In vitro photodynamic therapy of childhood rhabdomyosarcoma

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Abstract. Treatment of childhood rhabdomyosarcoma is limited by recurrent disease and the development of multidrug resistance. Therefore, novel treatment options are desirable. Photodynamic therapy (PDT) using the photodynamic agent hypericin is proposed as an alternative approach for intra-operative visualization and treatment of this disease. The aim of this study was to investigate *in vitro* effects of hypericin on childhood rhabdomyosarcoma and to evaluate photodynamic therapy as a possible basis for treatment. Rhabdomyosarcoma cells and fibroblasts (control) were incubated with increasing concentrations of hypericin. *In vitro* uptake and visualization of hypericin was evaluated by fluorescence microscopy and FACS. For photodynamic therapy, cells were exposed to white light for different time periods. Cytopathologic effects were assessed using standard histology. Cancer cells were investigated for cell viability (MTT assay), proliferative activity (Ki-67 assay), and apoptosis (TUNEL test). A 100% uptake of hypericin was found within the population of rhabdomyosarcoma cells. Uptake of hypericin in the fibroblasts was much less than in rhabdomyosarcoma cells. Hypericin without exposure to white light had no effect on tumor cell viability. After irradiation, PDT resulted in a nearly complete inhibition of cell proliferation of rhabdomyosarcoma cells with a corresponding increase in the frequency of apoptosis. In fibroblasts, PDT was less effective compared to tumor cells. Our data suggest hypericin as a novel tool for visualization and photodynamic therapy of childhood rhabdomyosarcoma.

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Abbreviations: ALA, 5-aminolevulinic acid; MDR, multidrug resistance; MTT-assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide-assay; PDT, photodynamic therapy; RMS, rhabdomyosarcoma; TUNEL test, terminale desoxyribosyl-transferase mediated dUTP nick-end labeling test

Key words: rhabdomyosarcoma, hypericin, *in vivo* visualization, photodynamic therapy

Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children. About two thirds of all sarcomas and 7-8% of all solid malignant tumors in childhood are rhabdomyosarcomas (1). The two main histopathological subtypes of this malignancy in children are embryonal and alveolar RMS (2). Specific genetic alterations (3) such as the translocation t(2;13)(q35;q14) which occurs in 55% of all cases and t(1;13)(p36;q14) in 22% contribute to the diagnosis of alveolar RMS (1). No specific genetic alterations are found in embryonal rhabdomyosarcomas (1).

The prognosis of these tumors is still poor and therapy is limited due to recurrent disease, development of metastases and multidrug resistance. Radical surgery plays a key role for the prognosis of these tumors. Mutilating surgery is often necessary for survival. Surgical procedures are complicated due to a lack of possible visualization of these tumors *in vivo*.

In vivo visualization of childhood rhabdomyosarcoma can be performed using fluorescent proteins. Tumors and their behaviour can be studied *in vivo* using this imaging tool (4). Up to now, fluorescent proteins are mainly a basic research tool and have not been used in humans. Therefore, the investigation of other treatment modalities is of increasing interest.

Photodynamic therapy (PDT) can be performed after local or systemic administration of a photodynamic drug. Exposure to light and the presence of oxygen result in tissue damage through development of oxygen radicals (5). Hypericin is a hydroxylated phenanthroperylenequinone derivate and is found in plants of the species *Hypericum* (5,6). The most common species is *Hypericum perforatum* (5). Hypericin produces singlet oxygen efficiently after light irradiation with a quantum yield of 0.73 (5,6).

The aim of this study was to investigate *in vitro* effects of hypericin on childhood rhabdomyosarcoma cells. The potential of *in vitro* tumor visualization was investigated and possible photodynamic therapy was evaluated as a basis for further treatment studies in animals and humans.

Materials and methods

Cell lines and culture conditions. The alveolar rhabdomyosarcoma cell line Rh 30 (DSMZ, Braunschweig, Germany) and the embryonal rhabdomyosarcoma cell line A204 (ATCC, USA) were cultured in DMEM medium (Gibco, Berlin, Germany) supplemented with 10% fetal calf serum, 1% L-Glu and 2.5% HEPES in a humidified atmosphere containing 10% CO_2 at 37˚C. The NIH3T3 fibroblasts were kindly provided by Olaf Heydenreich (Department of Cell Biology, University of Tuebingen, Germany). The fibroblasts were cultured under the same conditions as the tumor cell lines. These cells served as a control group. All cells were mycoplasma negative.

Hypericin experiments. Alveolar and embryonal rhabdomyosarcoma cells (Rh 30, $5x10^4$; A204,$3x10^4$) and fibroblasts (NIH3T3: $5x10^4$) were seeded in 8-well chamber slides (Falcon-Becton Dickinson Labware, Franklin Lakes, NJ, USA, #354108, 200 μl/well) and were cultured as described above. After 24 h, medium was removed and hypericin (Phytochem, Neu-Ulm, Germany) dissolved in DMSO was added in increasing concentrations (0, 10, 15, 25 and 50 μM). Cells were incubated for 2 h in a humidified atmosphere containing 10% CO_2 at 37˚C without exposure to light. After 2-h incubation, the hypericin solution was replaced by standard medium. Hypericin uptake by tumor cells and fibroblasts was then evaluated by fluorescence microscopy [Zeiss AxioVision, Jena, Germany, Filter set 15, #488015-0000, excitation 546 nm (+12/-12 nm), emission 570-650 nm, emission 590 nm].

For photodynamic therapy, cells were incubated with hypericin as described above and exposed to white light - considering the mandatory spectrum - (1000 Lux, Osram, Germany) under a clean bench (Heraeus, HLB 2472 GS, Germany) for 0, 10, 20 and 30 min, respectively. Cells were then cultured in the dark until day-5. At day-5, cell counts were performed after trypsination using a Neubauer counting chamber and light microscopy (magnification x40, Zeiss). Experiments were performed in triplicate.

FACS analysis. Cells were incubated with hypericin at different concentrations for 2 h as described above until trypsination. All experiments were performed in the dark. Hypericin uptake was analysed using a FACS CANTO flow cytometer (BD Heidelberg, Germany) and Flow Jo software (Tree Star, Inc., Stanford, USA). Fluorescence intensity of hypericin was detected using a blue-green laser with excitation at 488 nm and emission at 575 nm. The percentage of labelled cells and mean fluorescence intensity of labelled cells were calculated using the unlabelled cells as background. The hypericin uptake was estimated by the mean fluorescence index (MFI) which was the ratio between the mean fluorescence of the positive cell population and of the negative control.

Cell viability. Cell vitality after 5 days was assessed by an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-assay (Biomedica EZ4U, Biozol, Germany) (13). According to the manufacturer's guidelines, substrate was dissolved in activator solution and 20 μl was added to each well. Incubation was performed for 5 h and absorption was then measured using an ELISA reader (Tecan Spectra Mini, Grödig, Austria) at 450 nm against a reference at 620 nm.

Cell proliferation. For cell proliferation studies, a Ki-67-immunocytochemistry assay was performed on tumor cells at day-5. A maximal hypericin concentration of 25 μM was used for these experiments due to complete destruction of tumor cells caused by higher hypericin doses. Cells were therefore rinsed in PBS and then fixed with Roti-Histofix (Roth, #2213.3, Germany) for 10 min. After fixation, cells were again rinsed in PBS for 5 min. Blocking was performed using 1.5% normal goat serum (Dako, #X0907, Glostrup, Denmark) for 30 min followed by monoclonal mouse anti-human Ki-67-primary antibody-clone MIB-1 (DakoCytoformation, #M7240, Glostrup, Denmark, dilution 1:100) for 60 min at room temperature. Slides were rinsed for 3x5 min in PBS. Then, the secondary FITC-conjugated goat anti-mouse antibody (Dianova, #115-095-062, Hamburg, Germany, dilution 1:100) was added for 30 min at room temperature, followed by a 3x5-min PBS washing step. For counterstaining of the nucleus, DAPI was used for 1 min (Sigma-Aldrich, #D-9452, Germany, dilution 1:10000) followed by 3x5 min in PBS. Slides were mounted with mounting medium (Dako) and analyzed using fluorescence microscopy (Zeiss AxioVision).

TUNEL test. For investigations on induced apoptosis in tumor cells, a Terminal desoxyribosyl-transferase-mediated dUTP nick-end labeling test (TUNEL) was performed at day-5. Different concentrations of hypericin (0, 10, 15, 25 and 50 μM) and a constant irradiation time of 20 min were used for these experiments. Therefore, chambers were removed and slides were rinsed in PBS. Cells were fixed with Roth-Histofix (Roth, Munich, Germany) and again rinsed in PBS (3x). Slides were then incubated in 0.1% Triton/PBS (Merck, Darmstadt, Germany) on ice for 2 min and rinsed with PBS. TUNEL reaction solution (50 μl/well, Roche Diagnostics, #1684795, Pinzberg, Germany) was added for 60 min at 37˚C. For positive controls, cells were incubated with DNase I (1:10 in PBS, 15 min at room temperature, Roche Diagnostics). The TUNEL reaction solution was then added as described above. For negative controls, 50 μl/well of labeling solution (Roche Diagnostics, #1684795) was added. Slides were rinsed in PBS (3X). For nuclear-counterstaining, DAPI was used for 1 min (Sigma-Aldrich, #D-9452, Munich, Germany, dilution 1:10000) followed by 3x5 min PBS. Slides were mounted with mounting medium (Dako) and analyzed using fluorescence microscopy (Zeiss AxioVision).

Statistical analyses. Statistical analysis on cell number and the MTT assay was performed between the groups using one-way Anova on the ranks test and Student's t-test. All numeric data are expressed as mean ± SEM. Significance was assumed for all results with $p<0.05$. FACS data were analyzed by linear regression and slope confidence intervals of the MFI vs. concentration curves using GraphPad Prism (version 4.00 for Windows, GraphPad Software, San Diego, CA, USA).

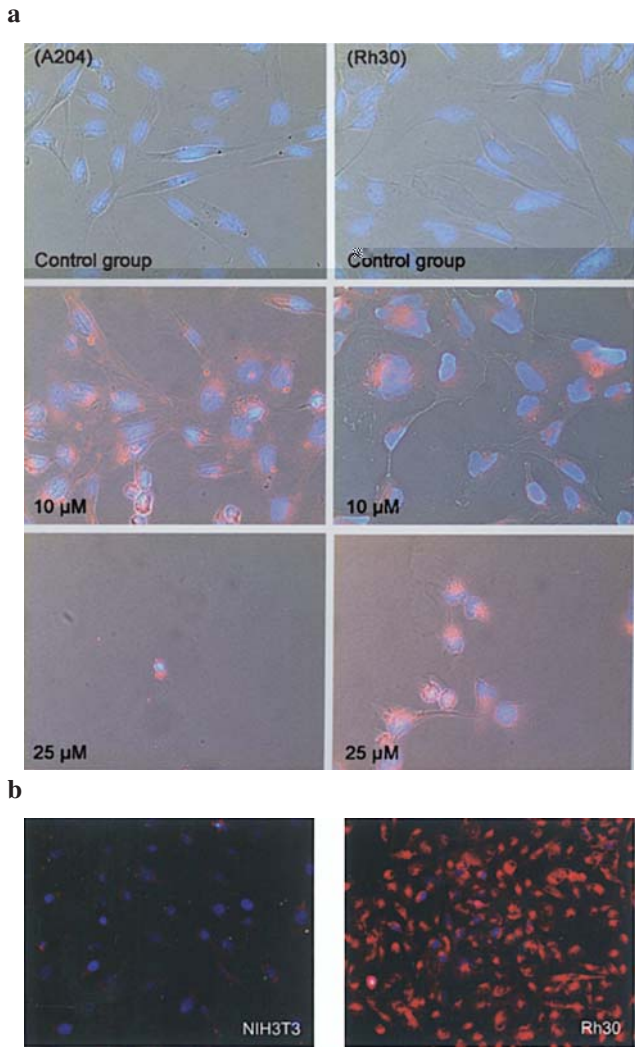

Figure 1. a, changes in tumor cell morphology at different concentrations of hypericin (irradiation time 20 min) in embryonal and alveolar rhabdomyosarcoma (blue, DAPI staining of cell nucleus; red, hypericin uptake, x40): shrinkage of tumor cell cytoplasm and nucleus. b, uptake of hypericin (10 μM) in NIH3T3 fibroblasts compared to alveolar rhabdomyosarcoma Rh30 showing a much weaker fluorescence signal in fibroblasts.

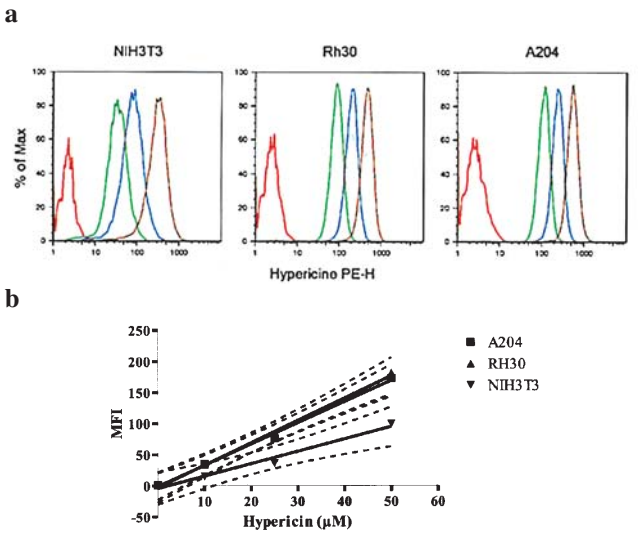

Figure 2. Hypericin uptake into rhabdomyosarcoma cells and fibroblasts. a, FACS-analysis of untreated control cells (red line) and cells treated with hypericin 10 μM (green line), 25 μM (blue line), or 50 μM (brown line). Increase of fluorescence intensity with hypericin concentration was observed. b, linear dependency of the mean fluorescence index (MFI) of hypericin concentration is shown as linear regression with a CI of 95%. Uptake of hypericin into rhabdomyosarcoma cells is significantly increased compared to fibroblasts.

Results

Biodistribution and cell morphology. Application of hypericin resulted in a complete uptake of hypericin in all tumor cells leading to strong fluorescence signals. In comparison, uptake of hypericin in fibroblasts was also found in all cells, but the fluorescence signals were definitely weaker (Fig. 1). Quantitative assessment of cellular hypericin fluorescence using FACS analysis revealed significant differences between fibroblasts and tumor cells (Fig. 2). Both tumor cell lines showed significantly higher hypericin induced fluorescence compared to fibroblasts as revealed by the slope of the MFI vs. concentration curve. The confidence intervals (CI of 95%) were 2.656 to 4.146 for A204, 2.727 to 4.444 for RH30 and 1.051 to 2.897 for NHI3T3. For estimation of possible changes in cell morphology, cells were examined using light microscopy. After hypericin treatment without light exposure, the cellular morphology as well as plasma to nucleus ratio were similar compared to untreated rhabdomyosarcoma cells. Exposure to light (20 min) and application of hypericin resulted in a shrinking of tumor cell nuclei and cytoplasm starting at a dose of 10 μM hypericin. Fibroblasts showed nearly no changes in cell morphology (Fig. 1).

Cell numbers. In embryonal rhabdomyosarcoma, exposure of tumor cells to light without application of hypericin had no effect on the total number of tumor cells. Application of hypericin combined with light exposure led to reduced tumor cell numbers. Photodynamic therapy (PDT) with a minimal concentration of 25 μM hypericin combined with a minimal exposure time to light of 20 min resulted in a nearly complete disappereance of the cells after 5 days. At 50 μM hypericin no viable tumor cells were present (Fig. 3a).

In alveolar rhabdomyosarcoma, a higher concentration of hypericin or a longer duration of photodynamic therapy was required to obtain similar effects as observed in embryonal rhabdomyosarcoma. Application of 25 μM hypericin with exposure to light for 30 min led to similar results as observed in embryonal rhabdomyosarcoma with 20 min. In both groups only the combination of white light for more than 20 min and hypericin concentration higher than 25 μM resulted in sufficient PDT (Fig. 3b).

Cell viability. The application of hypericin to embryonal rhabdomyosarcoma cells without light treatment led only to a partial reduction of viable cells (maximum 23% inhibition). Application of light alone did not influence cell viability. Combining hypericin and light resulted in a complete disappearance of viable cells depending on concentration and time. The percentage of non-viable cells was 92.8% (±0.9, $p<0.05$) after 20 min of PDT using 25 μM hypericin, 99.2% (±0.1, $p<0.05$) after 30 min of PDT and 25 μM hypericin, and 99.5% (±0.1; $p<0.05$) after 20 min PDT and 50 μM hypericin (Fig. 3c).

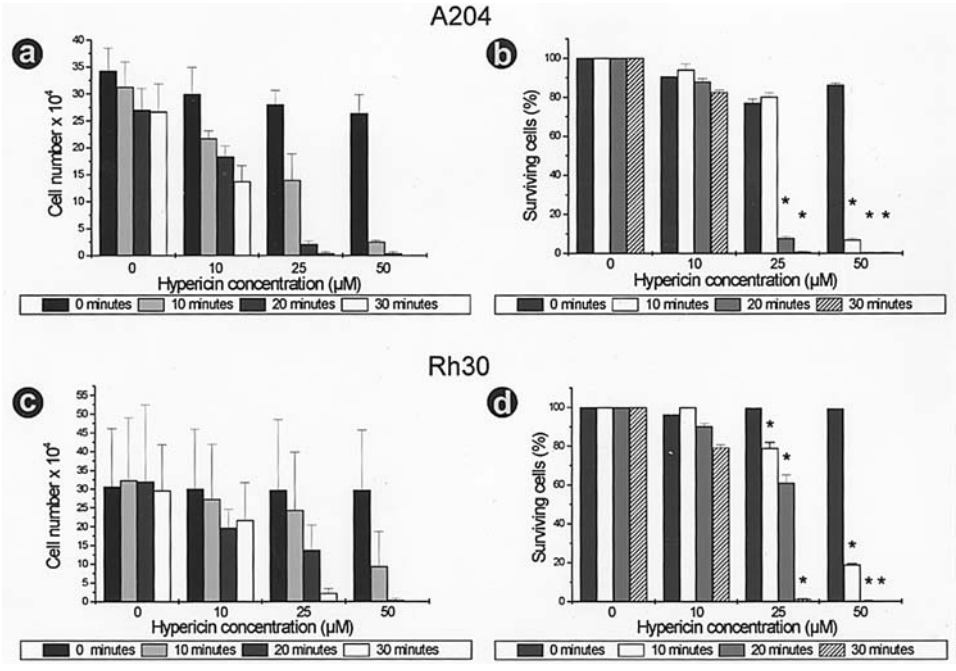

Figure 3. a, cell numbers at different concentrations of hypericin in embryonal rhabdomyosarcoma (cell line A204, see text for details). b, percentage of surviving tumor cells at different hypericin concentrations and different irradiation times in embryonal rhabdomyosarcoma (cell line A204, $^{*}p<0.05$). c, cell numbers at different concentrations of hypericin in alveolar rhabdomyosarcoma (cell line Rh30, see text for details). d, percentage of surviving tumor cells at different hypericin concentrations and different irradiation times in embryonal rhabdomyosarcoma (cell line A204, $^{*}p<0.05$).

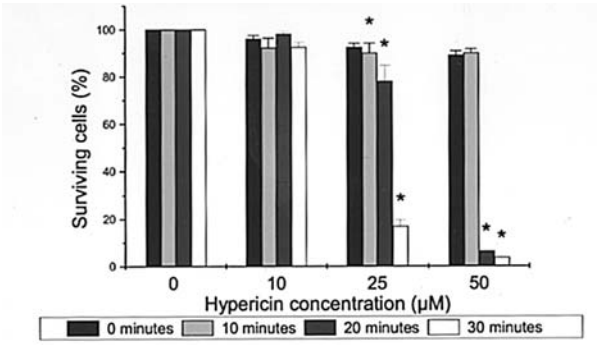

Figure 4. Survival rates of NIH3T3 fibroblast cell line after PDT at different hypericin concentrations and irradiation times ($^{*}p<0.05$).

In alveolar rhabdomyosarcoma, treatment with either hypericin or light did not have any influence on cell viability. However PDT for 30 min combined with 25 μM hypericin resulted in a nearly complete erradication (98.7 avital cells ± 0.1%, $p<0.05$) of viable cells. A complete disappearence of tumor cells was found using 50 μM hypericin and PDT for 20 min (99.7 non-viable cells ± 0.3%, $p<0.05$) (Fig. 3d).

Neither cell proliferation nor cell viability could be detected 96 h after PDT (30 min, 50 μM hypericin) in both tumor cell lines.

In fibroblasts, treatment with either hypericin or light did not have any influence on cell viability. PDT was less effective in fibroblasts than in tumor cells. PDT for 20 min combined with 25 μM hypericin produced only 22% non-viable cells. There were remaining viable cells at 50 μM hypericin and light exposure for 30 min (96.33 non-viable cells ± 0.33%, $p<0.05$) (Fig. 4).

Cell proliferation. Cell proliferation tests were performed to further evaluate the effects of PDT. In embryonal rhabdomyosarcoma, Ki-67 immunocytochemistry revealed a reduction of cell proliferation with higher concentrations of hypericin. At a concentration of 25 μM, hypericin resulted in nearly a complete reduction of proliferative activity (Fig. 5a). In alveolar rhabdomyosarcoma, there was still a slight proliferative activity noticeable at 25 μM hypericin (Fig. 5b).

Apoptosis. Induction of apoptosis in alveolar rhabdomyosarcoma was detected by the TUNEL assay. Apoptotic bodies were detected at hypericin concentrations at 10 μM (Fig. 6a). In embryonal rhabdomyosarcoma, the TUNEL test revealed apoptotic cells at 10 μM hypericin. At 25 and 50 μM hypericin, a nearly complete disappearance of tumor cells was found. In one case, a few apoptotic bodies were detected (Fig. 6b).

Discussion

Photodynamic therapy (PDT) is currently studied as a novel treatment approach in various malignancies producing relevant cytotoxic effects by the photochemical generation of reactive oxygen after light activation (7). Many substances such as 5-aminolevulinic acid (ALA) (8) and hypericin have been proposed for this purpose (9). Hypericin, is an extract from the St. John's wort, which is a herb occurring in Europe and Asia. ALA is an intermediate in heme biosynthesis in the body. ALA is rapidly bleached out, and exposure to light is limited to short illumination periods (9). In contrast, hypericin-induced fluorescence is stable and hypericin has a lower fluorescence clearence than ALA (10). Therefore, hypericine seems to be a promising photodynamic agent.

Hypericin has primarily been used for the treatment of depressive disorders (11). In recent years, hypericin has been

described for *in vivo* visualization of bladder cancer (10) and has been studied as a photodynamic agent in tumors such as transitional cell carcinoma of the bladder (12,13), colon cancer (14), squamous-cell carcinoma (15), glioblastomas (16), radiation-induced murine fibrosarcoma (17) and nasopharyngeal carcinoma (18). These studies revealed impressive anticancer effects regarding tumor growth and anti-angiogenic effects (16,18). Interestingly, hypericin had also anti-metastatic and anti-angiogenic effects in the dark (19,20).

Rhabdomyosarcoma is associated with poor survival in advanced tumor stages. Therapy is limited to surgery, chemotherapy and radiation therapy. In surgery, complete tumor resection is essential for survival and is one of the main prognostic factors to prevent local tumor recurrence. Thus, novel diagnostic and therapeutic options are desirable leading to investigation of alternative approaches such as hypericin PDT (1).

We studied hypericin effects using alveolar and embryonal rhabdomyosarcomas as well as fibroblasts (for control), with different concentrations of hypericin and exposure to white light at different times. A complete uptake of hypericin in all tumor cells, which was demonstrated by fluorescence microscopy and FACS analysis, was found. PDT with hypericin caused major changes in cell morphology. Fibroblasts showed a distinct weaker uptake of hypericin and showed no changes in cell morphology. Selective accumulation of hypericin in tumor cells was also reported by Hendrickx *et al* (7) and Du *et al* (21). Ritz *et al* reported a possible discrimination between glioblastoma cells and neurons; glioblastoma cells were discriminated from neurons by higher fluorescence intensity (22). Therefore, the use of hypericin for *in vivo* detection of childhood rhabdomyosarcoma should be analyzed. This could contribute to an improved identification of safety margins intraoperatively especially in difficult anatomical regions such as the bladder or pelvis.

PDT with hypericin resulted in a significant reduction of tumor cell number as well as a reduction of proliferative activity of cancer cells along with induction of apoptosis in a dose-dependent manner in childhood rhabdomyosarcoma. Our results correlate to previous animal models of hypericin PDT in which vascular damage and induction of apoptosis were reported (23). PDT seems to be more effective in tumor cells than in normal cells. PDT with hypericin could be easily used *in vivo* during surgery. After resection of the tumor, hypericin could be administered to the patient and light from the OR light system could be used for PDT. Therefore, PDT could be directly applied to the tumor and the surrounding tissue and could help to destroy unresected tumor cells.

Another common treatment problem in childhood rhabdo-

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on a human adenocarcinoma cell line. Tian *et al* described an induction of P-glycoprotein, which is contradictory to the above mentioned study (14). The analysis of hypericin PDT effects on MDR gene regulation in childhood rhabdomyosarcoma will have to be assessed in order to achieve improved chemotherapy results in further studies.

One major side effect of hypericin PDT is the so called hypericism, which is a severe photodermatitis in grazing animals caused by UV light exposure after ingestion of St. John's wort (25). On the other hand, hypericin is already safely used as a diagnostic and therapeutic tool in bladder cancer (5). Animal studies in mice showed no severe side effects of the drug if animals were kept in the dark (5,26,27). Hypericin is additionally useful in retroviral infections without major side effects (26,28). No mutagenic potential was found in *hypericum* extracts (27). Therefore, we believe that hypericin could be used for PDT in children suffering from rhabdomyosarcoma without severe side effects.

Finally, we conclude that hypericin is a promising novel tool for *in vivo* diagnosis and photodynamic therapy of childhood rhabdomyosarcoma *in vitro*. Hypericin seems to be more effective in tumor cells. This model allows further investigations in animals (using optical imaging) and humans and might help to establish novel treatment options in children. The use of the presented approach would be easily transferable to clinical conditions since normal OR light systems during surgery are sufficient.

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