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Use of Hypericin in Clinical Trials of Photodynamic Therapy

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Abstract: Hypericin is derived from St. John's wort and is a potent, naturally occurring photosensitizer used in photodynamic therapy (PDT) to treat cancer. PDT is based on the induction of a photochemical reaction between a non-toxic photosensitizer, light, and oxygen. In this technique, after systemic administration of a photosensitizing agent (PS), local necrosis of neoplastic cells, inflammatory lesions, or cells of microorganisms such as bacteria, viruses, and fungi are induced using light of a specific wavelength. The use of PDT allows a limited number of surgical procedures and often completes the treatment process. Hypericin is a potential clinical antitumor agent, as many studies have demonstrated its potent antitumor activity in vi-vo and in vitro after irradiation. This photosensitizer can induce apoptosis and necrosis depending on the concentration and dose of light. This article aims to provide a general overview of the use of hypericin in clinical trials of PDT therapy. The literature was searched for articles relevant to the topic of this review using the PubMed, Scopus, ScienceDirect, and Google Scholar databases using the following keywords: "Hypericin", "Photodynamic therapy," and “Nutrients”. The literature reviewed relies on the use of hypericin in PDT. Studies of photodynamic effects using hypericin may be helpful in the clinical search for the best treatments for skin cancer lesions, mainly in the youngest patients.

Keywords: Hypericin; photodynamic therapy; pediatric treatment.

1. Introduction

Photodynamic therapy (PDT) is a therapeutic method that, in addition to light and singlet oxygen, uses a photosensitizer as the most important component of therapy [1]. According to Międzybrodzka *et al*., the basic parameters characterizing the photosensitizer are chemical composition, availability, toxicity, selectivity, absorption range, solubility, and type of application. [2]. One of the commonly available and used photosensitizers is hypericin. It is obtained from the herbaceous plant Hypericum perforatum. In herbalism, known as St. John's wort [3].

It is characterized by high selectivity and cytotoxicity in cancer tissues and cells [4-7]. During the therapy, cells are localized in such places as mitochondria, Golgi apparatus, lysosomes, or endoplasmic reticulum [2]. The location of accumulation depends on the hydrophobic properties. Figure 1 shows the structure of hypericin.

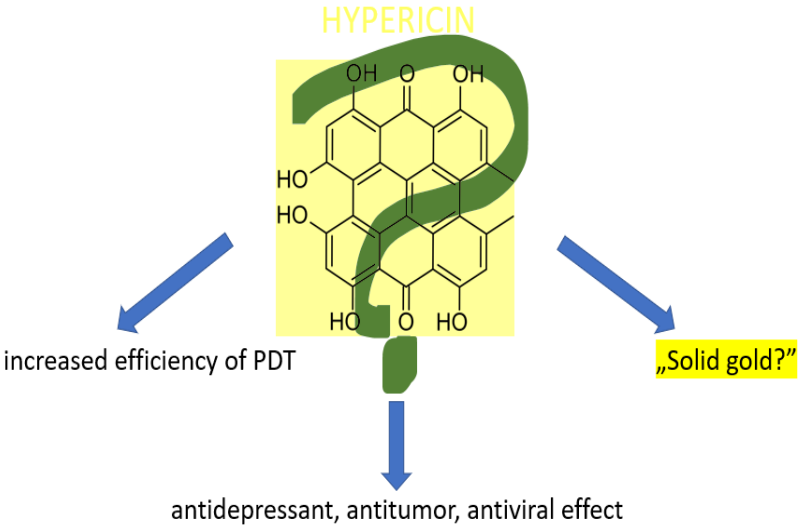

Figure 1. Structure of Hypericin.

The interest in hypericin as a potential anticancer agent is growing. The reason for this phenomenon is the increase in the number of conducted studies and, thus, the increased number of articles and publications describing its potent antitumor activity in vivo and in vitro studies after PDT therapy [8]. Hypericin has biological and physical properties support its effectiveness and efficiency in clinical trials. Table 1 shows the characteristics of hypericin. It is also possible to modernize this PS by using nanocarriers. Given the clear advantages of polymer nanocarriers in vivo, PDT study offers a promising new approach for improved treatment of localized tumors. The developed strategy could be extended to other photosensitizers currently in use in the clinic for photodynamic therapy.

Table 1. Characteristics of hypericin (HYP).

Characteristic	Description
Structure	Polycyclic quinone [9]
Origin	Extracted from Hyperforin perforatum (St. John's wort) [9].
General formula	$C_{30}H_{16}O_8$
Molecular weight	504.44
Use	Used as a drug treatment for depression and viral infections
Absorption peak	Has a maximum absorption peak of ~599 nm
Physical Properties	Has a substantial quantum yield, intense absorption spectrum in the visible region, low photobleaching, short half-life, and a wide excitation range
Biological Properties	Has high tumor-specific cytotoxicity and minimal side effects. HYP can induce vascular injury in tumor cell models through inhibition of mitochondrial function and can induce apoptosis in cancer cells through activation of the caspase-dependent pathway

PDT is a modern therapy method for inflammatory, bacterial, and cancer diseases. The advantages include relatively low invasiveness and selectivity [10,11]. The procedure begins with applying a photosensitizer and exposure to laser light. The whole mechanism is two-stage [12]. Type I reaction involves electron transfer, while Type II reaction involves energy transfer. Both types of reactions give rise to excited, reactive species that initiate cell death pathways. Type I reactions produce radical anion forms. On the other hand, in a type II reaction, singlet

oxygen ($^1O_2$) is generated. Both reactions can correlate with each other and occur simultaneously [13-22].

The main factors that affect the effectiveness of fighting cancer cells are the properties of the photosensitizer, which determine the type of laser light applied [23]. In addition, depending on the type of photosensitizer, it attaches to other elements of the immune system. The basic pathways of cancer cell destruction are necrosis, apoptosis, necrosis, and autophagy. It is possible to observe multiple pathways in one treatment regimen. For example, autophagy has been linked to both types of cell death, serving as a guide to cell survival or death [2,24].

2. Materials and Methods

PubMed, Scopus, and ScienceDirect were searched until November 2021 for clinical trials on the use of hypericin in the initial stage of treatment with PDT therapy. The search was carried out using the keywords "Hypericin" and "photodynamic therapy". The analysis includes academic publications that contain the phrase: "Hypericin supplementation; photodynamic therapy; pediatric clinical trials; skin neoplastic changes."

After reading the text of the article, a selection of available publications was made in accordance with the subject of our review. Articles for which full access was not obtained were disqualified. This review provides an overview of hypericin in PDT skin lesion treatment. As this topic is a relatively new one to explore, we decided to collect the available material and describe the use of hypericin in PDT.

3. Results and Discussion

3.1. Photodynamic therapy.

The number of superficial malignant skin lesions treated with PDT has increased significantly in the last 10 years. The cause of this phenomenon is believed to be the benefits of PDT, such as non-invasiveness, safety, and reduction in the occurrence and formation of scars [22]. These features make PDT with naturally occurring photosensitizers increasingly used in clinical trials of the youngest patients. The chemical composition of tissues is very diverse, resulting in biological structures of substances with selective absorption properties that interact with living matter with light [25]. Selective absorption is used in spectroscopic diagnostic techniques to characterize tissues and their chemical composition and detect various types of pathological changes.

3.2. Hypericin in clinical trials with PDT.

The cellular uptake of hypericin in tissues occurs mainly by diffusion. Another documented pathway was also membrane-associated translocation with albumin and/or low-density lipoproteins [26]. The hydrophobicity of hypericin predicts its localization in the cytoplasmic membranes of the endoplasmic reticulum and the Golgi complex. These membranes are also expected cellular targets of their phototoxic action. Moreover, hypericin may influence the function of mitochondria in a photo-dependent manner [27]. The hypericin molecule shows a maximum absorption peak at around 580-600 nm [28]. The reduction of the number of cancer cells administered with hypericin strictly depends on the presence of oxygen and light of different wavelengths. In turn, the high quantum efficiency of the produced reactive oxygen species is responsible for apoptosis and necrosis of cancer cells [26, 29]. Over the years,

it has been shown that when administered as a photosensitizing agent, hypericin inhibits the growth of various cultured cancer cells [30-34]. The sensitizing properties of hypericin have been known for a long time, and its stability in various cell lines has been documented for years [35,36]. Thomas *et al*. were among the first research teams to analyze hypericin in the in vitro cell line, confirming its destructive effect [37]. Koren *et al*. used hypericin in skin lesions in meta-static malignant mesothelioma for the first time [32]. Koren *et al*. administered hypericin superficially to the skin in synthesis with interstitial hematoporphyrin. Alecu *et al*. used PDT along with hypericin to treat non-melanoma skin cancer. Treatment consisted of multiple injections per shift followed by irradiation with visible light [38].

Schmitt *et al*. [39] described the limitations in his research on hypericin. The low concentration of hypericin used and the presence of common plant ingredients with the effect of scavenging free radicals decreased PDT therapy's effectiveness [39,40]. Another reason for the decrease in effectiveness may be hypericin's relatively high molecular weight (504 Da), which may not penetrate the skin effectively. For the drug to be delivered spontaneously through the skin, the optimal molecular weight should be less than 500 Da with adequate lipophilicity [41].

Research is currently underway to improve the effectiveness of the PDT method. Various strategies for the delivery mechanism of the photosensitizer to the skin have emerged in recent years. These strategies can be divided into passive and active methods. The passive approach involves optimizing the formulation to increase the skin's permeability to the formulation. However, passive methods do not significantly improve penetration when using drugs with a combined molecular weight greater than 500 Da (an example of which is hypericin).

On the other hand, active enhancement includes mechanisms such as iontophoresis or electroporation [42]. Another way is to chemically modify the photo-sensitizer to change its lipophilicity despite its high molecular weight [43]. The efficient penetration of hypericin deep into the skin and its mechanism of penetrating subsequent layers of the skin in order to accumulate in cancer cells are open questions and are still being analyzed [44].

Kleemann *et al*. [45] used hypericin to treat malignant melanoma cells. Together with the research team, they prepared a hypericin solution with a concentration of 50 μM, which they then diluted. The irradiation was carried out with UVA light with a wavelength in the range of 320-410 nm for about 6 minutes. As a result of PDT therapy, a decrease in the number of cancer cells by at least 50% was observed (in the images) [45]. The study also analyzed the distribution of the photosensitizer in the target cells. This is significant due to the short half-life and the short diffusion radius of reactive oxygen species responsible for PDT's cytotoxic dimension [46,47]. Photodynamic therapy with hypericin causes a significant increase in pigmentation in cells of all melanoma lines, which is an important observation due to the fact that cells of this tumor show sequestration of chemotherapeutic agents, creating a hypoxic environment by high oxygen consumption, which leads to bypassing cell death [48-50]. In vitro experiments with PDT with hypericin also indicate the mechanism of autophagy in melanoma malignum cells, which has a cytoprotective role, important for the effects of photodynamic therapy, but possible to overcome by increasing oxidative stress [51].

3.3. Mechanism of Hypericin in PDT.

It is generally accepted that PDT-induced apoptosis is mediated mainly by the mitochondrial pathway of caspase activation [52]. An early step in photosensitization is the release of cytochrome C from the mitochondria into the cytosol with subsequent cleavage and activation of pro-caspase 3 [53]. Caspase 3, which initiates apoptosis, is responsible for the cleavage of poly (ADP-ribose) polymerase, leading to DNA fragmentation, a hallmark of apoptosis. Several groups are currently exploring the signaling pathways to apoptosis in PDT mediated by hyper-icin. Reactive oxygen species (ROS) are also potential candidates to mediate cell death in hypericin-PDT. Figure 2. shows a process initiated by PDT with hypericin in cells.

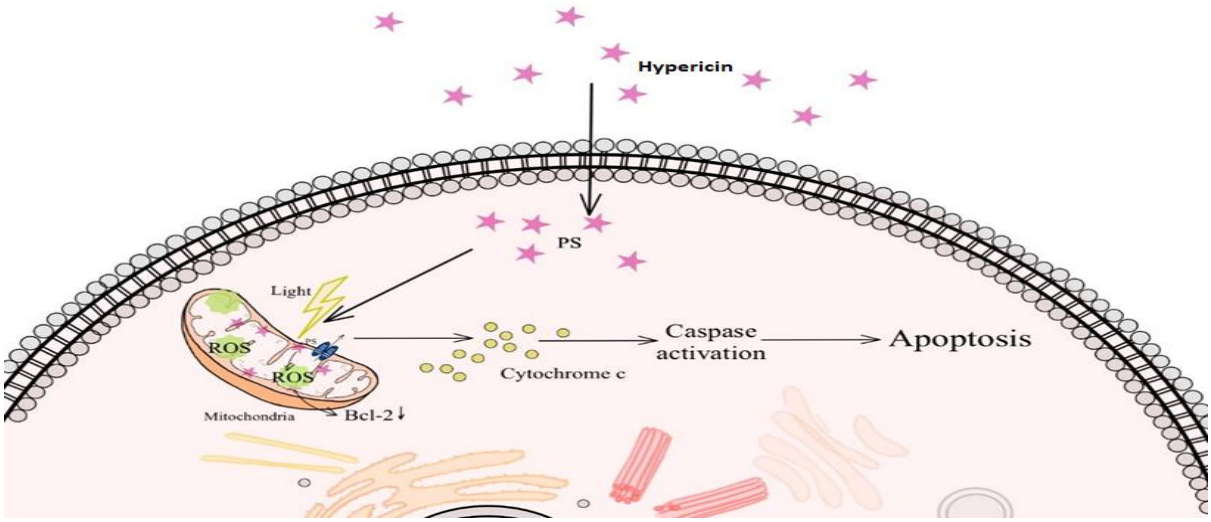

Figure 2. Diagram of activation of processes in cells as a result of PDT activity. Initially, PS penetrates the cancer cells and accumulates in the mitochondria. Upon activation of PS with laser light of a specific wavelength, ROS photogeneration and destruction of the apoptotic protein Bcl-2 occurs, which causes the permeabilization of the outer membrane of the mitochondria. Consequently, cytochrome c is released from the mitochondria into the cytosol, enhancing the apoptotic signal by activating caspases.

ROS initiates apoptotic death signaling directly and can induce oxidative stress, leading to cell necrosis [54]. There has been compelling evidence that ROS may participate in apoptosis either during the induction phase or contribute to the activation of caspases [55, 56]. Additionally, ROS directly damages DNA, proteins, and lipids and induces necrotic cell death through lipid peroxidation as well as protein and nucleic acid alteration [57]. Photoactivation of hypericin has been shown to cause a significant shrinkage in subcutaneous HK1 tumors [58]. There are two possible mechanisms responsible for tumor destruction induced by hypericin-PDT, viz., (a) direct killing of tumor cells by impairment of cellular membrane systems or mitochondria, and (b) indirect killing of tumor cells via destruction of the vascular system [59, 60]. This could explain the marked tumor shrinkage observed when hypericin concentration in the HK1 tumor tissue was maximal at the time of photoactivation. In addition, there was also a significant reduction in tumor size when light irradiation was performed at peak intravascular concentration. The finding that PDT is effective at high plasma drug levels suggests that vascular damage is also partly responsible for tumor destruction. However, vascular collapse following PDT is known to cause severe and persistent tumor hypoxia [61]. It limits the direct tumoral killing effect, as oxygen is an indispensable component in the PDT process. One of the major limitations of PDT is tumor recurrence, which is believed to be partly due to neovascularization. Recently, Zhou *et al*. [62] determined that combination therapy with PDT

and antiangiogenic agents was more effective in controlling tumor recurrence in a mouse model of poorly differentiated CNE2 NPC compared with PDT or antiangiogenic agents when administered alone. Lipid peroxidative stress was also observed to be significantly increased in HK1-treated tumors as compared with control tumors [58]. The malondialdehyde content in HK1 tumors was observed to increase by more than 60% compared with control tumors when measured 24 hours after PDT. Although the Cu-Zn SOD activity was not affected, there was a considerable reduction in GST levels following PDT [63].

3.4. PDT with hypericin in pediatric clinical trials.

Photosensitizers (such as hypericin, which is applied topically) are preferred in PDT dermatological therapy because of the reduced risk of prolonged skin photosensitivity, which often occurs after systemic administration [64]. Most of the research to date in the field of PDT in dermatology has focused on the use of aminolevulinic acid (ALA) as an early precursor of the heme biosynthetic pathway in which the endogenous photosensitizer protoporphyrin IX (PpIX) is formed [65-67].

In contrast to PIX, hypericin, as a broad-spectrum photoactive anthraquinone dye, is very photostable [68], and laboratory studies have shown that it has a pronounced pho-to-dependent antitumor activity [68, 69-73]. Thanks to these features, it can also be used to treat skin lesions in children and adolescents.

Boiy *et al*. demonstrated the potential of PDT with the use of hypericin in treating skin diseases [74-77] in their studies.

It is known that photosensitization of hypericin is toxic to cells. Ali *et al*. observed an increased apoptotic process after using hypericin irradiated with laser light [78]. It has also been observed that differential cell death in response to PDT is dependent on the cell type, light dose, and subcellular distribution of the photosensitizer [79-81].

An example of the use of hypericin in children in clinical trials is the research conducted by Seitz *et al*. They used PDT together with the photosensitizing agent hypericin to treat childhood rhabdomyosarcoma as an alternative treatment. In the experiment, 100% absorption was found in hypericin in rhabdomyosarcoma cells. The uptake of hypericin in fibroblasts was much less than in malignant tumor cells. Hypericin without exposure to white light did not affect the viability of cancer cells. After irradiation, PDT caused an almost complete inhibition of the proliferation of rhabdomyosarcoma cells with an increase in the incidence of apoptosis. The results of the research team led by Seitz suggest that hypericin is a new visualization tool and the therapy of childhood malignant neoplasm, which is rhabdomyosarcoma [82].

Hempfling *et al*. [83] also used PDT in combination with hypericin, this time in an in vitro study. As a result of PDT, there was a decrease in viability and a decrease in the proliferation of malignant tumor cells in rhabdomyosarcoma. As a result of the imposition of hypericin and the exposure of cells to laser light, the process of apoptosis was initiated [83].

In their research, Ocker *et al*. [84] also used an in vitro model for the pediatric treatment of rhabdomyosarcoma cells in children. The PDT treatment with hypericin showed a strong antitumor efficacy with positive cellular uptake and impairment of malignant cells at the metabolic and molecular levels. As a result of the PDT therapy, the number of living cells in the tumor was reduced [84]. The in vitro experiments are the beginning of applying this method of treatment in vivo, giving chances and hopes for curing children of malignant neoplasms and enabling children to live normal and healthy lives.

Gyenge *et al*. conducted studies in which, by means of hypericin and liposomal chlorin derivatives, they reduced the toxicity of the photosensitizer, thus reducing the undesirable phototoxic effects of PDT therapy [85]. A year later (in 2013), a research group also led by Besic Gyenge conducted research on the compilation of hypericin and a chlo-rine-based photosensitizer, this time in head and neck squamous cell carcinoma cells. The experiment showed that the mixture of hypericin and photosensitizer had a destructive effect on cancer cells. At the same time, the mechanism of apoptosis was observed. In addition, the immune system was activated related to the conducted mechanism of PDT therapy [86]. Liposomal preparations of hypericin were also studied by the research team of Plenagl *et al*. The preparations used solved the problem of poor solubility by making hypericin available as an aqueous preparation in therapeutic doses. Since hypericin tends to accumulate in the neoplastic tissue preferentially and is activated only by light, it is an indispensable element in treating skin lesions [87].

Research on using hypericin as a natural photosensitizer in pediatric studies contributes to expanding the current knowledge on the cellular response of epidermal cells to photodynamic therapy. Thanks to recent studies and reviews, it is possible to introduce the methodology into clinical trials in the field of pediatrics, which increases the effectiveness of future photobiological therapies. The varied results of the hypericin response in PDT underscore its efficacy and potential.

3.5. Future directions of hypericin-PDT treatment.

3.5.1. Leukemia.

Hypericin causes significant phototoxicity in human K562 leukemia cells, reducing cell viability in light intensity. Decreased cell population reduction and morphological changes are evidence of cell cytotoxicity, and the image of cells observed under the electron microscope clearly shows that hypericin was followed by apoptosis, which is responsible for cell death. Consistent with these structural changes in cells, cleaved caspase-3 and cleaved caspase-9 levels increased significantly after PDT [88,89]. Caspases are essential mediators of apoptosis. Hypericin showed a therapeutic approach in leukemia.

3.5.2. Bladder cancer.

Hypericin may be helpful in the treatment of bladder cancer, induced apoptosis or necrosis in a concentration-dependent manner, the mechanism being vascular destruction rather than cell destruction in these tumors [90]. The treatment has been shown not to damage the detrusor muscles or the underlying sphincter muscle layers. Hypericin, in combination with hyperoxygenation, was confirmed to destroy bladder cancer cells by apoptosis virtually completely [91,92].

3.5.3. Colon cancer.

Hypericin applied in vitro to colon cancer cells resulted in loss of cell viability in a light dose-dependent manner. In vivo, hypericin irradiation of tumors caused extensive vascular damage and tumor necrosis. Several studies have been performed to contribute to the discovery of the mechanisms of action of Hypericin PDT [93].

Phototoxic effects of hypericin loaded with superparamagnetic iron oxide nanoparticles were investigated. The results showed a complete arrest of cancer cell proliferation and induction of cell death. Other studies have indicated the possible ability to prevent tumors from returning [94,95].

3.5.4. Breast cancer.

In combination with other substances, hypericin inhibited cell proliferation and induced their apoptosis, decreased metabolic activity, and the viability of cancer cells [96]. In addition, photodynamic studies showed that hypericin inhibited cell colony formation, indicating a possible ability to prevent tumor recurrence. In various hybrid therapy experiments, remarkable inhibition of tumor growth as well as death of cancer cells have been proven [97]. 3 years later, Mokoena *et al*. used gold nanoparticles on which hypericin was adsorbed to increase the efficiency of PDT. The designed model was applied to the human breast cancer cell line MCF-7. The results confirmed and demonstrated that cell death characteristics emerged due to the therapy [98]. Advanced experiments are now combining nanotechnologies with hypericin in PDT. An example of research is the work of Zhou *et al*., who designed new theranostic nanoparticles based on dendrimers (manganese and polyester) for the treatment of breast cancer. The experiment showed that the use of the nanoparticle enhanced the uptake of the photosensitizer, which was hypericin, and also led to the enhancement of ROS. The entire process was imaged under the control of Magnetic Resonance (MRI) [99].

3.5.5. Cervical cancer.

Hypericin-induced photocytotoxicity in HeLa (cervical carcinoma cell line) cells is dependent on the number of cells, as the low cell density cultures were more sensitive to PDT than the confluent cultures. The increase in hypericin PDT-induced apoptosis of HeLa cells was confirmed, which may constitute the molecular basis for the development of new therapeutic strategies [100]. The hypericin complex with apomyoglobin (apoMb) was also studied, which, after irradiation, improved its effectiveness as a photosensitizer on HeLa cells by reducing the growth rate of the structure. The photodynamic effect of Hypericin-loaded lipid nanocapsules was also investigated. Promising results have been obtained for their use in cervical cancer [101].

3.5.6. Glioblastoma.

Hypericin can be used to regulate the proliferation of glioblastoma cells, and hypericin may be a promising phototoxic drug for the treatment of glioblastoma tumors [102,103]. Bassler *et al*., ocenili skuteczność akumulacji i penetracji hiperycyny w sferoidach komórek guza U-87. The authors analyzed hyperic distribution in cells. The experiment confirmed that the necessary incubation time is 30 minutes. This time is necessary to achieve PDT's most satisfactory therapeutic results [104]. Ng *et al*. used PDT activated by bioluminescence in combination with hypericin applied to human stage IV astrocytoma cells. The results confirmed that both bioluminescence-activated PDT and PDT combined with hypericin are able to effectively inactivate brain tumor cells (in this case, grade IV astrocytoma) [105].

3.5.7. Liver cancer.

Hypericin caused a decrease in cell viability, proliferative activity, and apoptosis in cells of the embryonic hepatoma (a malignant tumor of the childhood liver) and cells of the human liver cancer line [106]. The efficacy of hypericin associated with hepatocyte-specific antigen (anti-hypericin) was tested. The results confirmed the increased mortality of cancer cells compared to free Hipericin. In other studies, hypericin was used on cerium oxide nanorods coated with polydopamine, achieving specific targeting and good bio-compatibility [106].

3.5.8. Melanoma.

Hypericin decreased cell viability through various modes of cell death in melanoma cells associated with the absence (apoptosis) or the presence of a pigmented phenotype (necrosis) [107]. Moreover, hypericin PDT was shown to induce cell death after depigmentation significantly and to inhibit the growth of cancer cells [107] effectively. Another example is the work by de Morais *et al*., who published a study on using a copolymer in combination with hypericin in PDT in treating melanoma cells. The experiment confirmed that combining various substances into a polymer complex enables increased hypericin uptake by positively affecting PDT activity and effectiveness [108].

3.5.9. Lymphoma.

Hypericin was effective in prolonging survival in studies using a mouse lymphoma model by damaging the tumor vasculature as the primary mechanism of action. Treatment with hypericin decreased the viability of the human lymphoma cell line by altering the levels of proteins associated with apoptosis. In combination with visible light, hypericin significantly improved skin lesions in most patients [109]. Recently, a review by Liu *et al*. was published, presenting the latest discoveries and achievements in the field of photodynamic treatment of cutaneous lymphomas. The authors summarized many literature reports on PDT in the treatment of lymphomas. They compiled the data (molar extinction coefficient, uptake location, and penetration depth) in the performed PDT treatments using various photosensitizers, including hypericin. In the treatment of lymphomas, the effectiveness of PDT is high. However (as well as the authors of the review confirm the need for further studies (clinical as well as pre-clinical) examining different treatment regimens [110].

3.6. Limitations.

Despite the reduced toxicity and side effects of photoactive substances derived from medicinal plants [111] (hypericin), natural compounds are often difficult to create from complex extracts and maintain sufficiently high concentrations. Additionally, there are few plants from which natural photosensitizers can be obtained. Extracting phytochemicals from natural sources is difficult, expensive, and time-consuming. Therefore, using natural photosensitizers in clinical trials is uncommon and rarely conducted. This does not change the fact that the demand for plant production from which natural photosensitizers can be obtained in the future is growing more and more often [112].

However, PDT with hypericin successfully inhibits tumor growth via apoptosis and necrosis in various models and clinical trials [113-116]; what's more, it is much cheaper in comparison with the currently used photosensitizers.

Many literature reports are showing the effectiveness of PDT in combination with hypericin. However, further clinical experiments are still needed to analyze the effect of hypericin not only on the effectiveness of PDT but also on human physiology [117].

4. Conclusions

Neoplastic diseases are a constant challenge of modern medicine. Due to the constantly increasing incidence and mortality, alternative treatment methods are sought and improved. PDT, as a method without serious side effects, is gaining more and more popularity, mainly in pediatric studies in children with various types of cancer. The photodynamic effect of hypericin is directed at various subcellular organelles, primarily the mitochondria and the endoplasmic reticulum complex. Depending on drug administration and light conditions, the PDT effect leads to cell death, which occurs by induction of necrosis, apoptosis, or autophagy-related cell death. Overall, hypericin has excellent photosensitizing properties, tumorotropic properties, and low cytotoxicity, and it is one of the most effective photosensitizers extracted from plants.

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Conflicts of Interest

The authors declare no conflict of interest.

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