

The natural origins of cytostatic compounds used in rhabdomyosarcoma therapy

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D – writing the article; E – critical revision of the article; F – final approval of the article

Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online)

Adv Clin Exp Med. 2023;32(10):1179–1191

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Funding sources

None declared

Conflict of interest

None declared

Received on November 30, 2022

Reviewed on January 28, 2023

Accepted on February 12, 2023

Published online on March 15, 2023

Abstract

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children and represents a high-grade neoplasm of skeletal myoblast-like cells. About 40% of all registered soft tissue tumors are RMSs. This paper describes our current understanding of the RMS subtypes (alveolar (ARMS), embryonic (ERMS), pleomorphic (PRMS), and spindle cell/sclerosing (s/scRMS)), diagnostic methods, molecular bases, and characteristics. We also present the currently used treatment methods and the potential use of natural substances in the treatment of this type of cancer. Natural cytotoxic substances are compounds that have been the subject of numerous studies and discussions in recent years. Since anti-cancer therapies are often limited by a low therapeutic index and cancer resistance to pharmacotherapy, it is very important to search for new, effective compounds. Additionally, compounds of a natural origin are usually readily available and have a reduced cytotoxicity. Thus, the undiscovered potential of natural anti-cancer compounds makes this field of research a very important area. The introduction of model species into research examining the use of natural cytostatic therapies for RMS will allow for further assessment of the effects of these compounds on cancerous and healthy tissues.

Key words: rhabdomyosarcoma, natural compounds, anti-cancer therapy, muscle cells

Cite as

Lewandowski D, Szewczyk A, Radzka J, et al. The natural origins of cytostatic compounds used in rhabdomyosarcoma therapy. *Adv Clin Exp Med.* 2023;32(10):1179–1191. doi:10.17219/acem/161165

DOI

10.17219/acem/161165

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Introduction

Rhabdomyosarcomas (RMSs) are a heterogeneous group of malignant myogenous tumors. They are one of the most common soft tissue sarcomas considered a “childhood disease.” Soft tissue sarcoma in children represents a large group of malignant tumors, including RMS (approx. 40%).^{1–4} The importance of the problem is manifested by the establishment of global organizations that deal with rare diseases of soft tissue sarcomas, mainly in children. The 2 organizations in North America – the Intergroup Rhabdomyosarcoma Study Group (IRSG) and the Children’s Oncology Group (COG) – and the International Society for Pediatric Oncology (SIOP) in Europe strive to improve and support RMS treatment.^{2,5–7} They have developed a risk stratification system based on the primary localization of the tumor, pathology staging (occurrence of tumor-node-metastasis and anatomical localization), disease status after tumor resection, and histological assessment. These factors facilitate grouping cases into several subgroups, including low, standard, high, and very high-risk groups, and finally enable the selection of adequate treatment.^{7–9} According to the literature, RMS can develop in children with no family cancer history. However, the incidence of RMS is higher in children with parents or siblings who have had cancer, especially at a young age. Nevertheless, RMS is not considered a hereditary disease.¹⁰

Four main RMS subtypes are distinguished: alveolar (ARMS), embryonic (ERMS), pleomorphic (PRMS), and spindle cell/sclerosing (s/scRMS; Table 1^{9,11–31}). Based on microscopic observations, each RMS subtype is characterized by specific features. However, some researchers have suggested the use of a new molecular rather than histopathological RMS classification based on *PAX3/FOXO1* fusion oncogene expression. According to this system, RMSs can be classified as fusion-positive (FPRMS) and fusion-negative (FNRMS) tumors.¹¹ There are no genomic markers currently available (except *PAX/FOXO1* fusion) that can be used in RMS risk stratification. However, mutant *MYOD1* and *TP53* genes have recently been nominated as indicators of poor prognosis in FNRMS.¹²

The most frequent histological types of RMS observed in children and young people are ERMS and ARMS.¹³ Changes in ERMS patients are observed in the head and neck region, and in the genitourinary system. In contrast, ARMS occurs in the large muscles of the trunk, arms and legs, and has a high capacity for invasive growth and metastasis during the early stages of the disease.^{9,13,14} The PRMS is a rare tumor with aggressive behavior that occurs mainly in adulthood and very rarely in children. The most common primary sites of PRMS are the extremities, trunk wall and the genitourinary system. The PRMS may also give rise to cardiac metastasis.¹⁵ Clinical studies have also revealed PRMS in the liver with a hepatic cyst and in the pancreas.^{16,17} The s/scRMSs have a predilection

for paratesticular, head, neck, and limb sites in children and adults (Table 1).^{18,19}

Recent studies have revealed some genetic factors (age and biological sex) that can increase the risk for RMS. Studies have shown differences in the incidence of RMS by age, with peaks occurring during early childhood (children aged ≤ 4 years).²⁰ Additionally, a 2nd peak has been noticed during adolescence.¹⁰ It has also been shown that RMS is predominant in males; however, this discrepancy is consistent with most other pediatric cancers.^{21,22} More than half of RMS cases occur before 10 years of age, which indicates that in utero and early-life environmental exposures may play a large role in RMS etiology.²⁰ Martin-Giacalone et al. reviewed non-genetic factors increasing the risk of RMS, including parental age, recreational drug use by parents, prenatal diagnostic radiation, birth weight, allergies, hives, incomplete immunization, and breastfeeding for less than 12 months.¹⁰

Currently used cytostatics (i.e., doxorubicin, vinorelbine, vincristine, dactinomycin, or cyclophosphamide) cause numerous side effects. Therefore, it is important to find novel solutions to improve patient comfort. Natural origin compounds are shown to be as effective as standard cytostatics; however, they do not cause severe side effects. In this paper, we summarize the current knowledge on natural cytostatic compounds used in the treatment of RMS.

Signaling pathways in RMS

The effects of RMS therapies have been investigated in numerous areas of tumor cell activity considering various targets, including receptor tyrosine kinases (RTKs) and associated downstream signaling pathways, such as the Hedgehog (HH) signaling pathway, apoptosis, DNA damage response (DDR), cell cycle regulation, fusion proteins, and epigenetic modifications.²³

RTK signaling

Receptor tyrosine kinases are membrane-bound proteins involved in several physiological (e.g., embryonic development and wound healing) and pathological (signal transduction to tumor cells) processes.^{23,24} Since cancer cells produce and use growth factors, several RTKs, such as insulin-like growth factors (IGFs) and vascular endothelial growth factors (VEGFs), have been proposed as potential targets to treat RMS.^{23,25}

The IGFs 1 and 2 and insulin play crucial roles during skeletal muscle growth and differentiation, and act through the IGF 1 receptor (IGF1R). Furthermore, they are involved in the maintenance of adult muscle homeostasis. It has been found that IGF1R and its potent ligand IGF2 are widely overexpressed in childhood sarcomas, including RMS.^{26–31} The activity of IGF1R can be inhibited (via blocking its phosphorylation and downstream signaling)

Table 1. Comparison of 4 rhabdomyosarcoma subtypes: embryonic (ERMS), alveolar (ARMS), pleomorphic (PRMS), and spindle cell/sclerosing (s/scRMS)

RMS subtype	Characteristic features	Localization of neoplastic changes	Diagnosis methods	Molecular basis	Patients' age	References
ERMS	composed of primitive mesenchymal cells that show variable degrees of skeletal muscle differentiation; they are moderately cellular but in the typical pattern often contain both hypo- and hypercellular areas with a loose, myxoid stroma; perivascular condensations of tumor cells in the less cellular regions are common; sheets of small, stellate, spindled, or round cells with scant or deeply eosinophilic cytoplasm and eccentric, small oval nuclei with a light chromatin pattern and inconspicuous nucleoli	head and neck region including the nasal and oral cavities, as well as the middle ear and genitourinary system	immunodetection of MyoG; genotyping; vimentin in all cells (even most primitive); actin, myoglobin, myosin, and creatine kinase M staining in more differentiated cells	trisomy of chromosome 8 and loss of heterozygosity in the 11p15 chromosome region; inactivating mutations of TP53 and CDKN2A and activating mutations of RAS family genes	adolescents	9, 12, 18, 19, 20, 21
ARMS	cells form aggregates interrupted by fibrovascular septa that resemble alveoli of the lung with numerous aggregates and small, round scant in the cytoplasm of undifferentiated cells; nuclei of the cells are round with normal, dull, chromatin structures	large muscles of the trunk, arms and legs; genitourinary system	immunodetection of MyoG (>75% tumor cells), AP2 β and NOS1; genotyping; strong IHC staining	fusion in frame between undisturbed <i>PAX3</i> or <i>PAX7</i> gene on chromosome 2 or FOXO1 on chromosome 13; chromosomal translocations, including a frequent t(2;13) (q35;q14) or a variant t(1;13) (p36;q14); N-myc amplification is seen in 50% of cases (more aggressive cases)	children	9, 12, 18, 22, 23
PRMS	polymorphic, spindle-shaped, multinucleated, large cells with acidophilic cytoplasm	limbs, trunk wall, genitourinary system, cysts in the liver and pancreas, cardiac metastasis	positive immunocytochemical reaction for myoglobin, MyoD1, MyoG, fast skeletal muscle myosin, desmin; MSA and SMA	amplification of oncogenes JUN, MYC, CCND1, INT2, MDM2, and MALT; CGH reveals 8 highly amplified regions at 1p36.1-p36.2, 1p31-p32, 1q21-q31, 8q12-q21, 8q24-qter, 11q12-q13, 12q13-q14 and 18q12-q22	adults, very rarely in children	13, 14, 15, 18, 24, 25, 26, 27
s/scRMS	cells situated in sclerotic submucosa can be ribbon-shaped; neoplastic cells are arranged into microalveoli or lobules	head, neck, limbs	immunohistochemical diagnosis demonstrates positivity for desmin and vimentin; expression of myogenin (Myf-4), MyoD1 (Myf-3), myoglobin, SMA, and MSA	PAX fusion negative; recurrent <i>MyoD1</i> mutations; specific <i>MDM2/HMGA2</i> amplification at 12q13-15; loss of 10q22, loss of chromosome Y and gain of 18; in an adult cases: gain of chromosome 11 and loss of chromosome 22	children, adults	11, 16, 17, 18, 28, 29, 30, 31

MyoG – MoD1 myogenin; MSA – muscle-specific actin; SMA – smooth muscle actin; CGH – comparative genomic in situ hybridization; IHC – immunohistochemistry.

by picropodophyllin (PPP), which is a cyclolignan isolated from *Podophyllum* species.^{32,33} In vitro studies have revealed that PPP significantly blocks ERMS cell activity, especially migration and proliferation. Furthermore, RMSs increase their sensitivity to chemotherapy (e.g., vincristine and cisplatin) after PPP exposure. Additionally, the volume of the tumor is decreased after 2 weeks of PPP treatment in the human RMS xenograft model.³⁴ Importantly, PPP does not interfere with the insulin receptor (which is characterized by a high similarity to RTK) or RTKs.³⁵ Another advantage of PPP treatment is that it induces apoptosis and tumor regression, and interferes with microtubule assembly.^{33,35} The main side effect of vincristine is neurotoxicity.

Other side effects include a syndrome of inappropriate anti-diuretic hormone secretion, myelosuppression and alopecia.³⁶

The RAS/MEK/ERK and PI3K/AKT are signaling pathways promoting transcription, cell growth, motility, metabolism, and invasion. It has been shown that in RMSs, both pathways are characterized by abnormally increased activity. Therefore, they are frequent targets in ERMS treatment using doxorubicin, irinotecan, temozolomide, vinblastine, cyclophosphamide, or topotecan.^{23,35,37,38} Some in vitro studies have revealed positive effects when combining ERMS therapy with buparlisib (PI3K inhibitor), AEW541 (IGF1R inhibitor) and rapamycin (mTOR

inhibitor).³⁸ Similar effects have been observed for ERMS treatment with multiple PI3K/mTOR and MEK inhibitors (either trametinib or selumetinib).^{39–41} The combination of mTOR inhibitors and chemotherapy seems to be promising because it is well tolerated by pediatric, adolescent and young adult patients suffering from RMS.^{35,37}

HH signaling

The HH pathway is considered a key regulator of embryonic development and plays a crucial role in the adult organism in stem cell maintenance and tissue repair/regeneration.⁴² In humans, 3 HH proteins have been identified: Sonic (SHH), Indian (IHH) and Desert (DHH).⁴³ All of these proteins are ligands of Patched receptors (PTCH1, PTCH2). The binding between PTCH receptors and one of its ligands leads to the activation of smoothened (SMO) and prevents proteomic processing of GLI family zinc finger proteins (GLI1, GLI2 and GLI3). As a result, GLIs translocate to the nucleus, where they can act as transcription factors.⁴⁴ The HH signaling is necessary for many tumors, including ERMS, for their self-renewal and initiation. Gene mutation or deregulation results in alterations in this pathway.^{44–47} Since the HH pathway is constitutively activated in ERMS, blocking this pathway seems to have therapeutic potential. The inhibition of GLI1/GLI2 by GANT-61 causes a significant reduction in cell growth in RMS xenograft models, and this effect is increased by combined therapy with temsirolimus, rapamycin or vincristine.^{42,48}

Cell cycle and DDR

As inhibition of the cell cycle and DDR affects cell viability, targeted therapies could have potential anti-RMS applications.

Cell cycle

The cell cycle is a process strictly regulated by kinases. For example, cyclin-dependent kinases (CDKs), polo-like kinase 1 (PLK1) and Wee1 kinase are all involved in the regulation of cell cycle.⁴⁹

The CDKs play a crucial role during the whole cell cycle, and their activation is necessary for the progression of this process. Induced by Wee1 kinase, CDK1 phosphorylation leads to G2/M phase arrest and DNA repair. Therefore, CDK inhibitors may be useful in the treatment of RMSs when combined with other therapeutic agents. Indeed, promising results for ARMS have been observed with combined therapy using CDK, IGF1R or Wee1 inhibitors. However, combined treatment with palbociclib and doxorubicin has shown antagonistic effects in ERMS cells.^{50,51}

The PLK1 is involved in the G2/M phase transition. Since Wee1 kinase negatively regulates entry to the M phase, PLK1 is responsible for its phosphorylation leading to Wee1 degradation. Therefore, PLK1 inhibition can lead

to mitotic arrest and induce cell death.⁴⁹ In RMS cells, a higher level of PLK1 is observed, and ERMS is sensitive to PLK1 inhibition because it is involved in the activation of PAX3/FOXO1 expression.^{52–54} Interestingly, positive effects in RMS therapy have been observed with PLK1 inhibitors combined with anti-microtubule agents. Additionally, polo-box domain (a PLK1 catalytic domain) inhibitors combined with vincristine show a positive effect. However, the observed anti-cancer effects were less pronounced compared with monotherapies.^{52,54}

As mentioned above, Wee1 kinase is responsible for CDK1 phosphorylation leading to DNA repair. In vitro studies have shown that AZD1775 (Wee1 inhibitor)-combined therapy with cabozantinib or bortezomib is characterized by the highest efficiency in ERMS.⁵⁵ Moreover, patient-derived xenograft RMS models show a sensitivity to AZD1775 therapy, especially when combined with irinotecan and vincristine.⁵⁶

DDR

Poly (ADP-ribose) polymerase 1 (PARP1) is involved in single-strand break repair and is crucial for DNA protection.^{57,58} The PARP1 binds to damaged DNA, and this binding leads to the synthesis of poly (ADP-ribose) (pADPr) chains and recruits repair proteins. Since pADPr is involved in the release of PARP1 from DNA, the repair proteins can be attached (e.g., tyrosyl-DNA phosphodiesterase (TPD1)) to form a DNA repair complex with PARP. In vitro studies have revealed that olaparib treatment (PARP1 inhibitor) affects ERMS; however, this impact is indirect. On the other hand, combined therapy with irinotecan, melphalan, doxorubicin, and temozolomide increases the mortality of cancer cells.^{35,59,60} Additionally, irinotecan or rucaparib (PARP1 inhibitor) therapy combined with *TDPI* knockdown shows enhanced anti-cancer effects.⁵⁹

Apoptosis pathway

In anti-cancer treatment, therapies are mostly focused on inducing apoptosis, which leads to cell death. Some treatments are designed to activate apoptosis by extrinsic (through the death receptor) or intrinsic (through mitochondria) pathways. Apoptosis is induced by the caspase cascade activation that occurs after membrane-bound tumor necrosis factor (TNF)-related apoptosis-inducing ligand receptors (TRAILR1 or TRAILR2) connect with their ligands, namely TRAIL. The intrinsic mitochondrial pathway requires the release of mitochondrial cytochrome c and Smac to the cytoplasm. Released cytochrome c induces caspase-9 activation. On the other hand, Smac antagonizes survivin, which is one of the inhibitors of apoptosis protein (IAP).⁶¹

In vitro studies have revealed that TRAIL1 agonistic antibodies used in monotherapy or in combination with

IAP inhibitors do not affect RMS cells. However, TRAIL2 monotherapy shows dose-dependent cell viability. This treatment is more effective in combination with IAP inhibitors in ERMS cells.⁶¹ Additionally, ERMS in vitro therapy with YM155 (survivin inhibitor) reduces cell viability. Similarly, combined in vitro and in vivo therapies with cisplatin influence ERMS cells.⁶²

Current RMS therapies

Over 90% of patients with low-risk localized disease can be cured with multi-modal therapy, but the overall survival rates of patients with metastatic or recurrent disease remain dismal at 21% and 30%, respectively.^{63,64} There is no evidence of an improvement in the survival outcomes for metastatic or recurrent RMS in the past 30 years. Therefore, there is a need to develop new treatment strategies.

Currently, all RMS risk groups are treated in a multi-modal method, with the use of chemotherapy, surgical resection and/or radiation therapy (RT). In North American countries, chemotherapy includes treatment with natural origin substances such as vincristine, actinomycin D and cyclophosphamide (VAC).^{65–67}

Vincristine belongs to a group of drugs known as the vinca alkaloids. The source of these alkaloids is the Madagascar periwinkle (*Catharanthus roseus*). Vincristine acts by binding tubulin and inhibiting the formation of microtubules. Vinca alkaloids depolymerize microtubules and disrupt mitotic spindles, leading to cell cycle arrest.⁶⁸ Another mechanism of action of vincristine includes interfering with nucleic acid and protein synthesis by blocking glutamic acid utilization.⁶⁹

Actinomycin D is one of the oldest anti-cancer drugs and the first antibiotic to show anti-cancer activity.⁷⁰ It was isolated from *Actinomyces* species bacteria. This substance can inhibit transcription by binding DNA at the transcription initiation complex and preventing elongation of the RNA chain by the polymerase.⁷¹

In European countries, chemotherapy is carried out with the use of ifosfamide, vincristine and actinomycin D (IVA).⁷² The mechanism of action of ifosfamide is based on its reaction with DNA, with which it forms cross-links. This reaction leads to a blockage of the cell cycle. For this to occur, the biologically inactive form of ifosfamide must be metabolized into the active alkylating drug. This is done with the help of oxidases contained in the liver's cytochrome P-450.⁷³

Current research has shown that children with low-risk ERMS treated with multi-modal therapy have very promising results (90% of patients do not have relapses).⁷⁴ Primary tumor elimination is accomplished by surgery and/or RT with chemotherapy. Radiation therapy is part of the first-line treatment of virtually all ERMS patients.⁷⁵ According to the European Pediatric Soft Tissue Sarcoma Study Group (EpSSG), in patients with high-risk ERMS, the use of cyclophosphamide/vinorelbine (maintenance chemotherapy)

improves overall survival.⁷⁶ Since the EpSSG publication on treatment, maintenance chemotherapy has become the standard treatment for high-risk ERMS patients.⁷⁶ The long-term use of RT may cause toxicity.⁷⁵ The approach used to reduce toxicity proposes to include intensity-modulated RT (IMRT) or proton beam RT. In addition, it is recommended to use brachytherapy in areas that are assumed to reduce late toxicity (skeletal muscles, soft tissues), such as the vagina or bladder.^{77–80}

In the case of patients with ERMS metastatic disease, treatment with the multi-modal method does not tend to bring about the expected results. This method does not consider local therapy in the treatment of metastatic sites, such as in bone marrow. It has also been shown that high-dose chemotherapy, characterized by using very high doses of cytostatic drugs, does not improve patient outcomes in the same way as standard chemotherapy.⁸¹ Patients with low-risk ERMS who have relapsed are treated with chemotherapy drugs such as vincristine or irinotecan.⁸²

The identification and targeting of pathways responsible for ERMS invasion and metastasis are crucial for effective therapy. Our current knowledge on ERMS treatment failures caused by either metastatic disease, inadequate treatment or local tumor invasion forced us to plan different therapies. Given the limited number of patients with this disease, it is important to prioritize treatments that will bring about the greatest clinical benefits. The marked differences between RMS subtypes require more personalized treatments. In this context, one can mention the PAX3/FOXO1 oncogene, which is associated with enhanced FPRMSs metastasis, as an example.^{83,84} It has been shown that PAX3/FOXO1 fusion oncogene expression can be decreased by siRNA-mediated gene silencing.⁸⁵ Currently, preclinical and clinical trials are focused on small molecule inhibitors such as JQ1, which selectively disrupt the interaction between PAX3/FOXO1 and BRD4, disintegrating the fusion gene.⁸⁶ This approach has allowed for clinical success in some types of cancers, including lung carcinoma (inhibition of EML4-ALK1) or leukemia (inhibition of BCR-ABL).^{87,88} Another approach is to target the regulatory networks of PAX3/FOXO1 by targeting the regulatory kinases that are responsible for the stability or activity of the fusion protein. A recent study has highlighted RTKs as the target for small molecule inhibitors or immunotherapy, such as mAb CAR-T. A multi-track approach for RTK inhibition has proven to be an efficient strategy for refractory RMS.⁸⁹ A combination of ganitumab (an IGF1R monoclonal antibody) and dasatinib (a SRC family kinase inhibitor) is effective in blocking pathway substitutions and reducing tumor progression.^{90,91} A powerful but still limited treatment method is cancer vaccines based on human cytotoxic lymphocytes T that are capable of lysing HLA-B7+ RMS tumor cells.⁹² Biomarkers must be identified so that the most effective treatment method can be selected for each patient. One such factor could be *miR-486-5p*, which is increased in exosomes derived

from FPRMS patients.⁹³ Another interesting candidate as a possible therapeutic target is CD147, which is considered a cancer metastasis indicator expressed within FPRMS- and FNRMS-derived exosomes.^{94–96}

Limitations in current RMS treatment

As outlined above, the standard treatment for RMS is a combination of chemotherapy, radiotherapy and surgical resection of the tumor, adjusted to the stage of the disease. Commonly used drugs (e.g., vinca alkaloids) cause alkylation, leading to single- or double-stranded breaks in the DNA helix, preventing the cell from correct replication. However, the activity of alkylating compounds is not related to the cell cycle. Alkylating drugs target cancer cells that divide more often compared to other cells. As a result, the target of these substances is also normal cells that are characterized by frequent division (e.g., bone marrow).^{97–99} The other group of anti-cancer chemicals, antimetabolite drugs, are highly toxic to cancer cells, but also to normal cells. These drugs can damage the bone marrow and gastrointestinal mucosa and, in high doses, can be nephrotoxic and neurotoxic.¹⁰⁰

Although anti-cancer agents and RT have demonstrated many benefits in patients, these therapies in RMSs have many disadvantages, including the induction of multidrug resistance protein activity and the appearance of toxic side effects. It is also noteworthy that almost all anti-cancer agents affect not only cancer but also healthy cells.^{89,101} Moreover, most cytostatic compounds are only approved for the treatment of adults and not for pediatric cancers. Despite this, chemotherapy is still one of the most widely used treatments for all kinds of RMS at every stage of cancer progression.¹⁰²

Natural cytostatic substances in cancer therapies

Natural cytostatic substances are a group of compounds that have been under intense research for many years. The sources of anti-cancer natural compounds are arthropods, marine invertebrates, higher vertebrates, plants, and fungi. Numerous natural products exhibit anti-cancer activities, including anti-proliferative, pro-apoptotic, anti-metastatic, and anti-angiogenic effects, and also regulate autophagy, balance immunity, and enhance chemotherapy both in vitro and in vivo.^{103,104} Since anti-cancer therapies are often limited by a low therapeutic index and cancer resistance to pharmacotherapy, it is very important to search for new, effective compounds. The most common mechanisms for the anti-cancer activity of these compounds include, but are not limited to, migration, proliferation and cell death pathways, such as apoptosis and autophagy.

Their influences on the embryonic developmental signaling pathways (Notch, Wnt and HH) are especially advantageous for childhood cancers, such as ERMS.^{105,106} More than half of the anti-cancer drugs in use today have their origins in natural substances. In addition, naturally occurring chemicals and molecules often serve as a model for designing more active or more specific synthetic analogs.^{107,108} In many cases, a natural compound's absorption, distribution, metabolism, and excretion parameters can be improved by additional chemical modifications. Natural products, such as phytochemicals, minerals and vitamins, are also used in combination with anti-cancer drugs to facilitate treatment efficiency and minimize side effects.^{105,106,108–111} Moreover, natural compounds are usually easily accessible and have a reduced cytotoxicity (Table 2). The use of natural resources also offers a chance to find multi-target active compounds, allowing for a more effective way to treat cancer. The potential existing in as yet undiscovered natural anti-cancer compounds makes this field of research a very important area.

Tubulin-binding agents

An important group of natural anti-cancer therapeutics is comprised of tubulin-binding agents. Tubulin is the building block of microtubules, an essential part of the cytoskeleton that plays a vital role in the cell cycle. The mechanism of action of these chemicals is based on interference of the mitosis process and influence on the interphase, directing cells to the apoptotic pathway.¹¹² The main classes of drugs influencing the microtubules include the vinca alkaloids and taxanes.¹¹⁰

Alkaloids, such as vincristine, vinblastine and vindesine, are commonly used to treat hematological and lymphatic neoplasms, as well as several solid tumors. Structural modifications of these compounds led to the synthesis of vinorelbine, which is used to treat several cancers.¹¹³ The vinca alkaloids are effective drugs; however, they cause some side effects, such as myelosuppression and neurotoxicity.^{110,114}

The most common taxanes, paclitaxel and docetaxel, are obtained through semi-synthesis of a chemical compound (10-deacetylbaccatin III) obtained from the needles of the European yew tree (*Taxus baccata*). Several new drugs with improved toxicity and efficiency profiles have been tested (e.g., abazitaxel, paclitaxel poliglumex, paclitaxel+endotag, and polymeric-micellar paclitaxel).¹¹⁵ Taxanes are used in the treatment of metastatic breast cancer and as an adjuvant in chemotherapy.¹¹⁶

Other microtubule-destabilizing agents acquired from different natural sources, such as cyanobacteria (cryptophycins), marine mollusks (dolastatins) and Japanese sponge (halicondrins), have also been characterized. Their modified derivatives are undergoing clinical trials, and some of them show promising anti-cancer potential and good safety profiles.¹¹⁷ On the other hand,

Table 2. List of drugs/drug candidates (at different stages of development) used/assessed (in alphabetical order) for the use in the treatment for rhabdomyosarcoma and mentioned in the article

Drug	Origin	Mode of action	Compound ID number
Actinomycin D	antibiotic from <i>Streptomyces parvulus</i> spp.	DNA helix intercalating agent	457193
AEW541	pyrrolo[2,3-d]pyrimidine	IGF1R inhibitor	11476171
AZD1775 (adavosertib)	piperazine	Wee1 inhibitor	24856436
Beta-lapachone	benzochromenone from pink lapacho (<i>Handroanthus impetiginosus</i>)	redox disrupting agent	3885
Buparlisib	synthesis, aminopyridine	PI3K inhibitor	16654980
Camptothecin	alkaloid from the Chinese tree (<i>Camptotheca acuminata</i>)	topoisomerase inhibitor	24360
Cisplatin	synthesis, platinum coordination complex	alkylating agent	5460033
Combretastatin A4	combretastatin from the South African bushwillow tree (<i>Combretum caffrum</i>)	tubulin-binding agent	5351344
Cyclophosphamide	synthesis, nitrogen mustard-originated compound	alkylating agent	2907
Daunorubicin	anthracycline antibiotic from the bacterium <i>Streptomyces peucetius</i> var. caesius	topoisomerase inhibitor	30323
Docetaxel	tetracyclic diterpenoid from Pacific yew tree (<i>Taxus brevifolia</i>)	tubulin-binding agent	148124
Doxorubicin	anthracycline antibiotic from the bacterium <i>Streptomyces peucetius</i> var. caesius	DNA helix intercalating agent	31703
Epipodophyllotoxin	lignan from the Indian wild mandrake plant (<i>Podophyllum peltatum</i>)	topoisomerase inhibitor	105111
Etoposide	derivative of podophyllotoxin from the wild mandrake plant (<i>Podophyllum peltatum</i>)	topoisomerase inhibitor	36462
Exatecan mesylate	agent related to camptothecin (<i>Camptotheca acuminata</i>)	topoisomerase inhibitor	6918249
Flavopiridol	flavonoid from the pithraj tree (<i>Aphanamixis polystachya</i>)	CDK inhibitor	5287969
GANT-61	hexahydropyrimidine	GLI1/GLI2 inhibitor	421610
Geldanamycin	rapamycin analogue (macrolide lactam from <i>Streptomyces hygroscopicus</i> spp.)	mTOR inhibitor	5288382
Gimatecan	analogue of camptothecin (<i>Camptotheca acuminata</i>)	topoisomerase inhibitor	9577124
Homoharringtonine	cephalotaxine-derived alkaloid from Japanese plum yew (<i>Cephalotaxus harringtonia</i>)	80S ribosome-binding agent	285033
Irinotecan	pyranoindolizinoquinoline (carbamate ester) from camptothecin (<i>Camptotheca acuminata</i>)	topoisomerase inhibitor	60838
Isofasfamide	synthesis, nitrogen mustard-originated compound	alkylating agent	3690
Karenitecin	agent related to camptothecin (<i>Camptotheca acuminata</i>)	topoisomerase inhibitor	148202
Lurtotecan	analogue of camptothecin (<i>Camptotheca acuminata</i>)	topoisomerase inhibitor	60956
Paclitaxel	tetracyclic diterpenoid from Pacific yew tree (<i>Taxus brevifolia</i>)	tubulin-binding agent	36314
Palbociclib	synthesis, pyridopyrimidine	CDK inhibitor	5330286
Picropodophyllin (PPP)	cyclolignan alkaloid from mayapple plant family (<i>Podophyllum peltatum</i>)	IGF1R inhibitor	72435
Rapamycin	macrolide lactam from <i>Streptomyces hygroscopicus</i> spp.	mTOR inhibitor	5284616
Rucaparib	synthesis, tricyclic indole	PARP1 inhibitor	9931954
Selumetinib	synthesis, 1-methyl-1H-benzimidazole	PI3K/mTOR and MEK inhibitor	10127622
Temozolomide	synthesis, triazene analog of dacarbazine	alkylating agent	5394
Temsirolimus	ester analog of rapamycin (macrolide lactam from <i>Streptomyces hygroscopicus</i> spp.)	mTOR inhibitor	6918289
Teniposide	derivative of podophyllotoxin from the Indian podophyllum plant (<i>Podophyllum peltatum</i>)	topoisomerase inhibitor	452548
Topotecan	quinoline-based alkaloid extracted from the Asian tree (<i>Camptotheca acuminata</i>)	topoisomerase inhibitor	60700
Trametinib	synthesis, pyridopyrimidine	MEK inhibitor	11707110
Vinblastine	vinca alkaloid from Madagascar periwinkle (<i>Catharanthus roseus</i>)	tubulin-binding agent	13342
Vincristine	vinca alkaloid from Madagascar periwinkle (<i>Catharanthus roseus</i>)	tubulin-binding agent	5978
Vindesine	vinca alkaloid from Madagascar periwinkle (<i>Catharanthus roseus</i>)	tubulin-binding agent	40839
Vinflunine	semisynthetic vinca alkaloid from Madagascar periwinkle (<i>Catharanthus roseus</i>)	tubulin-binding agent	10629256
Vinorelbine	semisynthetic vinca alkaloid from Madagascar periwinkle (<i>Catharanthus roseus</i>)	tubulin-binding agent	5311497
YM155 (sepantronium bromide)	synthesis, organic bromide salt	surviving inhibitor	11178236

CDK – cyclin-dependent kinase. The table includes compound identifiers (CIDs) to facilitate access to the chemical molecule database, PubChem (<https://pubchem.ncbi.nlm.nih.gov>), which provides information on the structure and characteristics of the compounds.

microtubule-stabilizing compounds can also be effective against cancer. Some substances obtained from natural sources can bind microtubules and preserve them from dynamic reorganization. One example is epothilones obtained from myxobacterium (*Sorangium cellulosum*) and their chemical derivatives, such as ixabepilone.^{110,118}

Topoisomerase inhibitors

A 2nd group of important, natural anti-cancer chemicals are the topoisomerase inhibitors. Topoisomerase is a nuclear enzyme responsible for proper DNA replication and cell division. Its activity is highly increased in intensively dividing cancer cells. The DNA topoisomerases are well-known targets for anti-cancer therapies relying on enzyme poisoning. Such an approach leads to replication arrest and double-strand break formation. This mechanism of action is potentially dangerous since it brings the risk of therapy-related cancer and cardiotoxicity.¹¹⁹ Among the topoisomerase inhibitors, we can distinguish the camptothecins and their synthetic analogs. Camptothecin is an active compound present in an extract from the Chinese tree *Camptotheca acuminata*, and its derivatives include lurtotecan, exatecan mesylate, karenitecin, and gimatecan. These compounds are currently undergoing different phases of clinical trials.^{107,120}

A 2nd important group of topoisomerase inhibitors are the epipodophyllotoxins extracted from the wild mandrake plant (*Podophyllum peltatum*). Among the synthetic chemical derivatives, 2 compounds have been found to be active, namely etoposide and teniposide. Several side effects of both etoposide and teniposide have been observed, including hypersensitivity. However, some of these side effects have resulted from the use of adjuvants.¹⁰⁷

The 3rd prominent group of inhibitors specific to topoisomerase includes the anthracyclines. These compounds are derived from the *Streptomyces peucetius* bacterium. The most commonly used anthracyclines in clinical practice are doxorubicin and daunorubicin. This class of compounds exhibits a wide spectrum of anti-tumor activity, but at the same time, severe toxic side effects, such as cardiomyopathy and the induction of secondary cancers, can occur. The mentioned drugs and their analogs are effectively used in approaches combining immunotherapy and chemotherapy. The anthracyclines have been shown to induce immunological response.^{107,121}

Other natural anti-cancer compounds

Other natural anti-cancer compounds include active substances found in plants, microorganisms and marine organisms. At present, traditional plant-based medicines (e.g., flavopiridol, homoharringtonine, β -lapachone, and combretastatin A4) are still prevalently used as medical treatments around the world. These compounds exhibit a wide range of mechanisms of action. For example, flavopiridol

is a cyclin-dependent kinase inhibitor, homoharringtonine inhibits protein synthesis and blocks cell cycle progression, β -lapachone is a DNA topoisomerase I inhibitor, and combretastatin A4 inhibits tumor blood vessel growth. Other plant secondary metabolites, such as alkaloids, diterpenes, triterpenes, and polyphenolic type compounds, also exhibit great anti-cancer potential.^{110,122,123}

Microorganisms that thrive in diverse environments are also sources of novel anti-cancer compounds, such as rapamycin and geldanamycin. Rapamycin possesses immunosuppressing and anti-neoplastic activity. Geldanamycin, a rapamycin analog, has the ability to suppress the protein kinase activity of mTOR. Its chemical derivatives also show a potential to prevent cancer cell line proliferation.¹²⁴ The tumor-inhibitory features of the bacterial enzyme L-asparaginase are widely known. Since L-asparaginase inhibits protein biosynthesis in lymphoblasts, it is used to treat acute lymphoblastic leukemia.¹²⁵

Marine organisms, including plants, algae, bacteria, actinomycetes, fungi, sponges, and soft corals, are also sources of many chemical products. The most important compounds with the anti-cancer activity that have been isolated from marine organisms include peptides, polyphenols, polysaccharides, and alkaloids.¹²⁶

Natural cytostatic substances in RMS therapy

A standard treatment scheme based on chemo- and radiotherapy with tumor resection is still the most common for patients with RMS. However, high tumor malignancy combined with the young age of the patients limits successful application of the current treatment methods. The genetic and molecular pathways activated in RMS oncogenesis may constitute an efficient target for novel and effective tumor therapy development.⁸⁹ Flavonoids, which are phytochemicals produced in fruits, nuts and vegetables, exhibit anti-oxidant activities and protect against cancer development. It has been shown that flavonoids inhibit cancer cell growth and migration. In RMS, the fusion oncogene PAX3/FOXO1 transcription factor and G9a1 (a histone methyltransferase) are believed to be highly pro-oncogenic factors. Expression of both genes is regulated by the NR4A1 nuclear receptor.¹²⁷ A study conducted by Shrestha et al. revealed that the flavonoids kaempferol and quercetin bind to the ligand binding domain of NR4A1 and act as its antagonists in RMS cells.¹²⁸ These flavonoids also inhibit the expression of G9a, PAX3/FOXO1 and other pro-oncogenic NR4A1-regulated genes/pathways. Complementary results both in vitro and in vivo have demonstrated that kaempferol and quercetin are NR4A1 ligands acting as antagonists in RMS cells and mimic the effects of NR4A1 knockdown by RNAi. The authors suggested that NR4A1-active flavonoids can be repurposed for clinical applications in the treatment of RMS or other cancers where NR4A1 is a potential drug target.

The well-known Mediterranean herb *Rosmarinus officinalis*, commonly known as rosemary, also has therapeutic properties. Rosemary extract has been well-studied in animal models and has been shown to have anti-mutagenic and nontoxic properties.¹²⁹ Extracts from the leaves of *R. officinalis* possess a variety of bioactivities, including anti-oxidant, anti-tumor, anti-inflammatory, and anti-human immunodeficiency virus (HIV) properties.¹³⁰ *Rosmarinus officinalis* leaves contain numerous bioactive compounds, such as flavonoids, phenolic diterpenes, triterpenes, and caffeic acid esters.¹³¹ The RMS anti-cancer therapy can include doxorubicin or vinblastine separately, or a combination of these chemotherapeutics, with rosemary extracts. Research has shown that the use of rosemary combined with doxorubicin or vinblastine in anti-cancer therapy reduced their toxic effects.¹³¹ However, Kakouri et al. revealed the cytotoxic effects of *R. officinalis* extract on RMS cell lines.¹³² The rosemary extract used in the experiment contained a high phenolic content and showed strong anti-oxidant activity. According to the authors, *R. officinalis* extract is a potential alternative source of bioactive compounds which could be used in the future against RMS.

An extract from ginger (*Zingiber zerumbet*) also has promising therapeutic effects in the treatment of pediatric RMS cells. Zerumbone, a substance obtained from ginger, exhibits anti-tumor and anti-inflammatory properties. Evidence obtained so far indicates also that zerumbone has chemoprotective and chemotherapeutic effects on various cancers. Interestingly, it has been shown that the exposure of RMS cells to zerumbone results in cell growth inhibition, decreased proliferation and induction of apoptosis. The authors also showed that the treatment of pediatric RMS cell lines with zerumbone extract induces strong inhibitory and apoptotic effects through increased caspase 3/7 activity and increased reactive oxygen species (ROS) production, as well as through modulation of the nuclear factor kappa B (NF- κ B) pathway.¹³³

Curcumin, derived from the rhizomes of the turmeric *Curcuma longa*, also exhibits anti-tumor effects on pediatric RMS. Extracts from this plant induce apoptosis, inhibit cell proliferation and efficiently act on signaling pathways influencing tumor development. Studies carried out by Sorg et al. showed that curcumin decreases cell viability in RMS cell lines in a concentration-dependent manner, and enhances the effects of the cytotoxic drugs vincristine and dactinomycin, leading to reduced migration and increased cell apoptosis.¹³⁴

The in vitro cytotoxic activity of various plant extracts on RMS human cell lines (RD) was assessed by Maqsood et al.¹³⁵ The authors collected plant material from 6 plant species. The results showed that all of the plant extracts had a cytotoxic tendency towards RD cells after 48 h of incubation. Additionally, every plant extract showed more efficient cell-killing activity compared to 10 μ M cisplatin.

It has also been demonstrated that the natural isoquinoline alkaloid berberine exhibits anti-tumor activity in RMS

cell lines. Berberine is present in various medicinal plants, such as the Amur cork tree (*Phellodendron amurense*), which has been used as a traditional Chinese herb. Besides berberine, these plants synthesize a series of protoberberine-type alkaloids, such as palmatine, coptisine and jatrorrhizine. All of these compounds are believed to have anti-bacterial, anti-diabetic, anti-inflammatory, anti-oxidative, cardiovascular protective, and neuroprotective effects. The anti-tumor effects of berberine and palmatine have been studied on 3 human embryonal RMS cell lines: ERMS1, KYM1 and RD. It was observed that the intracellular incorporation of berberine in every RMS cell line was relatively higher than that for palmatine. Berberine significantly inhibited the cell cycle of all RMS cells at the G1 phase, whereas palmatine only suppressed the growth of RD cells.¹³⁶

The RMS is a highly malignant cancer most frequently found in children, accounting for 5% of all pediatric tumors. In the past decades, the survival rates for high-risk patients have not improved. At present, standard treatments, including chemotherapy, radiotherapy and surgical removal of the tumor, do not bring about the expected results and are often insufficient to avoid cancer development.¹³⁷ Therefore, the use of natural origin cytostatics may bring many benefits in RMS anti-cancer treatment. It is noteworthy that natural products can be obtained from 4 main sources: plants, animals, marine organisms, and microorganisms.¹³⁸ According to Mushtaq et al., natural substances are the foundation of novel therapeutic compounds with minimal side effects.¹³⁹ This is because of the presence of tremendous biodiversity among plants, animals, marine organisms, and microorganisms. The process of drug discovery from natural sources is slow and monotonous, and is associated with uncertain results. However, with the help of recent advancements, such as proteomics, genomics, transcriptomics and genetic modification, natural products can be screened for their bioactivity, which may contribute to future drug development.

Perspectives

There have been a lot of studies on natural compounds for RMS treatment and, in recent years, interest in these agents has increased. Most studies have been conducted in vitro and there are only scant results from the use of animal models. In vitro tests have their limitations and do not reflect the processes taking place in a living organism. Thus, the introduction of model species into RMS anti-cancer studies using natural cytostatic compounds will allow for a further assessment of their effects on cancerous and healthy tissues.

Among the vertebrates, zebrafish (*Danio rerio*) are a very good and commonly used model in cancer research.^{140–144} The zebrafish model is characterized by conserved

physiology and anatomy with mammals. A large number of progeny, rapid ex vivo development and transparent larvae allowing for real-time imaging of all developmental stages are the most valuable advantages of using this model organism.¹⁴⁵ The zebrafish model is also convenient for cancer research due to time- and cost-efficient genetic manipulations. Additionally, cancer tumors developed in zebrafish show a high histological and molecular similarity to human ones.¹⁴⁶

It is worth emphasizing that numerous mutant and transgenic zebrafish have been generated for studies of human cancers. One of them is the casper mutant, a transparent organism (because of a lack of melanocyte and iridophore cell populations) that allows for the investigation of labeled cancer cell growth in embryos or adult individuals.¹⁴⁷ In contrast to the mouse model, the transplantation of human tumor cells into zebrafish larvae does not require immunosuppressive drugs because the *D. rerio* adaptive immune system only becomes fully functional at 3 weeks post-fertilization.¹⁴⁸ The xenotransplantation of human cancer cells into zebrafish is a widely used method to analyze tumor biology and has enormous potential for the further evaluation of cancer progression and drug discovery. The xenotransplantation zebrafish model provides a unique opportunity to monitor cancer proliferation, tumor angiogenesis, metastasis, cancer stem cell self-renewal, and in vivo drug responses in real time.¹⁴⁹ Thus, zebrafish may be a valuable and efficient tool to evaluate novel therapeutic strategies for cancer and can contribute to new insights into tumor biology and cancer drug development.

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