Increasing the efficiency of hyperthermic intraperitoneal chemotherapy (HIPEC) by a combination with a photosensitive drug in pediatric rhabdomyosarcoma

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December 21, 2021

Abstract

Background: Cytoreductive surgery (CRS) in combination with hyperthermic intraperitoneal chemotherapy (HIPEC) is an option in advanced peritoneal sarcomatosis. Nevertheless, CRS and HIPEC are not successful in all patients. An enhancement of HIPEC using photodynamic therapy might be beneficial. Therefore, a combination of the photosensitizer Hypericin (HYP) with HIPEC was evaluated in an animal model. Procedure: An established HIPEC animal model for rhabdomyosarcoma (NOD/LtSz-scid IL2Rγnullmice, n=80) was used. All groups received HYP (100 µg/200 µl) intraperitoneally with and without cisplatin-based (30 or 60 mg/m2) HIPEC (37 or 42 °C, for 60 min) (five groups, each n=16). Tumor dissemination was documented visually and by using HYP-based fluorescence guidance. HYP-based photodynamic therapy (PDT) of the tumor was performed. Finally, tissue samples were evaluated regarding proliferation (Ki-67) and apoptosis (TUNEL). Results: HYP uptake even in smallest tumor nodes (< 1 mm) was found. HYP-based fluorescence guidance allowed a better tumor detection in comparison to visual inspection. Immunohistochemistry revealed HYP penetration across the tumor surface. HYP-based PDT without HIPEC induced marginal apoptotic effects at the tumor surface. Combining HYP with HIPEC revealed cisplatin concentration dependent decrease in proliferation capacity and induction of apoptosis across determined cell layers of the tumor surfaces. Conclusion: HYP as fluorescent photosensitizer offers an intraoperative diagnostic advantage detecting intraperitoneal tumor dissemination. The combination of HYP and cisplatin-based HIPEC was feasible in vivo showing enhanced effects on tumor proliferation and apoptosis induction across the tumor surface. Further studies combining HYP and HIPEC will follow to establish a clinical application.

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This paper has been presented at the 53rd Annual Congress of the International Society of Paediatric Oncology 2021 and was the winning abstract of the Jack Plaschkes Price of the International Society of Paediatric Surgical Oncology (IPSO).

Word Count for:

a) Abstract: 247

b) Main Text: 3575

The number of:

a) Tables: 1

b) Figures: 5

c) Supporting Information files: 1

Running title

Combination of Hypericin PDT with HIPEC

Keywords

Alveolar rhabdomyosarcoma, Hypericin, photodynamic diagnostic (PDD), photodynamic therapy (PDT), hyperthermic intraperitoneal chemotherapy (HIPEC), cisplatin, cytoreductive surgery (CRS), animal model

Abbreviations

5-ALA	5-aminolevulinic acid
CRS	Cytoreductive surgery
HE	Hematoxylin and eosin staining
HIPEC	Hyperthermic intraperitoneal chemotherapy
HYP	Hypericin
HYP-PVP25	Hypericin-Polyvinylpyrrolidone 25
ICG	indocyanine green
i.p.	intraperitoneal
PCI	Peritoneal carcinomatosis index
PDD	Photodynamic diagnosis
PDT	Photodynamic therapy
PS	Peritoneal sarcomatosis

5-ALA	5-aminolevulinic acid
PVP	Polyvinylpyrrolidone
RMS	Rhabdomyosarcoma
ROS	Reactive oxygen species
vs.	*versus*

Abstract

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Procedure: An established HIPEC animal model for rhabdomyosarcoma (NOD/LtSz-scid IL2Rγnullmice, n=80) was used. All groups received HYP (100 µg/200 µl) intraperitoneally with and without cisplatin-based (30 or 60 mg/m^2) HIPEC (37 or 42 °C, for 60 min) (five groups, each n=16). Tumor dissemination was documented visually and by using HYP-based fluorescence guidance. HYP-based photodynamic therapy (PDT) of the tumor was performed. Finally, tissue samples were evaluated regarding proliferation (Ki-67) and apoptosis (TUNEL).

Results: HYP uptake even in smallest tumor nodes (< 1 mm) was found. HYP-based fluorescence guidance allowed a better tumor detection in comparison to visual inspection. Immunohistochemistry revealed HYP penetration across the tumor surface. HYP-based PDT without HIPEC induced marginal apoptotic effects at the tumor surface. Combining HYP with HIPEC revealed cisplatin concentration dependent decrease in proliferation capacity and induction of apoptosis across determined cell layers of the tumor surfaces.

Conclusion: HYP as fluorescent photosensitizer offers an intraoperative diagnostic advantage detecting intraperitoneal tumor dissemination. The combination of HYP and cisplatin-based HIPEC was feasible *in vivo* showing enhanced effects on tumor proliferation and apoptosis induction across the tumor surface. Further studies combining HYP and HIPEC will follow to establish a clinical application.

Introduction

Peritoneal sarcomatosis (PS) in rhabdomyosarcoma (RMS) is a rare and the most advanced tumor stage with wide intraperitoneal tumor spread in children. Due to promising clinical reports, cytoreductive surgery (CRS) in combination with hyperthermic intraperitoneal chemotherapy (HIPEC) has been established as therapeutic option for PS . Nevertheless, this treatment is still experimental in children and not successful in all patients. The prognosis is sobering due to high probability of tumor recurrence . In a retrospective study on CRS and HIPEC (including seven cases of PS of RMS) the median over-all survival was 7.1 to 31.4 months after succeeded tumor resection whereas the diseases-free survival was described by 19.9 to 34 months depending on peritoneal cancer index (PCI). After incomplete cytoreductive surgery, no patient survived longer than 15 months . The evaluation of the intraperitoneal tumor spread and the achievement of complete CRS plays an integral role and might determine the therapeutic success . An enhancement of HIPEC by additive modalities such as photodynamic therapy (PDT) might be beneficial. The principle of PDT is a photosensitive agent, which ideally accumulates selectively in tumor cells. After illumination with light of the appropriate wavelength in the presence of oxygen the photoactivated sensitizer generates highly reactive oxygen species (ROS) leading to critical cytotoxicity and destruction of tumor cells via apoptosis or necrosis . Furthermore, photosensitizer are known to show fluorescence enabling visualization and focused resection of tumor tissue during photodynamic diagnosis (PDD) . The natural photosensitizer Hypericin

(HYP), an extract from the St. John's wort, represents an ideal and promising agent for the challenging treatment of PS of RMS: HYP with its strong self-fluorescence and low photobleaching has been used successfully as photosensitizer for *in vitro* and *in vivo* visualization and PDT of pediatric RMS . Up to now there are no data on combining HYP and HIPEC in RMS *in vivo* . Therefore, we tested a combination of the photosensitizer HYP with HIPEC in a recent established murine HIPEC animal model of intraperitoneal disseminated pediatric RMS.

Methods

Cell Culture Conditions

The human alveolar rhabdomyosarcoma cell line RH-30 (No. ACC-489, DSMZ, Braunschweig, Germany), which has a positive PAX3-FKHR fusion status , was used. Tumor cells were cultured in Dulbecco's modified Eagle's medium plus Ultraglutamine 1 (Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS) (PAN Biotech GmbH, Aidenbach, Germany) and 1% antibiotic-antimycotic solution (Gibco, Paisley, UK). All cells were mycoplasma negative and kept under humidified conditions at 37 °C and 5% CO_2 atmosphere.

Rhabdomyosarcoma xenograft model

Eighty NOD/LtSz-scid IL2Rγnullmice, with an age of 8-11 weeks and a weight of 25-30 g, were used. This immunodeficient mouse strain was initially sourced from Jackson Laboratories (Bar Harbor, ME, USA) and further obtained from own animal facility. Animals were kept under specific pathogen-free conditions, fed an autoclaved standard diet and given free access to sterilized water. The animal experiments were approved by the government ethics committee for animal studies (Regierungspräsidium Gießen, V54 – 19 c 20 15 h 01 MR 20/14, No. G38/2017). The RMS xenotransplantation procedure was described previously . Briefly, RMS tumor induction was carried out by intraperitoneal (i.p.) injection into the lower left flank of the animals of a total number of 2 x 10^6 RH-30 tumor cells solved in a 0.9% sterile sodium chloride solution (Ecolav® B.Braun, Melsungen, Germany).

Water solubilization of Hypericin

Since HYP must be water-soluble for *in vivo* application, it was complexed to Polyvinylpyrrolidone 25 (PVP) by hydrogen bonds without losing its characteristic absorption spectrum and photosensitizing properties . The water-soluble Hypericin- Polyvinylpyrrolidone 25 complex (HYP-PVP25) was prepared according to the protocol of Kubin*et al.* (2008): HYP (10 mg, HWI pharma Service GmbH, Rülzheim, Germany) was dissolved in EtOH (2.5 ml) and sonicated for 2 min. PVP25 (1 g, Sigma Aldrich, St. Louis, USA) was dissolved in water (8 ml) and added to the HYP. The solution was stirred for 5 min at 70 °C and for 10 min after adding water (5 ml). The solvent was cautiously removed under reduced pressure resulting in a solid red mass. HYP-PVP25 complex was dissolved in sterile water (Aqua ad iniectablia, B. Braun Melsungen AG, Melsungen, Germany) to form a final HYP-PVP25 working solution of 100 µg/ 200 ml, which was stored at 4 °C under light protected conditions.

HIPEC and Hypericin-based PDD and PDT

Treatment was performed 21 days after xenotransplantation (RH-30). An overview about the experimental setup is shown in Figure 1A. HYP-PVP25 was injected i.p. over the left lower abdomen in all mice, four hours prior to PDD/PDT. The mice were kept in the dark preventing premature HYP-based cytotoxicity. Whereas the first group received no HIPEC, the remaining mice underwent i.p. lavage over 60 minutes with cisplatin (30 or 60 mg/m^2) heated up to 37 or 42 °C three hours after the HYP-PVP25 injection (four groups). All groups (each group n=16) with the corresponding treatment, dosages and temperatures

are shown in Table 1. The HIPEC treatment was performed according to our established murine HIPEC model . Four hours after the injection of HYP-PVP25 or at the end of HIPEC, a median laparotomy was performed. Tumor dissemination was documented visually using the peritoneal carcinomatosis index (PCI) according to the principle of Jacquet and Sugarbaker and adapted for the animal model (Figure 1C). Visual examination of the tumor spread was repeated under HYP-PVP25-based fluorescence guidance (PDD) with blue light and recorded as PDD-PCI. Subsequently HYP-PVP25-based photodynamic therapy (PDT) was performed. Therefore, one tumor node was resected immediately after PDD (Non-PDT) followed by PDT of a representative tumor bulk under white light for ten minutes. The light source for PDT was firmly positioned three centimeters above the tumor. HYP fluorescence detection and photodynamic therapy were performed using KARL STORZ D-light C System, a 3 mm 0° laparoscope with a 300 Watt short-arc lamp and filter options for white light (400 – 700 nm) and fluorescence excitation (390 – 420 nm). Additionally, a long pass filter (> 450 nm) was integrated in the eyepiece of the laparoscope blocking the reflected blue excitation light without blocking the red fluorescence of HYP. Tumors with and without PDD and its corresponding regions were photographed*via* laparoscope camera.

Immunohistochemistry and Ki-67 proliferation marker detection

Tissues (tumor, liver, spleen, peritoneum) were harvested after laparotomy for the histological work-up as described previously : Tissues were fixed in 3.7% formalin, paraffin-embedded and cut into 3-5 µm thin sections. The sections were primary labeled with anti-Ki-67 (#M7240, Dako Cytoformation, Glostrup, Denmark, dilution 1:100, mouse-monoclonal) antibody and secondary labeled with a polymer-horseradish peroxidase (HRP) antibody (Dako Envision+ Kit; Dako, Glostrup, Denmark) with AEC (3-amino-9-ethylcarbazol). Sections were counterstained with Mayer's hemalum solution (Merck KGaA, Darmstadt, Germany) and digitalized as whole slide image (WSI) using the PreciPoint M8 scanning microscope and ViewPoint Light microscopy software (PreciPoint, Freising, Germany). The Ki-67 proliferation index was analyzed and recorded by the software Cognition Master Professional Suite (Ki67 Quantifier, VMscope GmbH, Berlin, Germany).

Analysis of tumor affine Hypericin uptake

HYP uptake was measured by red fluorescence. For mounting and nuclear counterstaining of HYP penetrated tissue sections a VECTASHIELD® HardSet Antifade Mounting Medium with DAPI (VECTOR Laboratories, Burlingame, USA) was applied to the formalin-fixed, paraffin-embedded and 3 µm thin cut tissue sections. Fluorescence microscopic images were captured on a wide field microscope (Leica DM5500, Leica, Wetzlar, Germany).

TUNEL assay

For investigations on induced apoptosis TUNEL assays were performed on 3 µm thin tissue sections as reported previously using the terminal desoxyribosyl-transferasemediated dUTP nick-end labeling test (In Situ Cell Death Detection Kit, Fluorescein, Roche Diagnostics GmbH, USA) . Control tissues were only treated with the labelling solution without enzyme as negative control and with 0.3 mg/ml DNase I (Roche Diagnostics GmbH) as positive control. Tissue sections were mounted and nuclear counterstained with VECTASHIELD® HardSet Antifade Mounting Medium with DAPI (VECTOR Laboratories, Burlingame, USA). Fluorescence microscopic images were captured on a wide field microscope (Leica DM5500, Leica, Wetzlar, Germany).

Statistical analysis

All numerical data sets were expressed as means +- standard deviations (SD). Statistical significance was determined by the unpaired student's t -test or one-way ANOVA (P values: ***P $<$ 0.001;

**P < 0.01; *P < 0.05) using the software Microsoft Excel 2017 and Graphpad Prism Version 8 (http://www.graphpad.com/scientific-software/prism/).

Results

Initially, 80 NOD/LtSz-scid IL2Rγnullmice were used for this study. Three weeks after xenotransplantation intraperitoneal sarcomatosis could be induced in 68 mice. No tumor occurred in five mice, while extraperitoneal tumor bulks were seen in seven mice after failed i.p. injection. No mouse was prematurely sacrificed prior to the treatment due to tumor progression. In one mouse HYP-PVP25 was injected subcutaneously. Five mice died prematurely during treatment. The remaining 62 mice were included into the statistical analysis.

Anesthesia, HIPEC and application of HYP-PVP25 were feasible without acute side effects in 93% of cases. Five mice died during treatment due to respiratory failure. Low body weight was noted in these five mice. Due to strict monitoring of body temperature, usage of a physical heat source and fast heat loss in anesthetized mice hypothermia (body temperature 34 – 36 °C) or hyperthermia (body temperature > 39 °C) during HIPEC did not occur. After i.p. injection of HYP-PVP25 all mice were observed regarding acute or delayed adverse reactions, which did not occur. No mouse died after the application of HYP-PVP25 or during HYP-PVP25-based PDD/PDT.

Conventional laparotomy after treatment revealed an extensive peritoneal dissemination of RMS in 81% of the cases. Tumor bulks could be identified by their specific morphology. Without PDD a median PCI of 7 (+/- 2.8) was measured. Due to its strong red fluorescence under blue excitation light the selective uptake of HYP in the tumor bulks, which already have been documented without PDD, could be proved. Healthy tissue did not show any HYP-PVP25 affinity. HYP-PVP25 showed stable and selective accumulation in the tumor nodules showing the full extent of the intraperitoneal tumor spread. Even smallest tumor nodules of less than one millimeter could easily be detected only by the red fluorescence. Using the KARL STORZ D-light C system surgical and photodynamic procedures were feasible without any difficulties. Clear PDD guided tumor identification and resection followed by PDT were carried out by easily switching between the illumination modes if needed. Under HYP-PVP25-based fluorescence guidance more tumors could be identified and distinguished from surrounding healthy tissue than without PDD. Consequently, we documented a statistically significant higher median PDD-PCI of 8.5 (+/- 2.9) (***P < 0.001).

The histological examination confirmed RMS cells in the observed tumors. The most and largest tumor masses (> 3 mm size) were typically found in the epigastric region on the greater curvature of the stomach followed by smaller tumors (1 – 3 mm size) in the right and left upper region, perihepatic and perisplenic. Under PDD a massive tumor affection by numerous small tumor nodules (< 1 mm size) occurred at the mesentery of the small and large intestine as well as at the peritoneum of the abdominal wall (Figure 1B).

Besides the macroscopic visible selective uptake and accumulation of HYP in tumor bulks we could demonstrate the penetration of HYP across the tumor surface using immunofluorescence microscopy. Thereby we observed a pronounced accumulation of HYP at superficial tumor cell layers followed by decreased intensity towards inner layers (Figure 2A).

Proliferation and apoptotic effects on the tumor were measured before and ten minutes after PDT. Early apoptotic effects at the outer tumor surface could be detected *via* TUNEL-assay (Figure 2B). Immunofluorescence microscopy revealed an increase in apoptotic cell layers after PDT. Superficial cell layers of 30 µm depth have been affected by photoactivated-HYP. Evaluation of tumor proliferation by Ki-67 immunohistochemistry and digital image analysis showed early PDT-dependent effects (Figure 2C and D). The statistical analysis revealed a significant reduction of tumor proliferation after ten minutes of PDT compared to the proliferation index of tumor harvested before PDT (***P < 0.001) (Figure 2D).

The combination of HIPEC and i.p. applicated HYP-PVP25 was feasible. The up taken HYP persisted after

HIPEC in the tumor nodules independent from the cisplatin dosage in all groups (Figure 3). Additionally, hyperthermia of 42° C leaded to deeper penetration and better distribution of HYP across the tumor surface with still decreased intensity towards inner cell layers. Whereas the outer tumor surface has been affected by HIPEC without PDT, increased early apoptotic effects to deeper cell layers were seen after ten minutes of PDT (Figure 4). Especially PDT in combination with 60 mg/m² cisplatin and hyperthermia of 42 °C led to scattered sectors of apoptosis with lost tumor structure. Sections showed outstanding untypical alterations in the Ki-67 staining, which was estimated as reduction of tumor proliferation and seen in the first 9 to 11 cell layers of the surface. The affected cell layers appeared as sharply bounded stripe. This observation was made exclusively in the PDT exposed tumor area in all groups combining HIPEC and PDT (Figure 5). Statistical analysis did not show any significant difference between the HIPEC subgroups with or without PDT.

Penetration depth of PDT after HIPEC was measured to be 35 to 50 µm depending on cisplatin concentration combined with hyperthermia (Supplemental Figure S1A). Statistical analysis revealed a significant difference in penetration depth of PDT following HIPEC of 60 mg/m² cisplatin at 42 °C compared to PDT after HIPEC with lower cisplatin dosage given with lower temperature (30 mg/m² cisplatin at 37 °C*versus* (*vs* .). 60 mg/m² cisplatin at 42 °C, *P = 0.013; 30 mg/m² *vs* . 60 mg/m² cisplatin at 42 °C, ***P < 0.001; 60 mg/m² cisplatin at 37 °C *vs* . 42 °C, **P < 0.007). Consequently, the deepest penetration depth of PDT was seen after HIPEC with cisplatin concentration of 60 mg/m² heated up to 42 °C. (Supplemental Figure S1C)

By HE-staining, no cell morphological changes were evident in the tissues of the tumors (Supplemental Figure S2 and S3) and the representatively examined control organs (liver, spleen, and peritoneum) after treatment (Supplemental Figure S4).

Discussion

PS of RMS is rare in children and its therapy represents an oncological challenge . HIPEC (in combination with CRS) represents a therapeutic but experimental option for PS of RMS . The exact extent of PS and succeeded tumor control (whether by CRS or HIPEC or both) are crucial for the outcome. Due to the still sobering prognosis an optimization of HIPEC by photodynamic therapy might be beneficial. Here, we tested the combination of HIPEC with the promising photosensitizer HYP in pediatric RMS for the first time.

Based on previous studies with HYP and RMS, we used our HIPEC mouse model of intraperitoneal disseminated pediatric RMS to evaluate the feasibility and benefits combining HYP and HIPEC *in vivo* . Besides reliable intraperitoneal tumor growth of human alveolar RMS and good tolerance of HIPEC and i.p. HYP-PVP25 we could demonstrate a selective uptake and accumulation of HYP in RMS even after HIPEC. Under fluorescence guidance with blue light tumor bulks exhibited strong red fluorescent signals with clear contrast between tumor margins and surrounding healthy tissue. The performance of HYP-PVP25-based PDD resulted in an optimized tumor detection and consequently significant higher PDD-PCI compared to the evaluation of PCI without PDD. Compared to our study Urla *et al* . described an even better HYP-PVP25-based tumor detection ratio (1.6 - fold via laparotomy following laparoscopy*vs* . 1.2 - fold laparotomy) coming from complicated measuring of the PCI via laparoscopy in small dimension of a mouse compared to one via laparotomy . Using the KARL STORZ D-light C system surgical and photodynamic procedures could be carried out easily. Devised for the minimal invasive approach KARL STORZ D-light C system can be used for performing HYP-PVP25-based diagnostic laparoscopy. Staging laparoscopy enhanced by HYP might represent a new method for the pre-operative assessment of peritoneal surface malignancies to prevent unnecessary laparotomy and reduce postoperative morbidity in children .

Besides the macroscopic visible selective HYP uptake we could proof a HYP penetration across the tumor surface. The accumulated HYP persisted in the tumor even after HIPEC and showed a strong signal at the periphery followed by decreased intensity towards inner cell layers. Knowing that HYP fluorescence signal can be detected after 20 minutes of incubation with accumulation in tumor tissue after 60 minutes in*in vitro*

studies the i.p. application of HYP-PVP25 was performed four hours before PDD/T in our mouse model . Whereas Urla *et al.*injected HYP 24 hours before PDD intravenously in a similar RMS mouse model we see the i.p. application with fast intraperitoneal distribution more suitable for PS of RMS .

Although the HYP uptake is thought to be tumor-selective a negligible HYP evidence was seen only microscopically in pancreas and fatty tissue ingrown by the harvested tumor. Similar findings were seen in other studies regarding liver and necrotic tissue . The lipophilic molecular structure of HYP with strong avidity to cholesterol (as one of the most essential part of natural cell membranes) is the major hypothesis of the selectivity for HYP in these tissues . The apparent accumulation of HYP in RMS might derive from intracellular co-transport of HYP with cholesterol during generally high metabolic activity in neoplastic tissue . Our study supports the anti-tumor effect of HYP-PVP25-based PDT, which already has been reported in various studies for different malignancies . In addition to HYP other photosensitive agents such as indocyanine green (ICG) and 5 - aminolevulinic acid (5-ALA) should be mentioned at this point: Whereas ICG seems to have primarily diagnostic properties for tumor visualization, Ritz *et al.* and Urla *et al.* demonstrated the superiority of HYP *vs.* 5-ALA regarding phototoxicity and visualization in medulloblastoma and RMS .

Using TUNEL-assay we demonstrated early apoptotic effects at the outer tumor surface by HIPEC without PDT, increased to deeper cell layers after PDT. This effect was seen especially combining PDT with 60 mg/m^2 cisplatin and hyperthermia of 42 °C. 9 - 11 cell layers with reduced proliferation appeared as sharply bounded stripe of the tumor surface in the corresponding PDT exposed sector only after HIPEC. The deepest penetration depth of PDT after HIPEC (50 µm) was seen after the application of 60 mg/m^2 cisplatin heated up to 42 °C. The observed penetration depth of PDT after cisplatin-based HIPEC was 10 µm deeper than after cisplatin-based HIPEC without PDT (40 µm, Wagner *et al* .) but considerably more superficial as those demonstrated by Goodman*et al* (1 - 3 mm) . This study was published regarding peritoneal malignancies in adults without having included pediatric RMS . Additionally, as our experiment ended immediately after PDT it was not possible to capture late anti-tumor effects after PDT with or without HIPEC knowing that cellular signal cascades and apoptosis pathway need more time . Nevertheless, we could demonstrate the promising diagnostic and synergistic anti-tumor properties of HYP-PVP25 combined with HIPEC*in vivo* . Moreover, the additional application of HYP-PVP25 is not to be expected reducing the tolerability of HIPEC. HYP has a low side effect profile not having showed severe side effects in other mouse models or in the experimental treatment of glioblastoma or bladder cancer in clinical studies . Photodermatitis, as major side effect caused by hypericism, can appear only at higher systemic HYP concentration typically during antidepressant therapy . The HYP doses (intraperitoneal or intravenous), which have been used for intraoperative PDD/T by Ritz *et al.* or in our study, were considerably lower than in the therapy of depression (0.1 mg/kg body wight *vs.* 1000 mg) . Therefore, the photosensitizer HYP also in combination with HIPEC should be tolerated without adverse side effects in children.

Several limitations should be pointed out. Due to ethics committee approval CRS before HIPEC or PDT was not performed. Focusing the feasibility of combining HYP-based PDD/T with HIPEC *in vivo* , we did not do any cytoreductive surgery compared to existing clinical HIPEC studies. Nevertheless, the observed intraperitoneal tumor spread in our study might be comparable to a situation of incomplete tumor resection in children, in which HYP-PVP25-based PDD/T could offer additional enhancement. One major limitation of the concept of PDT is the restriction to the outer surface of tissue as light permeates only into superficial cell layers. The full therapeutic potential of HYP can only be unfolded after activation by an appropriate illumination . As HYP shows a good penetration with distribution even to deeper cell layers of the tumor it can be utilized as carrier system, coupled with agents . To overcome the limitation of PDT, HYP was successfully radioiodinated revealing additional anti-tumor properties in RMS treated with targeted radiotherapy (^{131}I-HYP) *in vitro,* so further*in vivo* studies are required .

As translational treatment approach, HYP-PVP25 could be combined with CRS and HIPEC for PS of RMS without major changes: After preoperative i.p. application of HYP-PVP25 the photosensitizer accumulates in tumor tissue allowing better detection of the exact tumor dissemination also facilitating tumor resection under fluorescence guided PDD. Combined with HIPEC, PDT of incomplete resected tumors or its margins

might enhance local tumor control due cytotoxicity of photoactivated HYP. Based on this study the possible enhancement of HIPEC in RMS by a combination with HYP-PVP25 could improve the existing treatment strategy and consequently the outcome of the patient.

In summary, HYP as fluorescent photosensitizer offers an intraoperative diagnostic advantage detecting the exact intraperitoneal tumor dissemination. The combination of cisplatin-based HIPEC with HYP-PVP25 was feasible in the designed animal model for PS of RMS. TUNEL-method and Ki-67 staining could demonstrate promising synergistic anti-tumor properties of HYP-PVP25 combined with HIPEC. As a safe translation to clinical treatment of children is limited, further studies combining HYP, its coupled derivates and HIPEC are required to get additional insights on the possible efficiency of this approach.

Conflicts of Interest

The authors declare no conflict of interest.

Authors Contributions

GS conceived the project, edited, and revised the manuscript. BW, AA and LH performed the research and analyzed the data. BH prepared HYP-PVP25 under OV supervision. BW, AA and GS wrote the article. All authors have read and approved the final version of the manuscript.

Acknowledgement

This work was supported by KARL STORZ SE & Co. KG, Tuttlingen, Germany, which provided us laparoscopic equipment and technical assistance. We wish to thank Dr. Katrin Roth (Core Facility for Microscopy, Institute of Molecular Biology and Tumor Research / Center for Tumor and Immunobiology, Marburg University Medical Center) for support in wide field microscopy. We thank Dörthe Sönnecken (Department of Pediatric Surgery, University Hospital Marburg), Viktoria Wischmann and Mareike Maier (Department of Pathology, Marburg University Medical Center) for technical assistance in the experiments.

Availability of data

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Legends

Fig. 1 (A) Schematic illustration of the HIPEC and HYP-PVP25-based PDD and PDT treatment procedure. Treatment experiments were performed 21 days after RH-30 tumor cell xenotransplantation. Four hours prior PDD and PDT, HYP-PVP25 (100 µg/200 µl) was injected i.p.. The first group received no HIPEC, the remaining four groups underwent i.p. lavage with a perfusion rate of 180 ml per hour over 60 minutes with cisplatin (30 or 60 mg/m^2) heated up to 37 or 42 °C three hours after the HYP-PVP25 injection. Immediately afterwards, a median laparotomy was performed and tumor dissemination was documented visually using the peritoneal carcinomatosis index (PCI) according to the principle of Jacquet and Sugarbaker *et al.* (1996) and repeated under HYP-PVP25-based fluorescence guidance (PDD) with blue light and recorded as PDD-PCI. Subsequently HYP-PVP25-based photodynamic therapy (PDT) was performed for 10 minutes. **(B)** Representative images of native and Hypericin-fluorescence guided (PDD) tumor and lymph node detection. As a characteristic of the RMS tumor model used, which was described previously in detail, the tumor foci are always found at intra-abdominal sites, which are preferentially located on the large curvature of the stomach,

peri-hepatic, peri-splenic, mesenteric and peritoneally on the abdominal wall . With aid of PDD, the tumor sites and metastases can be much better delineated and detected. **(C)**Overview about the used scheme for peritoneal carcinomatosis index (PCI) calculation. Total and PCI of the respective organs was recorded according to the principle of Jacquet and Sugarbaker *et al.*(1996), which has been previously adapted for the HIPEC animal model . PCI lesion size score: 0 = no tumor; 1 = tumor [?] 1 mm; 2 = tumor > 1 mm [?] 3 mm; 3 = tumor > 3 mm.

Fig. 2 Evaluation of the Hypericin-Polyvinylpyrrolidone 25-monotherapy. **(A)** Analysis of HYP-PVP25-uptake and distribution within the RMS tumor *via* red autofluorescence signal of HYP. Both HYP-PVP25-treated tumors with and without subsequent PDT treatment for 10 minutes showed a good HYP-uptake capacity into the deeper tumor layer. A somewhat more pronounced HYP-fluorescence signal in the outer layers of the tumor after PDT treatment is striking.**(B)** Terminal desoxynucleotidyl transferase (TUNEL)-labeled DNA fragmentation as a sign of apoptosis induction (green fluorescence signal) is visible in the marginal areas of the HYP-PVP25-based PDT-treated tumors (10 min), whereas HYP-PVP25-treated tumors without subsequent PDT (Non-PDT) showed no evidence of apoptosis induction. The nuclei were counterstained in blue with DAPI. **(C)**Immunohistochemical analysis of the proliferation marker Ki-67 (brown) showed no obvious changes in protein expression through the HYP-PVP25-monotherapy without (Non-PDT) and with 10-minute PDT.**(D)** Determination of the Ki-67 proliferation index using a Ki-67 quantifier software module (Cognition Master Professional Suite: Ki67 Quantifier, VMscope GmbH, Berlin, Germany) showed a significant (*** P value = 0.0003; unpaired t-test) slight reduction in the proliferation capacity during HYP-PVP25-based PDT.

Fig. 3 Analysis of HYP uptake and distribution within the tumor. The red fluorescence of HYP was determined by wide field microscopy. Red fluorescence signal is strong within the outer tumor margins and is seen down to the deeper layers of the tumor regardless of the combined HIPEC- HYP-PVP25 treatment with and without PDT. The cell nucleus was counterstained with Hoechst (blue).

Fig. 4 Investigation of apoptosis induction by terminal desoxynucleotidyl transferase (TUNEL)-assay after combined HIPEC treatment with HYP-PVP25-based PDT. TUNEL-labelled fragmented DNA as a sign of apoptosis induction is evident by a green fluorescence signal. Green fluorescence signals are evident in the outer margins of tumors treated with combined HIPEC- HYP-PVP25 therapy across multiple cell layers. The expression of which is dose- and temperature-dependent and moreover apoptosis induction is again significantly enhanced by addition of a 10-minute PDT. The cell nuclei were counterstained with DAPI (blue).

Fig. 5 Evaluation of changes in tumor cell proliferation in case of combinatorial HIPEC- HYP-PVP25 treatment by analysis of Ki-67 protein marker expression (brown signal). Immunohistochemical analysis of the proliferation marker Ki-67 revealed no obvious changes in protein expression through the individual treatments without addition of the 10-minute HYP-PVP25-based PDT (Non-PDT). Subsequent PDT reduced the expression capacity of the proliferation marker Ki-67 in the tumor margins by an average of 8-10 layers of tumor cells, depending on the temperature and dose of the previous HIPEC treatment.

TABLE 1. Overview of the treatment groups (each n = 16).

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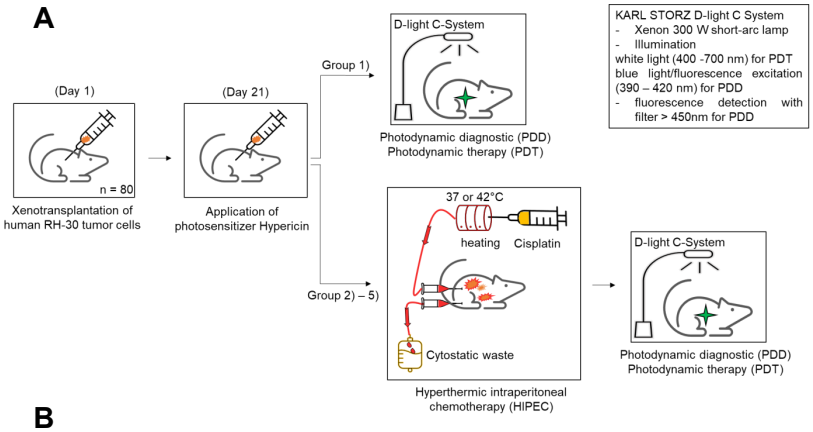
`Table 1.docx` available at https://authorea.com/users/410114/articles/550277-increasing-the-efficiency-of-hyperthermic-intraperitoneal-chemotherapy-hipec-by-a-combination-with-a-photosensitive-drug-in-pediatric-rhabdomyosarcoma

A



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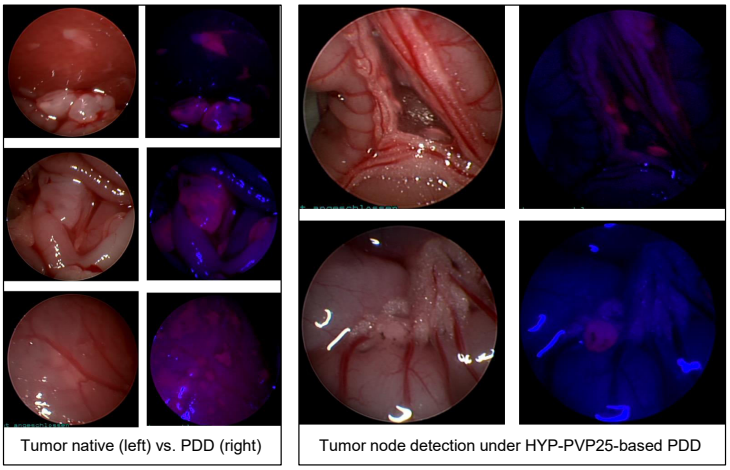

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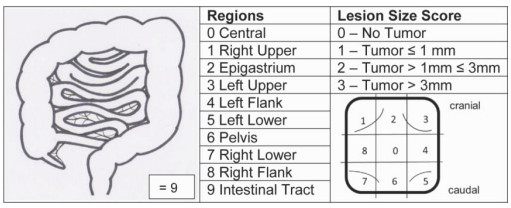

Regions	Lesion Size Score
0 Central	0 – No Tumor
1 Right Upper	1 – Tumor ≤ 1 mm
2 Epigastrium	2 – Tumor > 1mm ≤ 3mm
3 Left Upper	3 – Tumor > 3mm
4 Left Flank	
5 Left Lower	
6 Pelvis	
7 Right Lower	
8 Right Flank	
9 Intestinal Tract