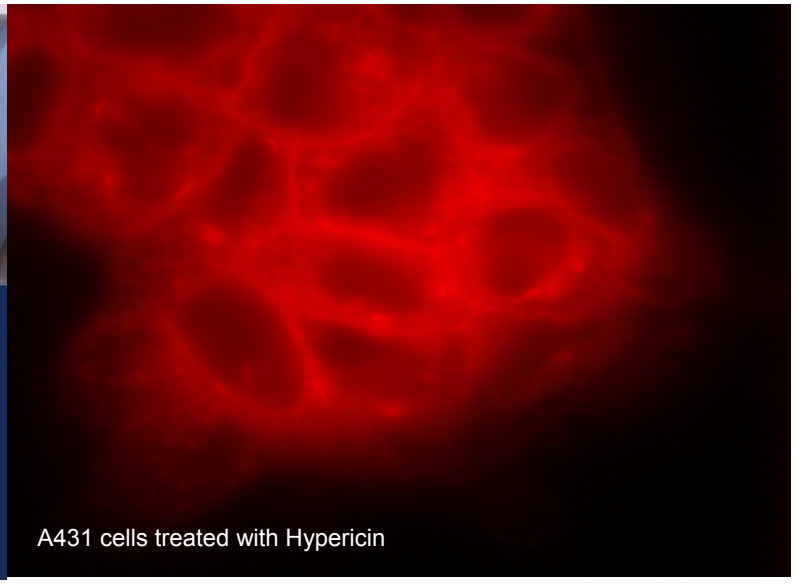
Detection of the Photosensitizer Hypericin

Tecan Ultra Evolution, Safire and GENios Pro


A431 cells treated with Hypericin

Introduction

Hypericin is a powerful, naturally occurring photosensitizer that is found in *Hypericum perforatum* plants, commonly known as St. John's wort. Hypericin (HY) is a polycyclic phenanthroperylenedione, which in cells binds mostly to the cellular membrane and can be metabolized rapidly in vivo with no toxic properties.

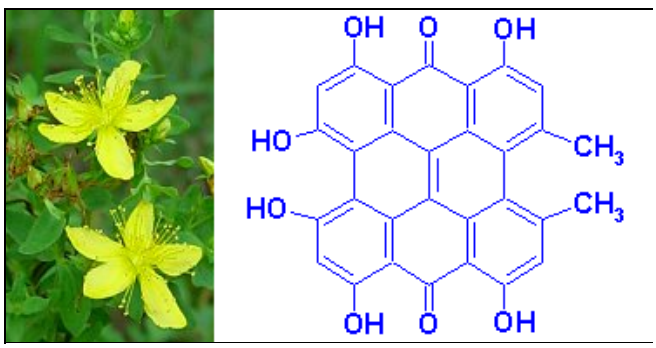


Figure 1: Plant of the genus Hypericum perforatum (left), chemical formula of Hypericin (right)

In recent years, there has been an increased interest in Hypericin as a potential clinical anti-cancer agent, since several studies established its powerful in vivo and in vitro anti-neoplastic activity when irradiated. Investigations of the molecular mechanisms underlying Hypericin photocytotoxicity in cancer cells have revealed that this photosensitizer can induce both apoptosis and necrosis in a concentration and light dose-dependent fashion. Photodynamic therapies (PDT) with Hypericin result in the activation of multiple pathways that can either promote or counteract the cell death program. (1, 2, 3)

Material and Methods

Cell culture: A431 cells (human epidermoid carcinoma cells, ATCC No. CRL-1555) were kindly provided by Prof. Barbara Krammer, University of Salzburg, and maintained in culture in 'standard medium' (DMEM high glucose, Sigma, D-5671) supplemented with 5 % heat inactivated fetal calf serum (FCS, PAA laboratories, Austria) in an atmosphere of 5 % CO_2 at 37 °C. For treatment with the photosensitizer Hypericin cells were grown in 96-well plates (COSTAR, black, transparent bottom; Szabo-Scandic, Austria, Cat. No.: 3603) at a density of 7500 cells/well.

Staining: After an overnight incubation, the culture medium was removed, the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS) and then treated with 100 µl of 50, 100 or 200 ng/ml Hypericin (Fluka) in DMEM without FCS at 3 different time points. Cells were incubated for 29 hrs in an atmosphere of 5 % CO_2 at 37 °C. Measurements were performed at 6 different time points .

Instruments: Tecan Ultra Evolution; Tecan GENios Pro; Tecan Safire.

Measurements: Hypericin uptake was measured after different incubation times at Ex 485 nm/ Em 590 nm.
Measurements were taken before and after replacing the medium (containing Hypericin) with PBS. Either single measurements (one measurement point per well) or multiple reads per well (circle, 4x4) were performed (see Figure 4 for geometrical view).
In addition the excitation and emission spectra of cells treated with Hypericin have been recorded with a Tecan Safire.

Microscopy: Localization of Hypericin was analyzed using cells grown on cover slips which were incubated with 200 ng/ml Hypericin in DMEM without FCS for 3 hrs (Figure 2). Hypericin accumulation in cells was then visualized using oil immersion and a 1000x magnification on an inverted fluorescence microscope (Olympus IX70). Pictures were taken using the appropriate filter block SWG (Ex 480 - 550 nm, dichroic filter 570 nm, Em 590 nm) and a Spot II CCD camera (Diagnostic Instruments).

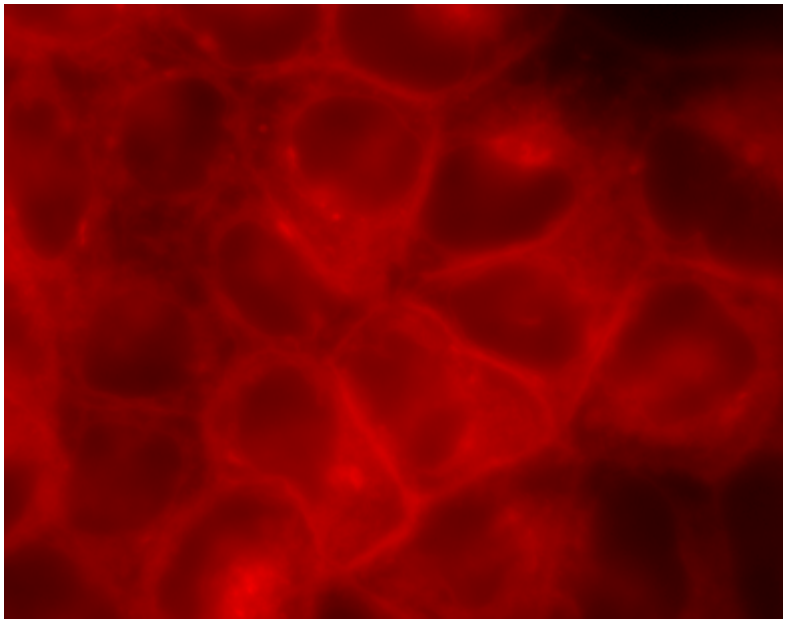


Figure 2: Localization of Hypericin in cells. Picture was taken 3 hrs after adding 200 ng/ml Hypericin

Results

Spectra: Excitation and emission spectra of Hypericin have been recorded (Figure 3). The excitation peaks were found at **~ 480 nm** and **~ 560 nm**, the emission maximum at **~ 610 nm**. Based on these findings the excitation filter 485 nm and the emission filter 590 nm were selected for further measurements.

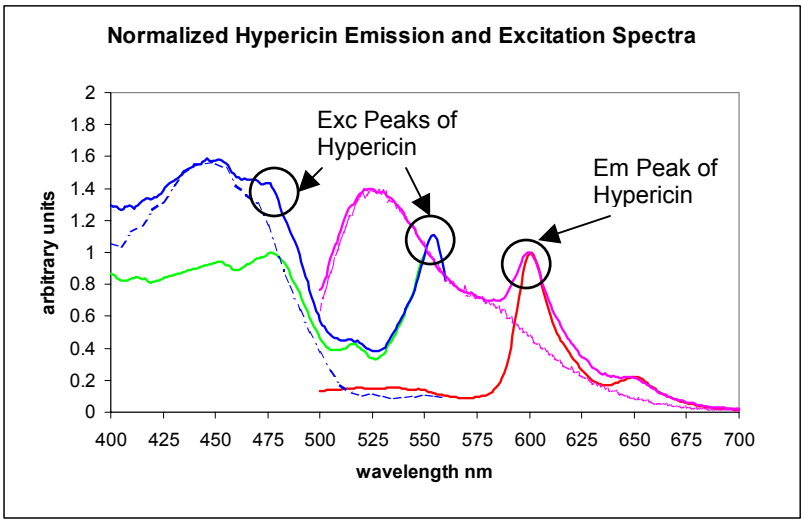


Figure 3: Normalized excitation and emission spectra of Hypericin treated cells in different media (Excitation spectrum of Hypericin in medium; Excitation spectrum of Hypericin in PBS; Emission spectrum of Hypericin in medium; Emission spectrum of Hypericin in PBS), the dashed blue line shows the excitation spectrum and the dashed pink line shows the emission spectrum of the medium (without Hypericin).

Comparison single measurement vs. multiple reads per well: Figure 5 shows the results of a single measurement versus a multiple reads per well measurement of cells kept in PBS. Measurements were taken with a Tecan Safire after 5.5 hrs incubation time. The results are comparable with those of Tecan Ultra Evolution and GENios Pro. The measurements were performed with the following measurement parameters: bottom reading: a) single measurements: excitation at 485 nm (20 nm bandwidth except Safire: 12 nm); emission at 590 nm (20 nm bandwidth except Safire: 12 nm), 40 µs integration time, 10 flashes; b) multiple reads per well: same as a) but circle, 4x4; 3 flashes per measurement point (see figure 4 for geometric view).

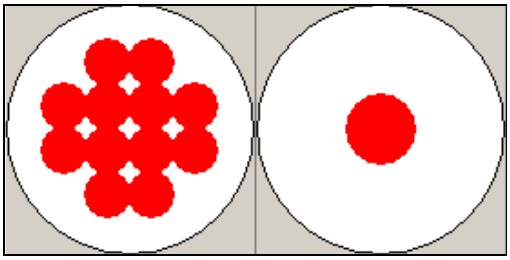


Figure 4: The picture on the left hand side shows a schematic view of the distribution of measurement points when using the multiple reads per well function (circle, 4x4). On the right side a single measurement is displayed.

Comparison Medium vs. PBS: Cells treated with Hypericin were measured either kept in medium or maintained in PBS. The measurements were performed with the following measurement parameters: bottom reading: excitation at 485 nm (20 nm bandwidth except Safire: 12 nm); emission at 590 nm (20 nm bandwidth except Safire: 12 nm), 40 µs integration time, multiple reads per well (circle, 4x4), 3 flashes. Figure 6 shows the results of a Tecan Ultra Evolution after 3.5 hrs incubation time. The measurements with Tecan Safire and GENios Pro showed comparable results.

Hypericin signal over time: Cells were measured after replacing standard medium with PBS after 5.5, 6.5, 7.5 hrs and after overnight incubation after 27, 28 and 29 hrs. The measurements were performed with the following measurement parameters: bottom reading: excitation at 485 nm (20 nm bandwidth except Safire: 12 nm); emission at 590 nm (20 nm bandwidth except Safire: 12 nm), 40 µs integration time, multiple reads per well (circle, 4x4), 3 flashes. The results of Tecan GENios Pro over a time course of 29 hrs are displayed in Figure 7.The result is representative for Tecan Ultra Evolution and Safire.

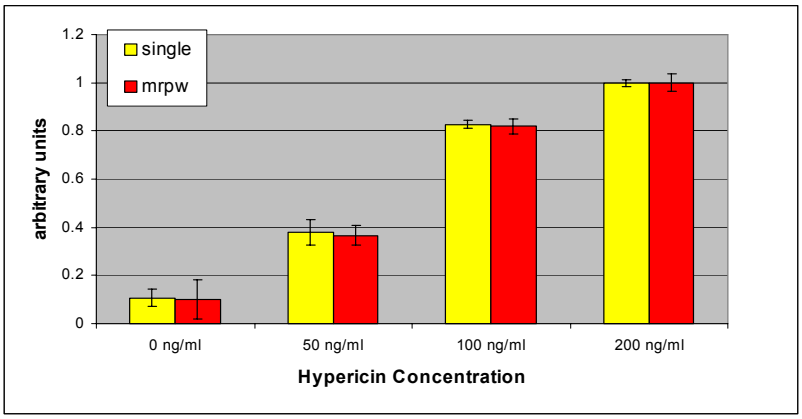


Figure 5: Comparison of plates measured with single measurements (single, yellow column) or multiple reads per well (mrpw, red column). Measurement was performed with a Tecan Safire after 5.5 hrs incubation. Data are normalized to get rid of intensity differences due to different gain settings.

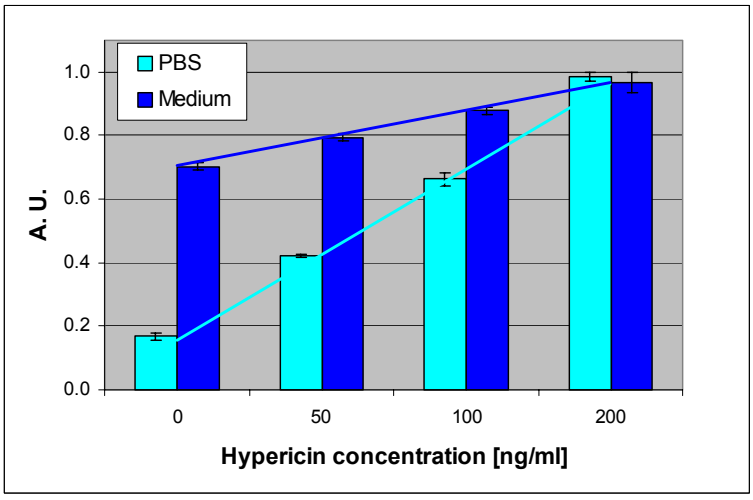


Figure 6: Comparison of plates measured with medium (dark blue) or PBS (blue) with a Tecan Ultra Evolution after 3.5 hrs incubation. Data were normalized to get rid of intensity differences due to different gain settings. The lines indicate a linear regression showing the different slopes of the increasing signal.

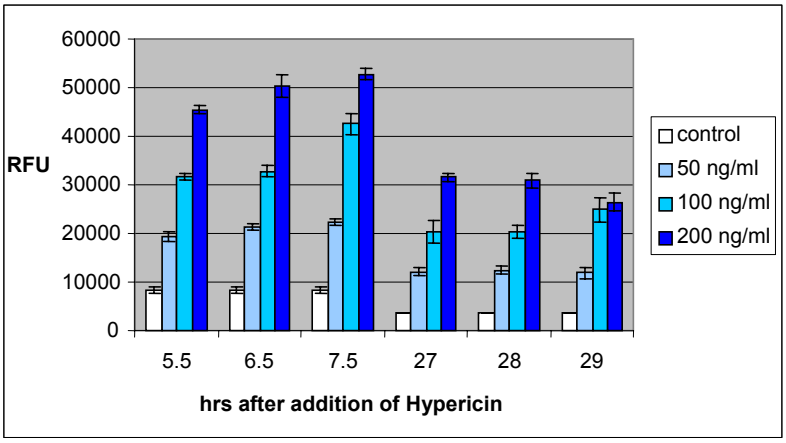


Figure 7: Hypericin signal over time measured with a Tecan GENios Pro.

Discussion

The data above clearly show the ability of Tecan Ultra Evolution, Safire and GENios Pro to detect the photosensitizer Hypericin with the bottom reading option of the instruments.

The excitation and emission spectra of Hypericin (Figure 3) indicate the influence of different media on the Hypericin signals. Figure 2 shows two of the three excitation peaks of Hypericin. The third excitation peak (at about 610 nm) is not shown because the emission wavelength was set to 600 nm for the scan measurements. A high level of background fluorescence was observed when measuring the cells with medium. This fluorescent background could be decreased when replacing medium with PBS. However the Hypericin uptake was detectable with both assay conditions, cells kept in medium or PBS (Figure 6).

The more or less homogenous distribution of cells in the wells can also influence the measurement results. The measurement mode 'Multiple reads per wells' is rather time-consuming but might improve measurement results especially when having inhomogeneous cell distributions or cell clustering. Multiple reads per well were therefore taken and compared with single reads per well (see Figure 4). There were no significant differences between the results indicating that for this type of measurements a single reading per well and therefore shorter measurement time is sufficient (Figure 5).

The time course of the uptake of different Hypericin concentrations is shown in Figure 7. However, the long incubation time of more than 24 hrs is not necessary. After about 8 hrs (50 ng/ml Hypericin) and 7 hrs (100 and 200 ng/ml Hypericin), respectively, the ratio of the signal of Hypericin-treated cells to control cells does not change any more (data not shown).

Acknowledgement

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(3) **Agostinis P, Vantieghem A, Merlevede W, de Witte PA**: Hypericin in cancer treatment: more light on the way. Int J Biochem Cell Biol 2002 Mar;34(3):221-41

Glossary

DMEM	Dulbecco's modified eagle's medium
DPBS	Dulbecco's phosphate buffered saline
Em	Emission
Ex	Excitation
FCS	Fetal calf serum
HY	Hypericin
mrpw	Multiple reads per well
PBS	Phosphate buffered saline
PDT	Photodynamic therapy

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