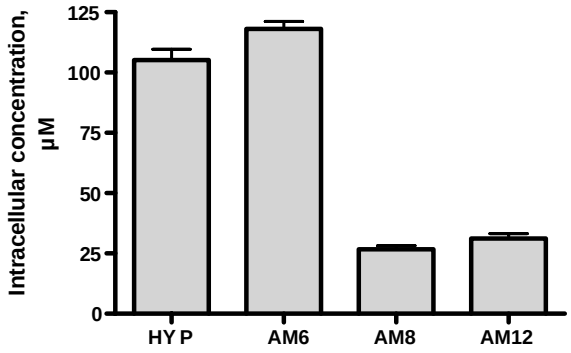


Figure 1. Cellular uptake of hypericin and derivatives. The results were calculated assuming a mean volume of 3 μL/10^6 cells. HYP: hypericin; AM6: hypericin acid hexylamide; AM8: hypericin acid octylamide; AM12: hypericin acid dodecylamide.

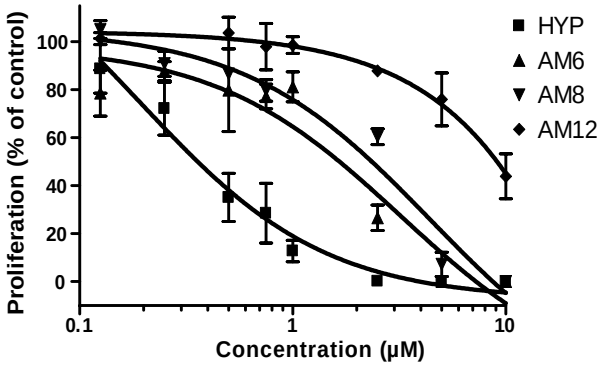
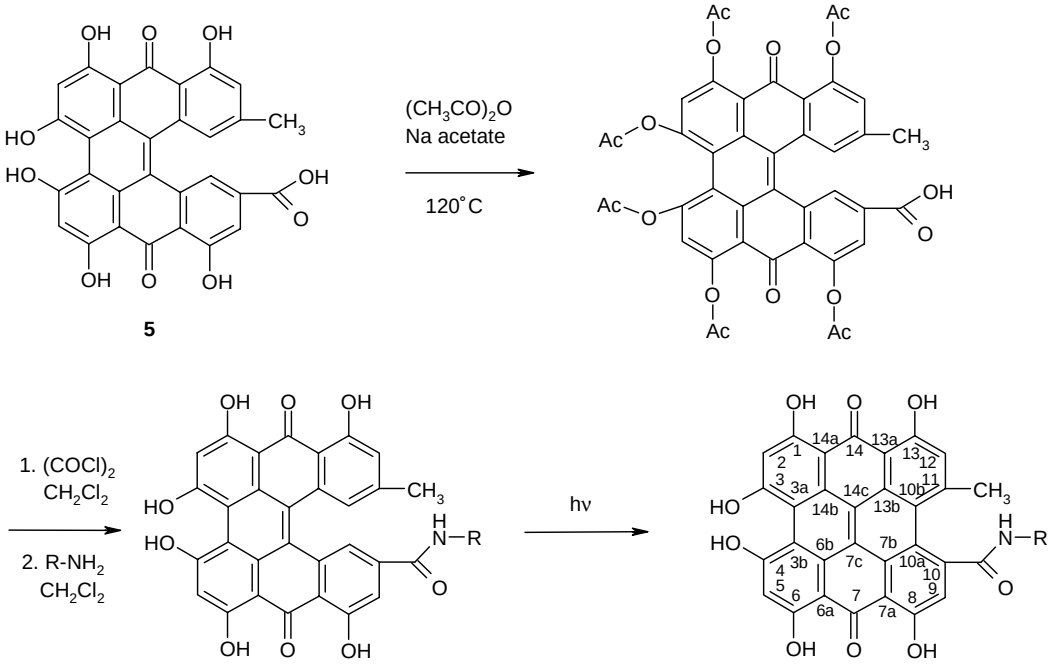


Figure 2. Photo-induced antiproliferative effect by hypericin and derivatives. HYP: hypericin; AM6: hypericin acid hexylamide; AM8: hypericin acid octylamide; AM12: hypericin acid dodecylamide.



9 R=-C_6H_{13} (yield 13%); **10** R=-C_8H_{17} (yield 15%)
11R=-$C_{10}H_{21}$ (yield 8%); **12**R=-$C_{12}H_{25}$ (yield 16%)

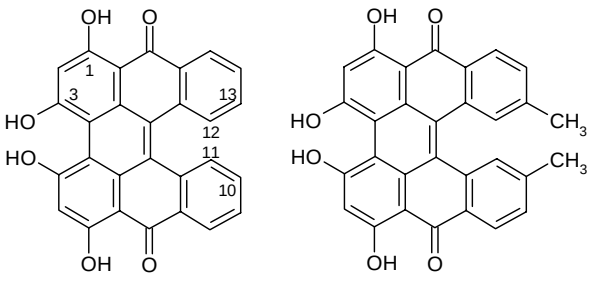
Scheme 3.

0.19, 2.49 μM ± 0.36 and >10 μM, respectively ($n = 3$). In all cases dark toxicity was not observed, at least at the concentrations used (maximally 10 μM).

3. Discussion

One way to obtain hypericin derivatives is the dimerization of emodin derivatives. Following this strategy, synthesis of different hypericin analogues substituted at the position of the methyl moieties was reported.[19–21] First, we attempted a similar reaction route and successfully synthesized emodic acid amides starting from emodic acid. However, dimerization as well as coupling with emodin only could be achieved with unacceptably low yields (unpublished results). As a result, our alternative approach was to compose first hypericin acid (**6**) followed by the coupling with the amines. Unfortunately, after protection of the phenolic groups, derivatization of **6** could not be achieved using either oxalyl chloride or dicyclohexylcarbodiimide (DCC). The most probable reason for the unsuccessful outcome is the steric obstruction of the targeted carboxylic acid group caused by the proximity of the bulky methyl group. The mechanism of hypericin acyl-chloride formation and its conversion into amide involves the formation of a tetrahedral intermediate that is highly sterically hindered. A bulky methyl group at C10 blocks the nucleophilic attack and lowers the yield on the acyl-chloride and consequently on the final product. Hypericin is already distorted in the so-called propeller conformation, with the torsion angle between C10–C10a and C10b–C11 bonds being 32.4°.[22] Further torsion might not be possible without a cleavage of the C10a–C10b bond. Likely, this steric effect is also the reason why the reaction with DCC was not successful. Instead of the expected amide formation, the intermediate *O*-acyl derivative has apparently rearranged into a stable *N*-acyl form[23] having a bad leaving group so that it cannot be substituted with an amine (unpublished results).

Helianthrone (**7**) and its dimethyl derivative (**8**) are structurally related to **3** having the same helical shape of C_2 symmetry as hypericin.[24] However, since the distance between the C atoms at positions 10 and 13 in **7** and **8** is greater than in corresponding positions 10 and 11 in **1**, the divergence from planarity is even more pronounced. Consequently, the methyl groups in **8** are further apart and steric obstruction is much less of a problem. The reason for such structural diversity is the absence of the 11–12 bond (see Scheme 4). This bond is also missing in **3** and **5**, and therefore it can be presumed that the bond lengths and distance between the substituents at C10 and C13 are similar. With the substituents at C10 and C13 not being so close as corresponding ones in **1**, it can also be expected that the steric obstruction is less pronounced and that the carboxyl group of **5** is accessible for derivatization.



Scheme 4.

As seen in Figure 3, it is worth noting that the retention times (RP-C18 HPLC-column) of the amide derivatives follow surprisingly well an exponential growth equation with an R squared value of 0.9971. This may allow us to predict the chromatographic behaviour and lipophilicity of derivatives with different chain lengths attached in the same manner.

Of interest for future work, the lipophilicity of hypericins does have a major impact on the cellular uptake. Whereas the more lipophilic amides **10** and **12** were taken up limitedly, the hexylamide **9** accumulated approx. as well as hypericin itself. Significantly, also on RP-C18 HPLC compound **9** showed a similar behaviour and therefore featured a comparable lipophilicity/hydrophilicity balance as **1**. Consequently, it looks like the influence of the hydrophilic carboxy substituent present in **6** can be neutralized by the lipophilic hexyl group reverting the acid in a hypericin-like compound.

The data from the cell proliferation assay revealed that **1** and **9** displayed potent photo-induced effects. On the other hand, **10** and **12** showed much less photo-activity. To some extent these differences can be explained by the individual differences seen in cellular uptake. Less active hypericin derivatives were generally poorly taken up by the cells. However, the differences in activities observed between **1** and **9** cannot be explained in these terms, since these compounds are taken up intracellularly to a similar extent (difference $P > 0.05$), while their antiproliferative effects are very significantly ($P < 0.01$) different. Since a similar situation exists for compounds **10** and **12**, it can be concluded that not only the cellular uptake, but also the light-induced activity is affected by the

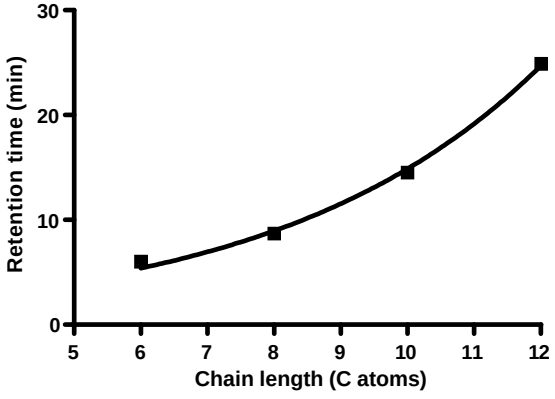


Figure 3. Retention times of the hypericin acid amide derivatives. *x*-axis: number of carbon units attached to hypericin acid, *y*-axis: retention times as assessed by RP18 HPLC.

introduced structural changes. It is clear that the mechanisms behind these observations are far from understood and require further detailed investigations.

4. Conclusion

The synthesis of new hypericin acid amides can be successfully achieved only when protohypericin acid is used as a precursor, not when hypericin acid itself with its highly inaccessible carboxyl group is the starting product. Afterwards the protohypericin acid amide can easily be converted into the hypericin acid amide by irradiation. We can also conclude that the hydrophilicity of the amide bond was balanced by the lipophilicity of the hexyl chain. In case of the other amide derivatives, the lipophilic character of the hydrocarbon chains prevailed and the compounds therefore turned out to behave more lipophilic than hypericin.

5. Experimental

Photochemical reactions were performed by irradiation with a 500 W halogen lamp. Electrospray mass spectrometry (ESMS) and nuclear magnetic resonance (NMR) spectrometry were used for structure confirmation. Mass spectrometry was also used for analysis of the reaction mixtures and chromatographic fractions. Accurate masses were obtained from high resolution electrospray mass spectra acquired in negative ionization mode on a quadrupole/orthogonal acceleration time-of-flight mass spectrometer (Q-Tof 2, Micromass, Manchester, UK). The peptide YGGFL was added as internal reference and used as lock mass compound.

NMR analysis was performed in deuteroacetone using a Varian Unity 500 (Varian, Palo Alto, CA, USA).

HPLC was performed on a Merck–Hitachi HPLC system (Schaumburg, USA) equipped with the Merck–Hitachi F-1050 Fluorescence Spectrophotometer and Purospher® RP-18 column (5 μm, 150–4.6 mm) (Merck, Darmstadt, Germany). Compounds were isocratically eluted with the acetonitrile, citrate buffer (pH 7), tetrahydrofuran mixture (50:30:20), using a flow rate of 1 mL/min. For all compounds, excitation wavelength was set at 470 nm and emission was measured at 600 nm.

Compound **1** was synthesized according to the previously published method.[3] ^{1}H NMR and ^{13}C NMR spectra were found to be identical to the previously described ones.[25]

Compound **6** was synthesized according to the previously published method.[21] ^{1}H NMR and ^{13}C NMR spectra were found to be identical to the previously described ones.[21]

Compound **5** was obtained as the intermediate in the synthesis of **6** and served as precursor for the hypericin acid amides.

5.1. General procedure for the synthesis of hypericin acid amides

The whole procedure was performed in subdued light conditions. Compound **5** (50 mg, 0.104 mmol) was dissolved in a mixture of acetic anhydride (5 mL) and sodium acetate (100 mg). The suspension was heated up to 120 °C and stirred for 3 h. After cooling down, an excess of water was added and the solution was extracted with ethyl acetate (20 mL). The water phase was discarded and the organic phase was washed twice with water. The ethyl acetate fraction was dried with anhydrous sodium sulfate and evaporated under reduced pressure. The reaction product contains a mixture of penta- and hexaacetoxy-derivatives, according to mass spectrometry analysis. The residue was dissolved in the mixture of dichloromethane (5 mL) and oxalyl chloride (2.5 mL, 2.9 mmol), dimethylformamide (two drops) was added and the mixture was stirred at room temperature for 5 h. Afterwards, the mixture was evaporated to dryness, re-dissolved twice in dry dichloromethane, evaporated and again dissolved in dichloromethane (5 mL). The amine (hexylamine, octylamine, decylamine or dodecylamine) (10 mmol) was then added and the mixture was stirred at room temperature for 14 h. The excess amine was extracted with buffer solution (5% NaH_2PO_4, pH 4.5), after which ethyl acetate was added to the mixture. The organic phase was washed twice with water, dried with anhydrous sodium sulfate and evaporated under reduced pressure. The residue was purified by column chromatography, using silica gel prepared in dichloromethane and eluted with dichloromethane/ethyl acetate (50:50), ethyl acetate and ethyl acetate/acetone (gradient, from 80:20 to 65:35). The relevant fractions were dissolved in acetone (0.2 mg/mL) and irradiated for 5 h. Pure amide derivatives were obtained after additional purification on a Sephadex LH20 column (Pharmacia, Uppsala, Sweden) eluted with a mixture of dichloromethane/methanol/acetone (gradient, from 55:30:15 to 35: 50:15). Purification afforded:

Compound **9**, 8 mg (overall yield from **5**: 13%) accurate mass calculated for $C_{36}H_{26}NO_9$: ($[M-H]^-$) 616.1607. Found: 616.1602.

Compound **10**, 10 mg (overall yield from **5**: 15%) accurate mass calculated for $C_{38}H_{30}NO_9$: ($[M-H]^-$) 644.1920. Found 644.1920.

Compound **11**, 5 mg (overall yield from **5**: 8%) accurate mass calculated for $C_{40}H_{34}NO_9$: ($[M-H]^-$) 672.2233. Found 672.2230.

Compound **12**, 12 mg (overall yield from **5**: 16%) accurate mass calculated for $C_{42}H_{38}NO_9$: ($[M-H]^-$) 700.2546. Found: 700.2543.

The NMR spectra for the different amide derivatives were almost identical, except for the overlapping signals at 1.29–1.27 ppm for HMR-spectra (hydrogens of the alkyl chains from β to ω-1) and about 29 ppm for CMR-spectra (carbons of the alkyl chains from δ to ω-3).

^{1}H NMR of **12** [$(CD_3)_2CO$]: δ = 14.75/14.73 (H1/H6 phenolic hydrogens), 14.53/14.41 (H8/H13 phenolic hydrogens), 7.85/7.27 (H9/H12 aromatic hydrogens), 6.32/6.28 (H2/H5 phenolic hydrogens), 2.9 (obscured by HOD, α-H alkyl), 2.82 (methyl at C11), 1.29-1.27 (alkyl hydrogens from β-H to [ω-1]-H), 0.85 (terminal methyl of the alkyl chain, ω-H). Assignments here were based mainly on literature values.[26]

^{13}C NMR of **12** [$(CD_3)_2CO$]: δ = 185.0/184.4 (C7/C14), 176.5/175.2 (C3/C4), 170.0/169.7 (C1/C7), 169.7 (overlapped, CO-amide), 163.6/163.1 (C8/C13), 146.1/143.1 (C10/C11), 128.3/128.2 (C6b/C14b), 123.1/122.2 (C7b/C13b), 120.4/119.5 (C7c/C14c), 119.6/118.4 (C9/C12), 119.6/119.3 (C3a/C3b), 112.7/109.8 (C7a/C13a), 106.5/106.3 (C2/C5), 103.5/103.2 (C6a/C14a), 40.9 (α-C alkyl), 32.6 ([ω-2]-C alkyl), 30 (overlapped signal, β-C alkyl), 29 (overlapped multiple alkyl C's from δ-C to [ω-3]-C), 28.0 (γ-C alkyl), 24.7 (methyl at C11), 23.3 ([ω-3]-C alkyl), 14.3 (ω-C alkyl) ppm.

5.2. 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 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) FCS, 1% (v/v) non-essential amino acids (Gibco-BRL, Paisley, Scotland, UK), 1% (v/v) antibiotic/antimycotic solution (Gibco-BRL, Paisley, Scotland, UK) and tylosine (60 μg/mL) (Eli Lilly, Bruxelles, Belgium).

5.3. Cellular accumulation

RT-112 cells were seeded onto 6-well tissue culture plates (Costar, Cambridge, MA, USA) at a concentration of 9×10^5 cells/well and incubated for 24 h at 37 °C. Subsequently, the medium was replaced with fresh medium containing 1 μM of hypericin, hypericin acid hexylamide, hypericin acid octylamide or hypericin acid dodecylamide and the cells were further incubated at 37 °C for 2 h. After the incubation, the cells were washed twice with PBS and harvested by treatment with trypsin solution (Gibco-BRL, Paisley, Scotland, UK). 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). After centrifugation (5 min, 6000*g*), the supernatant was collected and concentrated under reduced pressure. The residue was dissolved in 250 μL dimethylsulfoxide (DMSO) 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). Concentrations were calculated from calibration curves. The excitation and emission filters were set at 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, England, UK). The results were calculated assuming a mean volume of 3 μL/10^6 cells.[27] All experiments were performed in triplicate. The difference in the cellular uptakes was statistically evaluated using one-way ANOVA with Tukey–Kramer post test.

5.4. Photodynamic treatment

To determine the photocytotoxic effect of the compounds, RT-112 cells were seeded onto 96-well tissue culture plates (5×10^3 cells/well). After 24 h incubation, the medium was replaced with the fresh medium containing a range of concentrations (0.125–10 μM) of hypericin or analogues and the cells were further incubated at 37 °C for 2 h. Afterwards the cells were washed twice with PBS and irradiated for 10 min with a light dose of 1.8 J/cm^2, using a Rhodamine 6 G dye laser (375B, Spectra Physics, Mountain View, CA, USA) pumped by a 4 W Argon-laser (Spectra Physics) and coupled into a fibre optic microlens (Rare Earth Medical, West Yarmouth, MA, USA). The fluence rate at the surface of the tissue culture plate was measured with an IL 1400 radiometer (International light, Newburyport, MA, USA). The compounds were irradiated with a light of 595 nm wavelength. Afterwards cells were cultured for two more days and the relative proliferation (as compared to control) was determined with a MTT proliferation assay, based on the ability of mitochon drial enzymes to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide (Sigma, Steinheim, Germany) into purple formazan crystals. For this purpose, the medium was replaced by a 1 mg/mL solution of MTT in fresh medium and incubated for 4 h. Afterwards MTT solution was discarded and replaced with 200 μL DMSO. The concentration of formazan in the wells was determined by measuring the absorbance at 550 nm using a microplate reader. All experiments were performed in triplicate. After curve fitting with a non-linear regression model (GraphPad Prism, San Diego, CA, USA), the IC_{50} values (concentration which corresponds to the 50% of control value) were determined and statistically evaluated using one-way ANOVA with Tukey–Kramer post test. To establish activity in the dark, similar experiments were performed, except for the irradiation procedure.

Acknowledgments

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References and notes

1. Brockmann, H.; Pohl, F.; Maier, K.; Hashcad, M. N. *Justus Liebigs Ann. Chem.* **1942**, *553*, 1.
2. Brockmann H., Kluge F., U.S. Patent 2,727,704.

3. Falk, H.; Meyer, J.; Oberreiter, M. *Monatsh. Chem.* **1993**, *124*, 339.
4. Mazur Y., Bock H., Lavie D. U.S. Patent 5,120,412.
5. Rodewald, G.; Arnold, R.; Griesler, J.; Steglich, W. *Angew. Chem.* **1977**, *16*, 46.
6. Lavie, G.; Mazur, Y.; Lavie, D.; Meruelo, D. *Med. Res. Rev.* **1995**, *15*, 111.
7. D'Hallewin, M. A.; de Witte, P. A.; Waelkens, E.; Merlevede, W. J.; Baert, L. *J. Urol.* **2000**, *164*, 349.
8. Chen, B.; de Witte, P. A. *Cancer Lett.* **2000**, *150*, 111.
9. Kamuhabwa, A.; Roskams, T.; de Witte, P. A. M. *Int. J. Cancer* **2002**, *107*, 460.
10. Chen, B.; Roskams, T.; Xu, Y.; Agostinis, P.; de Witte, P. A. M. *Int. J. Cancer* **2002**, *98*, 284.
11. Liu, C. D.; Kwan, D.; Saxton, R. E.; McFadden, D. W. *J. Surg. Res.* **2000**, *93*, 137.
12. Hadjur, C.; Richard, M. J.; Parat, M. O.; Favier, A.; Jardon, P. *J. Photochem. Photobiol. B. Biol.* **1995**, *27*, 139.
13. Du, H. Y.; Olivo, M.; Tan, B. K.; Bay, B. H. *Int. J. Oncol.* **2003**, *23*, 1401.
14. Dougherty, J. T.; Gomer, J. C. C.; Henderson, B. W.; Jori, G.; Kessel, D.; Korbelik, M.; Moan, J.; Peng, Q. *J. Natl. Cancer Inst.* **1998**, *90*, 889.
15. Ochsner, M. *J. Photochem. Photobiol. B. Biol.* **1997**, *39*, 1.
16. Kamuhabwa, A.; Agostinis, P.; D'Hallewin, M. A.; Kasran, A.; de Witte, P. A. *Anticancer Res.* **2000**, *20*, 2579.
17. Kerb, R.; Brockmöller, J.; Staffeldt, B.; Ploch, M.; Roots, I. *Antimicrob. Agents Chemother.* **1996**, *40*, 2087.
18. Gruszecka-Kowalik, E.; Zalkow, L. H. *Org. Prep. Proced. Int.* **2000**, *321*, 57.
19. Falk, H.; Meyer, J. *Monatsh. Chem.* **1994**, *125*, 753.
20. Falk, H.; Schmitzberger, W. *Monatsh. Chem.* **1993**, *124*, 77.
21. Amer, A. M.; Falk, H.; Tran, H. T. N. *Monatsh. Chem.* **1998**, *129*, 1237.
22. Freeman, D.; Frolow, F.; Kapinus, E.; Lavie, D.; Lavie, G.; Meruelo, D.; Mazur, Y. *J. Chem. Soc., Chem. Comm.* **1994**, *33*(7), 891.
23. Wong, S. S. *Chemistry of Protein Conjugation and Cross-linking*; CRC Press: Boca Raton, Florida, 1993, p 199.
24. Rahimipour, S.; Palivan, C.; Barbosa, F.; Bilkis, I.; Koch, Y.; Weiner, L.; Fridkin, M.; Mazur, Y.; Gescheidt, G. *J. Am. Chem. Soc.* **2003**, *125*, 1376.
25. Kapinus, E. I.; Falk, H.; Tran, H. T. N. *Monatsh. Chem.* **1999**, *130*, 623.
26. Stalkos, D.; Tatsis, E.; Gerothanassis, I. P.; Troganis, A. *Tetrahedron* **2002**, *58*, 4925.
27. Lin, C. W.; Shulok, J. R.; Wong, Y. K.; Schanbacher, C. F.; Cinotta, L.; Foley, J. W. *Cancer Res.* **1991**, *51*, 1109.