REVIEW

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Challenges and potential for improving the druggability of podophyllotoxin-derived drugs in cancer chemotherapy

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Covering: up to 2020

As a main bioactive component of the Chinese, Indian, and American *Podophyllum* species, the herbal medicine, podophyllotoxin (PTOX) exhibits broad spectrum pharmacological activity, such as superior antitumor activity and against multiple viruses. PTOX derivatives (PTOXs) could arrest the cell cycle, block the transitorily generated DNA/RNA breaks, and blunt the growth-stimulation by targeting topoisomerase II, tubulin, or insulin-like growth factor 1 receptor. Since 1983, etoposide (VP-16) is being used in frontline cancer therapy against various cancer types, such as small cell lung cancer and testicular cancer. Surprisingly, VP-16 (ClinicalTrials NTC04356690) was also redeveloped to treat the cytokine storm in coronavirus disease 2019 (COVID-19) in phase II in April 2020. The treatment aims at dampening the cytokine storm and is based on etoposide in the case of central nervous system. However, the initial version of PTOX was far from perfect. Almost all podophyllotoxin derivatives, including the FDA-approved drugs VP-16 and teniposide, were seriously limited in clinical therapy due to systemic toxicity, drug resistance, and low bioavailability. To meet this challenge, scientists have devoted continuous efforts to discover new candidate drugs and have developed drug strategies. This review focuses on the current clinical treatment of PTOXs and the prospective analysis for improving druggability in the rational design of new generation PTOX-derived drugs.

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1 Introduction

More than 64% of new drugs are derived from natural products, and is the main pathway for drug discovery.[1] Traditional Chinese medicine plays a vital role in the comprehensive treatment of cancer. As an auxiliary and supplement of major treatment, modalities for cancer such as surgery, chemotherapy, and radiotherapy both clinical observations and biomolecular research have confirmed the therapeutic efficacy of traditional Chinese medicine in cancer,[2] including artemisinin and taxane.

Podophyllotoxin (PTOX) belongs to the arylnaphthalene class of lignans and is widespread in nature with high structural diversity. PTOX derivatives (PTOXs) are the main medicinal

components of traditional Chinese medicines including *Dysosma versipellis*, *Diphylleia sinensis*, and *Sinopodophyllum hexandrum*. PTOXs exhibit broad spectrum and high efficiency in antitumor, antiviral, and antibacterial activities, which could be an ideal target for new drug discovery. In 1990, WHO recommended 0.5% PTOX as a first-line drug for the treatment of condyloma acuminatum. The two main semi-synthesized podophyllotoxin glycosyl derivatives, namely, etoposide (VP-16) and teniposide (VM-26), were approved by FDA in 1983 and 1992. VP-16 was used in frontline cancer therapy against various cancer types, such as small cell lung cancer and testicular cancer.[3] VM-26 was mainly used for the treatment of acute lymphoblastic leukemia.[4] However, VP-16, VM-26, and other PTOX-derived drugs generally show serious side effects such as bone marrow suppression, hair loss, and neurotoxicity as they target fast-dividing cells. Several fast-dividing normal cells such as bone marrow cells and hair follicle cells are also under threat. In addition, large doses of drugs are required to circulate to non-targeted organs due to the clearing effect of the *in vivo* system.[5] The drug resistance was also triggered by new PTOX-derived drugs, leading to major side effects, for *e.g.*, metabolic disorder and secondary tumorigenesis.[6,7] To overcome the drawbacks in medicines, more attention has been invested for developing new efficient drug discovery strategies based on PTOX structural modification, clinical guidance, and diversity in partner drugs.

This review intends to focus on the druggability strategies for developing PTOX-derived drugs, including (i) predicting PTOX-responsiveness and structural optimization so as to select suitable clinical indications for overcoming toxicity; (ii) increasing the selectivity and sensitivity PTOXs for overcoming drug-resistance and toxicity; and (iii) exploiting drug delivery systems to enhance bioavailability and prevent adverse effects.

2 Podophyllotoxin is a classical natural lignan in drug discovery

2.1 Typical structural representative of natural lignans

Most of the chemical drugs in the market are obtained by the synthetic or semi-synthetic modification of natural products with a certain biological activity. As a class of plant secondary metabolites, lignans are derived from the oxidative dimerization of two phenylpropanoid units, dimers, trimers, or tetramers. To date, more than 200 classical lignans and 100 neolignans have been characterized in over 70 families in the plant kingdom.[8,9] Lignans are generally classified into six major subtypes: dibenzylbutanes, dibenzylbutyrolactones, arylnaphthalenes/aryltetralins, substituted tetrahydro-furans, 2,6-diarylfurofurans, and dibenzocyclooctadienes.[10] PTOXs is a class of lignans found in the podophyllum resin obtained from the roots of *Podophyllum* plants *Dysosma versipellis*, *Diphylleia sinensis*, and *Sinopodophyllum hexandrum*. The structure of PTOXs contains a dimeric skeleton that is formed by a β–β′-linkage between two phenyl propane units, some of them with a different degree of oxidation in the side-chain and a different substitution pattern in the aromatic moieties.[11] PTOXs present typical structural characteristics of lignans such as a carbocycle between the two phenylpropane units, which are attached by two single carbon–carbon bonds in the side chains

Associate Prof. Wei Zhao has studied and worked in Prof. Ya-Jie Tang's group to carry out research on the design, synthesis, and druggability of podophyllotoxin-derived drugs based on tubulin, topoisomerase II, or other targets for new drug development since 2007. Based on the study of the drug–tubulin complex crystal structure, a dual-target drug design strategy for podophyllotoxin derivatives was established. A method of tandem biotransformation was designed and developed to modify the molecular structure of podophyllotoxin for the directional synthesis of potential antitumor leading compounds. Wei Zhao is committed to research on druggability of podophyllotoxin derivatives.

Ying Cong started her doctoral study and scientific research under the supervision of Prof. Ya-Jie Tang in 2019. Her doctoral research focuses on the design, synthesis, and discovery of podophyllotoxin leading compounds with high activity and low toxicity by the strategy of specificity-targeting tumor cells or tissues.

Since 2005, Prof. Ya-Jie Tang's group has been committed to the discovery of new podophyllotoxin-derived anti-tumor drugs. A series of candidate drugs with nanomolar-potency antitumor activity were developed, which were superior to the approved tubulin-targeting antitumor drugs. Compared with the approved topoisomerase II-targeting anti-tumor drugs, other candidate drugs exhibited weaker DNA damage and millimolar-potency (low) toxicity. Prof. Ya-Jie Tang's group also focuses on the biomanufacture of podophyllotoxin-derived drugs in order to solve the problems of complex synthetic steps, enantiomers, environmental pollution, etc., during chemical synthesis processes.

of β–β′ positions (Fig. 1A). In contrast, PTOXs consists of five rings with four chiral centers, an aryl-tetralin-type skeleton, a *trans*-lactone, and four rings form a rigid skeleton (Fig. 1B), which is an important region embedded in the receptor protein. PTOXs contain almost all of the functional groups of aryl-naphthalene lignans. Thus, PTOXs are the most prominent lignans with significant pharmacological activities.

2.2 Broad spectrum bioactivity

The structural diversity of PTOXs is accompanied by many attractive pharmacological activities of the lignan family, such as antitumor, antioxidant, antibacterial, immunosuppressive, and antiasthmatic properties. PTOX cannot be used as an oral or injectable drug as it exhibits high toxicity *in vivo* but it has a special effect in the treatment of abnormal skin cell proliferation caused by human papillomavirus (HPV).[12,13] PTOX was effective in the treatment of anogenital warts in children and *Molluscum contagiosum*, which is generally a self-limiting benign skin disease that affects mostly children and young adults. PTOX has also proved effective in the treatment of rheumatoid arthritis as a result of the reduction it brings about in the activating system of the complement system. PTOX and some of its isomers have been tested for other activities such as insecticidal activity, phytogrowth inhibitory activity, and ichthyotoxic activity. PTOX showed significant ichthyotoxic, insecticidal, and phytogrowth inhibitory activities. The FDA-approved drugs VP-16 and VM-26 are glucopyranose derivatives of 4′-demethylepipodophyllotoxin (DMEP), which act on DNA–Topo II in clinical application. In 2017, the National Comprehensive Cancer Network (NCCN) showed that VP-16 was preferred for the treatment of high-dose chemotherapy for lymphoma, especially for some relapsed or primary refractory lymphoma after initial treatment in patients.[14,15] VP-16 also played an excellent role in the treatment of unresectable non-small cell lung cancer (NSCLC). The phase III clinical trial results of combination drug VP-16/cisplatin showed that the three-year overall survival (OS) of radiotherapy combined with VP-16/cisplatin was significantly higher than that of paclitaxel/carboplatin, and the median survival time was 23.3 months.[16,17]

Not only do the PTOX derivatives exhibit superior antitumor activity but also antiviral activity against various viruses or their complications. The antilung cancer drug VP-16 is also a WHO Essential Medicine and a powerful selective suppressor of activated T-cells and monocytes that reduces the production of inflammatory cytokines. Etoposide was used to treat the cytokine storm in critical complications patient of the coronavirus disease 2019 (COVID-19) in phase II (*i.e.*, ClinicalTrials NCT04356690) by conventional drug new use strategy on May 8, 2020.[18] The rationale for the use of VP-16 to treat the cytokine storm in COVID-19 is the high mortality associated with the hyperinflammatory response to the virus. To date, there are still no other drugs in the market except for VP-16, VM-26, and etopophos. PTOX is a classic leading compound for many developmental possibilities and has great advantages.

2.3 Diversity in antitumor mechanisms

Structurally diverse PTOXs exhibit different antitumor mechanisms. It was first discovered that PTOXs could target the colchicine binding domain of tubulin by the trimethoxybenzene unit of the E ring (Fig. 2). The tubulin inhibitor 4β-(1,2,4-triazol-3-ylthio)-4-deoxypodophyllotoxin (Tr-PTOX) showed synchronous binding to a site in αβ-tubulin that is distinct from the colchicine domain.[19] Comparing the structures of the complexes showed that the side chains of the T7 loop of β-tubulin flip outward and the T5 loop of α-tubulin changes its conformation. From the comparison of the crystal structures of tubulin–Tr-PTOX and tubulin–colchicine complexes, the β-subunit T7 loop was found to reversibly participate in resistance to straightening, which opposes the microtubule assembly by flipping in and out. Together with the biochemical results from Tr-PTOX, the structural data highlight the main contributors in the α-subunits and the colchicine domain β-subunits: the dual-target binding sites in the α-T7 loop and the β-H7-T7 loop of tubulin. Tr-PTOX can synchronously bind to αβ-tubulin. The
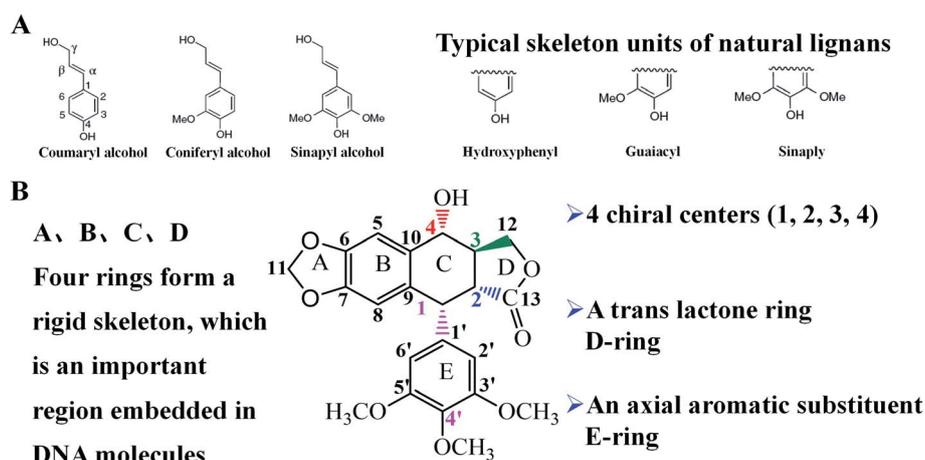
Fig. 1 The molecular structure of podophyllotoxin-like lignans. (A) The typical molecular skeleton of natural lignans. (B) The chemical characteristics of the molecular structure of podophyllotoxin.

structures also highlight common features for the design and development of novel potent microtubule destabilizing agents. There is a short helix at the α-T5 loop formed after the binding of Tr-PTOX to α-tubulin. Tr-PTOX inhibited microtubule polymerization, which may be a result of the interaction with α-T5, which was a significant factor in lateral tubulin interactions. The intramolecular interaction network that stabilizes the T5 loop helix of both α- and β-tubulin also forms in the microtubules in the absence of a ligand.

4′-Demethylepipodophyllotoxin (DMEP) is an important PTOX derivative, the configuration of whose C-4 cite is converted from α to β and the methoxy group of E-4′ is changed into the hydroxyl group. DMEP derivative can bind with topoisomerase II (Topo II) and DNA in the cells to form a ternary complex (Fig. 2), which indirectly inhibits the transcription and replication of DNA for inducing tumor cell apoptosis.[20] The crystal structure of the Topo II–DNA–etoposide ternary complex reveals that the bound etoposide interacts extensively with both the protein and DNA. All parts of the aglycone core contribute to drug–DNA interaction by being located between the base pairs. The A, B, and D rings also mediate drug–protein interactions. The E ring is anchored both by interactions with G478, D479, and L502, and by being sandwiched between R503 and the deoxyribose ring of the nucleotide. The interplay between the protein, the DNA, and the drug explains the structure–activity relationship of the etoposide derivatives and the molecular basis of drug-resistant mutations.[21]

In recent years, new targets of podophyllotoxin derivatives have been found. Picropodophyllotoxin (PPP) with a *cis*-lactone configuration has no inhibitory effect on the microtubules and apparently lacks cytotoxicity.[22] In contrast with etoposide and PTOX, PPP has no inhibitory effect on topoisomerase II and tubulin, and consequently does not cause any DNA breakage. After these observations, PPP has received little or no attention. Until 2004, PPP was identified as an effective and selective inhibitor of insulin-like growth factor-1 receptor (IGF-1R) tyrosine phosphorylation, which is an important role in the transformation and proliferation of malignant cells (Fig. 2). Surprisingly, PPP could not inhibit the insulin receptor (IR), which is highly homologous with IGF-1R and the beta subunit is 95 kD.[23] IGF-1R could maintain the malignant phenotype of the tumor cells and is involved in tumor cell protection against

antitumor therapy.[24] In contrast, IGF-1R is not an absolute requirement for normal cell growth. PPP could reduce pAkt and the phosphorylated extracellular signal regulated kinase 1 and 2 (pErk1/2), induced apoptosis, and caused complete tumor regression in xenografted and allografted mice by significantly blocking the IGF-1R activity. PPP did not affect the insulin receptor or compete with ATP in the *in vitro* kinase assay, suggesting that it may inhibit IGF-1R autophosphorylation at the substrate level.[25,26] In 2010, PPP was demonstrated to inhibit the growth of human glioblastoma cell lines and reduce the phosphorylation of IGF-1R and Akt. *In vivo* experiments have shown that PPP can not only cause significant tumor regression in subcutaneously transplanted tumors in mice but also shows obvious degenerative changes in transplanted tumors in the brain of nude mice, which indicates that PPP can cross the blood–brain barrier.[27] In addition, when interval radiation induces mouse glioma stem cells, the cells will produce radiation resistance through the upregulation of IGF-1R. The tumors formed by these cells have also been proven to be radiation resistant *in vivo* but they were significantly inhibited by PPP monotherapy. Surprisingly, PPP treatment also makes tumors sensitive to radiation. PPP was found to interfere with microtubule dynamics to suppress cancer in 2014, which is independent of the IGF-1R pathway. Clinical trials have shown that PPP has no neurotoxicity and the side effects are mainly neutropenia but the process is reversible.[28,29]

3 Challenges in the druggability of podophyllotoxin derivatives

3.1 The toxicity and toxicological mechanism

Although podophyllotoxin-derived drug is active in the treatment of many cancers and is widely used in therapy, it presents several limitations, such as moderate potency, poor water solubility, development of drug resistance, metabolic inactivation, and toxic effects by targeting rapidly-dividing tumor cells, such as bone marrow cells, digestive tract cells, and hair follicle cells.[30,31] Clinical studies have showed that normal cells are also affected by serious side effects, including bone marrow suppression (*e.g.*, decreased blood cells), mucositis (*e.g.*, gastrointestinal mucosal inflammation), hair loss, cardiotoxicity, neurotoxicity, and immunosuppression. VP-16 exhibited nephrotoxicity by triggering reactive oxygen species (ROS) generation and activating extracellular regulated protein kinases (ERK) in HK-2 cells. ROS promotes mitochondrial biogenesis and cytosolic ATP induction, which eventually enhance necrosis but not apoptosis. On the other hand, ERK activation causes caspase 3/7 activation, which in turn ruptures the nuclear envelope and eventually induces apoptosis. Furthermore, ERK activation is independent of ROS generation.[32–34]

o-Quinones are reactive metabolites of natural catechol products (*e.g.*, catechin, curcumin, and podophyllotoxin) that could be responsible for the cytotoxic/genotoxic and chemopreventive effects of the parent catechol.[35] Catechol can be readily oxidized to *o*-quinones by an oxidative enzyme, metal
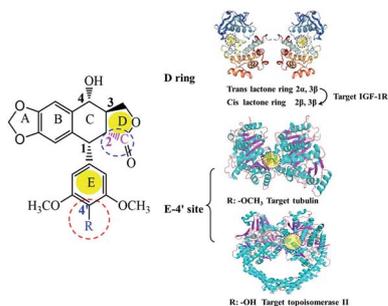
Fig. 2 Effect of the structural diversity of podophyllotoxin derivatives on the drug target diversity.

ion, or, in some cases, molecular oxygen. Based on the oxidation, a semi-quinone radical is initially formed, which is readily converted to the o-quinone. Random oxidative damage generates ROS and causes oxidative stress within the cells in the molecular oxygen process. The successive removal of two electrons (or, alternatively, an electron and a hydrogen atom) from catechol is usually catalyzed by cytochrome P450 or other oxidative enzymes, such as peroxidases.[35–37] Once formed, o-quinones have a variety of biological targets, including normal physiological metabolizing enzymes. The initial reaction with glutathione (GSH), a major non-protein thiol, leads to GSH depletion either through direct alkylation and/or through oxidation-generated oxidized glutathione.[38–40] A major metabolite of etoposide is produced by o-demethylation catalyzed by P450 that gives catechol, which is readily oxidized to an o-quinone. The o-quinone of etoposide causes depletion of GSH and oxidative stress within the cancer cells, thus inducing apoptosis. Similar reactions occur in normal cells, which contribute to side effects. The specific target of the etoposide o-quinone is topoisomerase and the etoposide o-quinone is considered to be a classical topoisomerase II poison. Other biological targets of etoposide o-quinone include stress proteins such as BiP, GST P1-1, and the JNK signaling pathway, and the IKK and the NF-κB signaling pathways.[41]

3.2   Drug resistance and its origin

The phenomenon of drug resistance in human malignancies is a multifaceted problem that severely limits the success of existing therapeutic agents. The phenomenon of drug resistance confers upon the malignant cells the ability to withstand exposure to lethal doses of many structurally unrelated antineoplastic agents, including VP-16 and VM-26.[42,43] Acquired or intrinsic drug resistance is one of the major handicaps in the success of podophyllotoxin-derived drug chemotherapy. Currently, there are two main mechanisms of etoposide resistance: (1) drug resistance has been characterized by the over-expression of a membrane-associated glycoprotein, the P-glycoprotein, which is a member of the large ATP-binding cassette family of proteins and appears to play a role in drug efflux.[44] The oral absorption of etoposide is mainly limited by its active efflux out of the cells by and metabolism by cytochrome P450 because etoposide is recognized as a dual P-glycoprotein and cytochrome P450 3A substrate drug with poor water-solubility.[45–47] The substrate recognition of etoposide usually occurs within the transmembrane (TM) domains in multiple-overlapping binding sites, in which could ATP energize the transfer of substrates from these binding sites on the P-glycoprotein to the outside of the cell.[48] (2) Similar to other topoisomerase inhibitors and DNA damaging agents, resistance to etoposide may arise as a result of alterations in the target expression and activity, increased drug efflux, and alterations in the DNA damage response mechanisms.[49,50] Mutations in Topo II have conferred resistance to Topo II-targeting anticancer drugs. These drug-resistant mutation sites can be classified into three groups on the basis of their potential mechanisms of resistance.[51] Group 1—clustering around the E ring (i.e., P501,

L502, R503, and E522) and glycosidic moiety (i.e., G776, E777, Q778, A779, and M782)—likely decreases drug-binding affinity by eliminating key drug–protein interactions or by introducing structural changes in the drug-binding pocket. Group 2 is composed of residues participating in DNA-binding, such as K505, L507, R510, H514, A668, P732, K814, Q922, A924, and V925. By reducing the enzyme's affinity towards DNA, these mutants likely produce less cleavage complexes and thus compromise drug action. Group 3 contains mutations that reduce the catalytic activity of Topo II; residues G465, K466, R496, and G550 may impair the communication between adenosine triphosphatase (ATPase) and the TOPRIM domain; P819 and Y821 are key active-site residues.[21] Notably, group 1 mutations may display specific resistance to etoposide. In contrast, mutations in groups 2 and 3 exhibit cross-resistance.

3.3   Low water solubility and bioavailability

Low water solubility and poor bioavailability are the most widespread problems in podophyllotoxin derivatives. Etoposide has a bioavailability of approximately 50% and is metabolized in the liver via the cytochrome P450 system.[52,53] About 50% to 60% of etoposide is excreted in the unaltered form by the kidneys and 40% to 50% through biliary excretion. Kidney disease is associated with increased (mainly hematologic) toxicity. Dose reductions of 25% and 50% are recommended for CrCl of 15 to 50 mL min^{-1} and less than 15 mL min^{-1}, respectively. Etoposide has been administered to HD patients (50% dose reduction either before or after dialysis). Also, a dose reduction of 50% is suggested in PD patients. Etoposide has to be administered in a formulation containing polyethylene glycol, Tween 80, ethanol, and benzyl alcohol due to its low solubility.[54] These excipients can increase the toxicity of intravenous infusions. Recently, etoposide phosphate was introduced to improve the solubility of etoposide. The phosphate analog prodrug of etoposide, viz., etopophos, was approved by FDA in 1996, and can be rapidly absorbed and completely converted to the parent compound in vivo.[55–57]

4   Ongoing efforts for improving the druggability of podophyllotoxin derivatives

4.1   The drug combination for enhancing the effectiveness and reducing adverse drug reactions

VP-16 is a first-line drug in the clinical treatment of cancer but the drug alone produces serious bone marrow suppression and genotoxicity. In actual clinical treatment, several drugs, or drugs and treatment methods are used in combination, which could significantly improve the curative effect or reduce the side effects; this strategy is known as drug combination. In clinical antitumor therapy, different types of drug combinations have been used clinically for improving the antitumor activity and reducing serious side effects of VP-16. There are sequential therapies used in combination therapy and combination therapies have also been applied simultaneously.

Drug combinations with different mechanisms of action can enhance the efficacy. For example, the combined use of alkylating agents and antimetabolites can often increase the effectiveness of treatment. Cyclophosphamide (CPM) is a classic alkylating antitumor drug. The combination of VP-16 and CPM is widely used in refractory and recurrent lymphoblastic leukemia and lymphoma in the pediatric population. The combination of VP-16 and CPM with the purine nucleoside antimetabolite AraG was used for the effective treatment of refractory or relapsed T-cell leukaemia or lymphoma in seven children. Five of them were completely relieved after 1–2 courses of treatment but showed certain neurotoxicity.[58] In addition, some researchers have co-administered VP-16/CPM with another purine nucleoside analog, clofarabine, in order to treat relapsed/refractory acute myeloid leukemia (AML). In all the 17 patients, a 41% response was obtained and 4 patients were completely relieved.[59] Unfortunately, this result is not superior to therapy, and clofarabine and cyclophosphamide pose a high risk of bone marrow aplastic anemia. VP-16 could also be used in combination with total body irradiation (TBI) as a prognostic therapy to reduce the risk of relapse and improve leukemia-free survival after hematopoietic stem cell transplantation for acute lymphoblastic leukemia. Compared with CPM/TBI, VP-16/TBI can significantly reduce the risk of relapse (Table 1).[60]

Based on the characteristics of cell growth cycle, another principle is to use combined drugs that act on different phases of the cell growth cycle in order to kill tumor cells at multiple links. VP-16 belongs to the antimetabolism class of drugs, which targets Topo II, inhibits mitosis, and stops cell division at the S phase or the G_2 phase. Paclitaxel (PTX) can act in the M phase, which plays an antitumor effect by interfering with the microtubule network necessary for the function of cell mitosis and interphase cell division. VP-16 and PTX were co-wrapped in PLGA microspheres to combine them for administration in the

treatment of SAOS-2 osteosarcoma and it was found that the 24 h cell survival rate was only 48%, which was significantly better than that of single administration.[61] Similarly, hybrid nanoparticles of PTX and VP-16 were prepared as a lipid polymer, which showed a significant tumor regression effect and the apoptosis rate was significantly higher than that of the single administration group (45% vs. 15%).[62]

For slow-growing solid tumors, non-cell cycle-specific drugs can be used to stop the proliferative phase and part of the G_0 phase cells in order to shrink the tumor body, to drive the G_0 phase cells into the proliferation cycle, and then to use cell cycle-specific drugs to kill them. Platinum drugs are the most typical type of non-cell cycle-specific drugs. VP-16/cisplatin is often used to treat inoperable non-small cell lung cancer. Another similar and commonly used combination is PTX/carboplatin. Studies have shown that compared to PTX/carboplatin, VP-16/cisplatin treatment showed superior survival advantage; the three-year survival rate reached 41.1%, while that of the latter was only 26%.[63] Most small cell lung cancer (SCLC) patients have a wide range of stages at the time of onset and the prognosis is still poor. Recently, researchers have conducted clinical trials on the basis of VP-16/cisplatin, in combination with the immune checkpoint inhibitor durvalumab, and have achieved exciting results. The median survival time is 13 months, and in 34% patients, the survival time exceeds 18 months.[64] When combined with radiotherapy, the median survival time of the VP-16/cisplatin group was approximately 3 months longer than that of the PTX/carboplatin group.[65]

Ovarian cancer is the main cause of death in patients with malignant gynecological tumors. About 75% of patients with advanced ovarian cancer eventually relapse. Almost all patients with recurrent disease will eventually develop platinum resistance.[66] For platinum-resistant ovarian cancer, non-platinum

Table 1 Multiple drug combination strategies

VP-16 combined with drugs	Indication	Pharmacology	Reference
Cyclophosphamide + AraG	Recurrent lymphoblastic leukemia; lymphoma	Sequential therapies; nucleic acid alkylation	58–60
Paclitaxel	Osteosarcoma	Sequential therapies; inhibition of different phases of the cell cycle	61 and 62
Cisplatin	Solid tumors; non-small cell lung cancer	Sequential therapies; G_2 and M cell cycle arrest	63
Cisplatin + durvalumab	Non-small cell lung cancer	Sequential therapies; G_0 and G_2 cell cycle arrest	64 and 65
Apatinib	Ovarian cancer; non-small cell lung cancer; small cell lung cancer	Combination therapies	66 and 67
Dexamethasone + cyclosporin A	Hemophagocytic lymphocyte histiocytosis	Combination therapies; inhibit the production of interferon-gs	68
Ciprofloxacin	Leukemia; acute myelogenous leukemia; acute myeloid leukemia	Sequential therapies; inhibits DNA synthesis and replication	69
Vorinostat	Leukemia; solid tumors; relapsed/refractory sarcomas	Combination therapies; G_1 or G_2 phase cell cycle arrest; inhibition of histone deacetylase	70

cell cycle-specific drugs, such as PTX or VP-16, are preferred. The antiangiogenic agent apatinib, in combination with VP-16, exhibited therapeutic effect and antidrug resistance in clinical trials against platinum-resistant ovarian cancer. One strength of this study that is to be noted is that both apatinib and oral etoposide were orally administered without the need for hospital admission or an infusion pump during a median progression-free survival of 8.1 months. The strategy of using apatinib in combination with VP-16 showed that the treatment regimen might have improved adherence and economic effectiveness for patients.[67]

Hemophagocytic lymphocyte histiocytosis (HLH) is a life-threatening hyper-inflammatory syndrome. In 1994, it was discovered that the combination of etoposide/dexamethasone can greatly improve the 5 year survival rate of patients (21–54%). Due to the high mortality rate from week 1 to week 8, the therapy was improved in 2004, with intensive treatment involving the pre-administration of cyclosporin A (CSA) aimed at increasing immunosuppression without causing additional bone marrow toxicity. In addition, CSA has been reported to inhibit the production of interferon-g (IFN-g), which is beneficial for the treatment of HLH. This clinical study was conducted in 2011, and the patient's median 5 year survival rate (5.2 years) was found to increase to 61%.[68]

PTOX has also shown an important position on the road to antivirals, which are the best treatment for AIDS-related Kaposi's sarcoma that is a common cause of morbidity and death in HIV-infected people. People have tried to use chemotherapy drugs combined with antiretroviral therapy to treat this disease, and bleomycin, PTX, VP-16, and other drugs were selected. Unfortunately, the VP-16 group did not work well and ended early. Ciprofloxacin significantly improved the growth inhibition activity of the standard antineoplastic drug VP-16 in a schedule-dependent manner and, therefore, may have an important role as an adjuvant therapeutic agent in a clinical setting.[69] VP-16 combined with vorinostat was used for obtaining a synergistic effect and the synergism became more pronounced when etoposide was given after vorinostat. Cell cycle analyses revealed that the sequence-dependent interaction of vorinostat and ara-C or etoposide reflected the arrest of cells in the G_1 or G_2 phase during vorinostat treatment and recovery into the S phase after the removal of vorinostat.[70]

Podophyllotoxin molecules actually possess many features of bioactivity that make them attractive subjects for the intensive analysis of the druggability of natural products.

4.2 Molecular structure modification for reducing the toxicity and overcoming drug resistance

A multidisciplinary approach for unveiling the physiological functions of podophyllotoxin will continue to provide valuable information for other natural products and medical studies. Such knowledge will improve the likelihood of successful and efficient discovery drug from natural products in various disease conditions. Despite the toxicity and other defects associated with the clinical use of podophyllotoxin derivatives, podophyllotoxin is still a good candidate for the design and

preparation of antitumor and antiviral drugs with more efficiency and safety. This section offers an overview of new podophyllotoxin drugs that might improve the druggability. The first category is the optimization of molecular structures based on the drug targets. In order to improve the activity of podophyllotoxin derivatives towards tumor cells and reduce their toxicity towards normal cells. Molecular structure modification strategy is an important strategy to reduce the toxicity and overcome drug resistance.

As shown in Fig. 3, the molecular structure diversity of the podophyllotoxin derivatives determined the drug target selectivity. The antitumor mechanism study indicated that podophyllotoxin-derived drugs could target a variety of drug targets: (i) molecular modeling analysis of previous studies has indicated that the introduction of a moderate substituent at the C-4 hydroxyl resulted in a significantly improved cytotoxic activity, which could be inserted into a hydrophobic cavity of α-tubulin.[71] Podophyllotoxin derivatives inhibit cell cycle arrest caused by microtubule polymerization (Fig. 3A). (ii) Podophyllotoxin-derived drugs etoposide and teniposide form a ternary complex with DNA and topoisomerase II, resulting in DNA damage (Fig. 3B). (iii) Etoposide-derived clinical phase II drug F11782 simultaneously targets topoisomerase II and I (Fig. 3C). (iv) Podophyllotoxin isomer picropodophyllotoxin is not the target tubulin but IGF1R, which inhibits the differentiation, proliferation, and migration of the tumor cells (Fig. 3D). (v) Podophyllotoxin-derived clinical phase II drug F14512 with alkane ammonia chain exhibited superior tumor cell selectivity by targeting the polyamine transport pathway (Fig. 3E).

Naturally occurring indole and thiophene derivatives have been reported to have strong antitumor activity. It has been found that adding thiophene or indole rings to drugs can significantly increase the cytotoxic activity of the drugs. A series of thiophene- or indole-substituted PTOX derivatives was designed for the screening of more potent PTOX derivatives. Among them, compound **3D** showed strong tubulin polymerization inhibitory effect on HepG2 and other cancer cell lines without damaging normal cells. In addition, western blotting and siRNA results showed that **3D**-treated HepG-2 cells down-regulated the Bcl-2 expression. As a guardian of microtubule integrity, Bcl-2 can respond to the damage to the microtubules.[72] The potential binding site of the colchicine domain near the αT5 loop-αH7 of tubulin is conducive for the introduction of affinity fragments. Therefore, they chose benzoheterocycles with a high affinity of αT5 ring-αH7 (*i.e.*, indole, indazole, and quinoline) as affinity fragments, and introduced it into the molecular structure of PTOX with an imine bond, with the aim of improving the binding affinity of tubulin. Among them, Compounds **3** and **6**, which are the derivatives of the indole ring and the indazole ring, have a strong inhibitory effect on four cancer cells including MCF-7 and HepG-2. Among them, the IC_{50} value of Compound **3** for MCF-7 reached the nanomolar level. Animal experiments show that Compound **3** can destroy the structure of solid tumors and has no lethal toxicity.[73]

DMEP-derived drug etoposide and teniposide could bind with Topo II and DNA in the cells to form a ternary complex
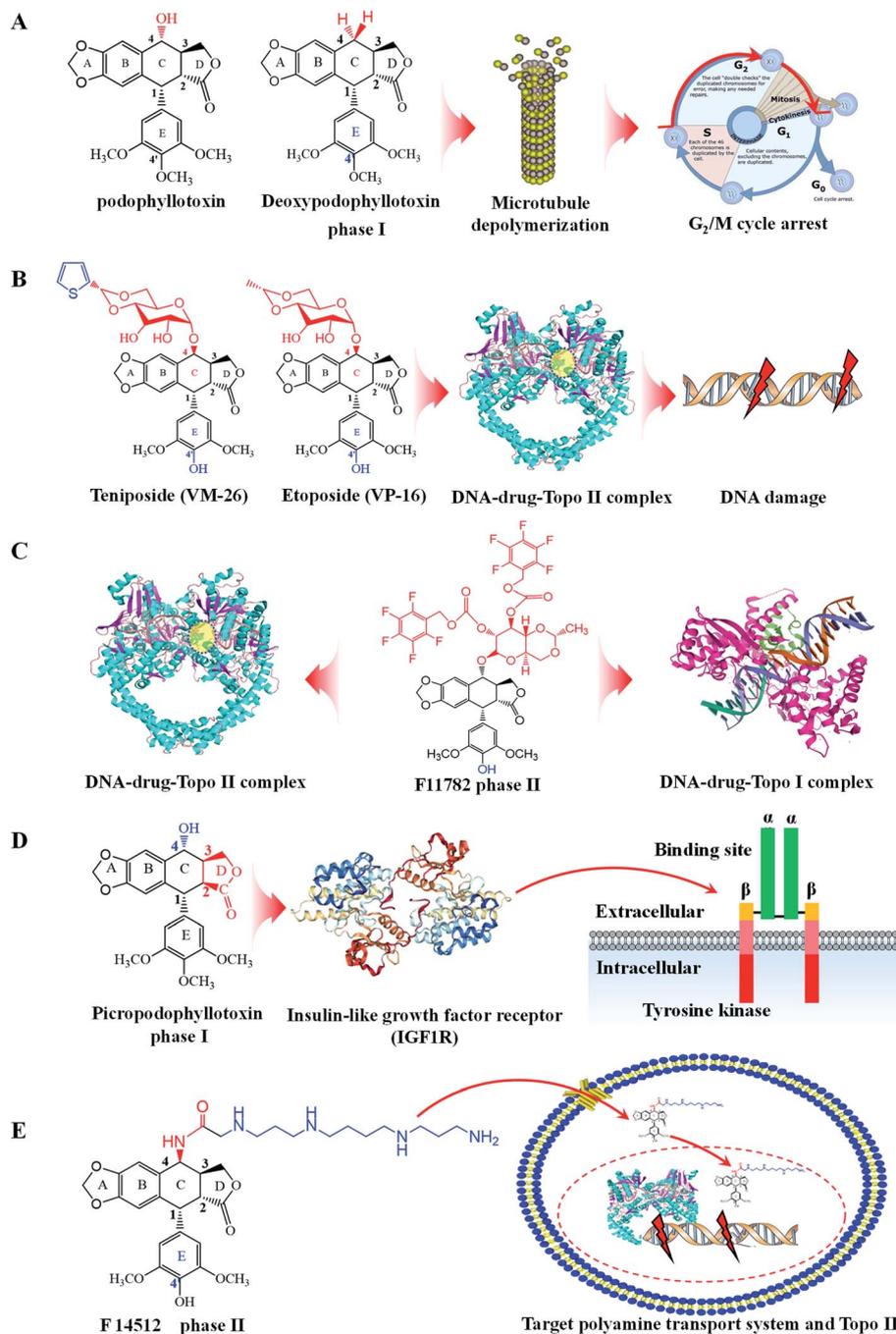
Fig. 3 Antitumor mechanism of podophyllotoxin derivatives. (A) Podophyllotoxin derivatives inhibit cell cycle arrest caused by microtubule polymerization. (B) Podophyllotoxin-derived drugs etoposide and teniposide form a ternary complex with DNA and topoisomerase II, resulting in DNA damage. (C) Etoposide-derived clinical phase II drug F11782 simultaneously targets topoisomerase II and I. (D) Podophyllotoxin isomer picropodophyllotoxin is not the target tubulin but IGF1R, which inhibits the differentiation, proliferation, and migration of tumor cells. (E) Podophyllotoxin-derived clinical phase II drug F14512 with alkane ammonia chain exhibited superior tumor cell selectivity by targeting the polyamine transport pathway.

(Fig. 3B), which indirectly inhibits the transcription and replication of DNA so as to induce tumor cell apoptosis.

The multidrug resistance of cancer is also a serious problem that limits the druggability. Compound **CIP-36** (Fig. 4) was obtained from 4-methoxyindole-substituted PTOX derivatives, whose outstanding contribution to inhibiting the activity of

Topo II was verified. **CIP-36** showed a significant inhibitory effect on the growth of ADM-resistant A562/A02 cells, with a GI_{50} value of 3.34 μM and a drug resistance index of 3.27, which are much higher than those of VP-16 (GI_{50} value of 33.85 μM) and ADM (GI_{50} value of 68 μM).[74] The Carin ring can stably bind to the tubulin Asn150. Moreover, the indole groups can also form
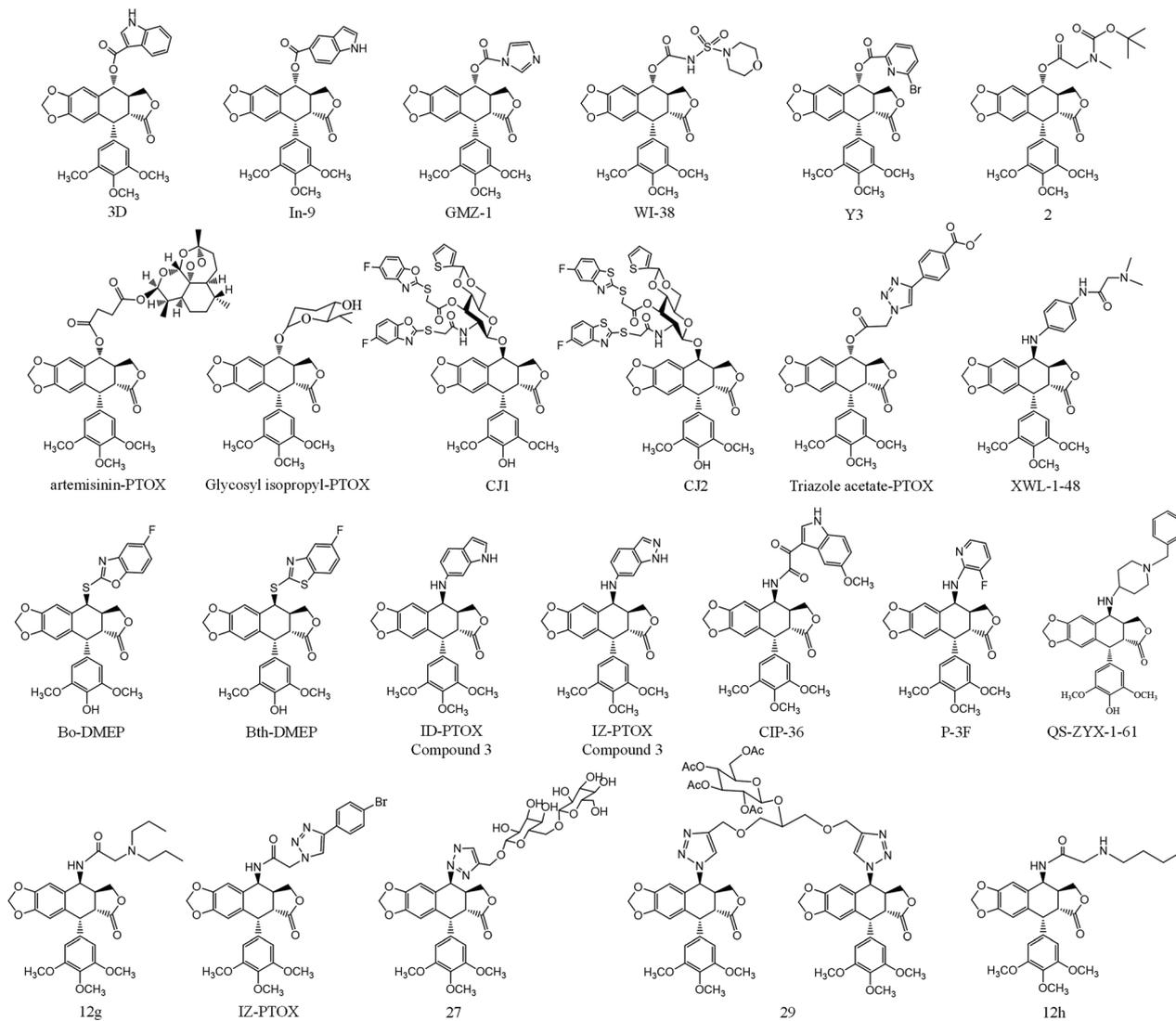
Fig. 4 Structures of podophyllotoxin derivatives.

hydrogen bonds with the bases on DNA. The natural plant extract indirubin is a compound formed by two indole groups connected together. It has antitumor, antibacterial, and other broad-spectrum activities. It is combined with podophyllotoxin by molecular hybridization strategy. The hybrid molecule shows an excellent effect for improving the anti-multidrug resistant tumor cells; the antitumor activity is 155 times higher than that of etoposide, and the IC_{50} value is the nanomolar scale.[75] Some functional modules containing nitrogen atoms have also been found to have good results. For example, **GMZ-1** (Fig. 4) was obtained by modifying the imidazole group to PTOX through an ester bond. The IC_{50} value of HeLa and other tumor cell lines was at the nanomolar level. Similarly, it also strongly inhibited the ADM-resistant cell line A562/A02 (IC_{50} 120 nM). The results of this study suggested that **GMZ-1** can induce apoptosis of K562/A02 cells *in vitro* and significantly reduce the expression of MDR1 within 24 h.[76] Similarly, the PTOX derivatives obtained by modifying *N*-morpholine sulfonamide also achieved good

results with nanomolar IC_{50} and showed low toxicity to the normal cell **WI-38**.[77] The natural product antharidin is also an effective antitumor active ingredient but it has relatively serious nephrotoxic and inflammatory side effects. Its derivative nor-cantharidin is relatively low in toxicity and is linked to podo-phyllotoxin, and the resulting hybrid molecule shows a balance between high activity and low toxicity.[78] Picropodophyllin (PPP) (Fig. 5), with a *cis*-lactone configuration, has no inhibitory effect on the microtubules and apparently lacks cytotoxicity. In contrast with etoposide and PTOX, PPP has no inhibitory effect on topoisomerase II and tubulin, and consequently, do not cause any DNA breakage.

Molecular structure modification of podophyllotoxin at the C-4 position was an important direction for improving the antitumor activity, reducing the toxicity, improving the water solubility, and avoiding drug resistance, as the linker replace-ment was based on the principle of bioelectronics and various non-glycosidic structures replaced the glycosidic structures
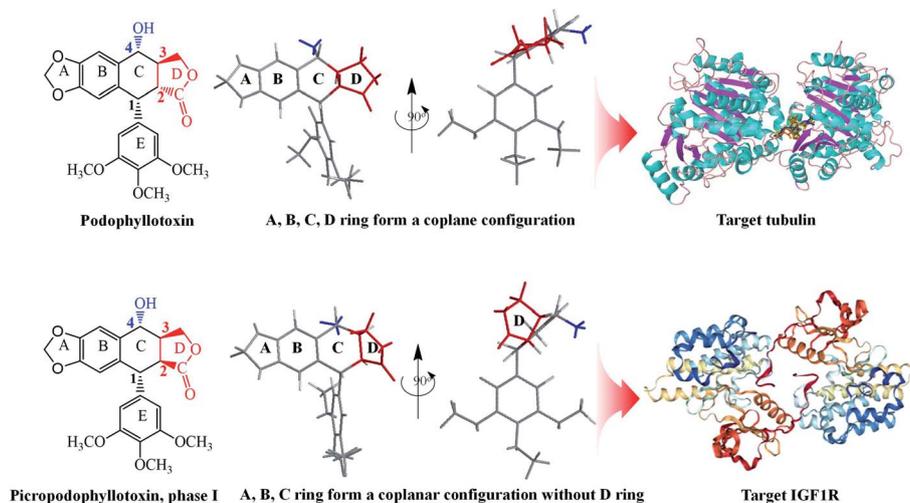
Fig. 5 Molecular framework, configuration comparison, and target transfer of podophyllotoxin and picropodophyllotoxin.

(Fig. 6). Halogen substituents on heterocycles can easily form stable interactions with potential electron-rich sites such as nitrogen, oxygen, and aromatic pi-electron systems in proteins, or may change the properties of drugs.[79] Among many halogen atoms, fluorine is the most potential one. The 3-F-pyridine-substituted DMEP derivative significantly inhibited the proliferation of HeLa cells (IC$_{50}$ value of 60 nM). The mechanistic study showed that the fluorine atom is more likely to inhibit the target protein activity, which increased the G53 expression, up-regulated the Bax expression, down-regulated the Bcl-2 expression, and activated caspase-3 to cause G$_2$/M phase arrest and apoptosis. Later, they found that replacing DMEP with PTOX resulted in **P-3F**, which exhibited a 297-fold enhancement in the antitumor activity compared to VP-16. Molecular docking results indicated that the 3-F-pyridine ring can penetrate deep into the hydrophobic pocket and form strong hydrogen bonds with Ala316 and Ala317. Mechanistic studies have shown that **P-3F** (Fig. 4) enhances the stability of P53, releases RPS27a from the nucleolus into the nucleoplasm, blocks the P53-mdm2 feedback loop, and thus increases P53 levels.[80] Some pyridine ester derivatives have also been reported to effectively reverse

tumor multidrug resistance, such as sorafenib and PAK-104P. PTOX has been combined with different picolinates or bromo-pyridates, and the screened out compound Y8. Y8 has excellent anti-K562/ADR cell proliferation performance (IC$_{50}$ value of 46 nM), which stimulated the ERK1/2 signaling pathway and reduced the expression level of P-gp protein in K562/ADR cells.[81] QS-ZYX-1-61, a derivative of VP-16, was significantly more potent than VP-16 in suppressing the viability of A549 cells. Treatment of cells with QS-ZYX-1-61 led to a DNA damage response and a dramatic increase of apoptosis.[82]

Molecular structure modification of the PTOX-derived drugs not only exhibited target selectivity but also affected the mechanism of action on the same target, such as topoisomerase II (Fig. 7). PTOX-derived candidate drug XWL-1-48, obtained by the structural optimization of GL-331, is more cytotoxic better bioavailability than its GL-331 parent, which showed strong inhibition of breast cancer cells.[83] Molecular docking results indicated that XWL-1-48 can bind to Topo II by forming two strong hydrogen bonds (Gln732, Asp436) and potential pi–pi interactions.[84] A hydrophobic side chain structure at C-4 may increase interaction with the hydrophobic pocket in the active site of Topo II; using *N*-acetylamino as a linker may increase water solubility. Molecular docking studies have shown that the active site of Topo II is occupied by small molecules of the compound, which causes cell death.[85] Many studies have shown that the introduction of protected or deeply protected amino acid groups can enhance the biological activity and selectivity of the antitumor drug. The sarcosine-substituted PTOX derivatives exhibited a strong inhibitory effect on the proliferation of A549 cells, with an IC$_{50}$ value of 9.5 nM, and an IC$_{50}$ value of 160 nM for normal cells L-02, indicating that the compound has strong tumor cell selectivity.[86] Nitric oxide is thought to reverse the multidrug resistance of tumor cells.[87] Nitric oxide-substituted podophyllotoxin derivatives showed 7 nM antitumor activity against K562/ADR cells, which was nearly 1000 times higher than that of vincristine tolerated by the cell line, which inhibited the expression of P-glycoprotein in drug-resistant cell lines
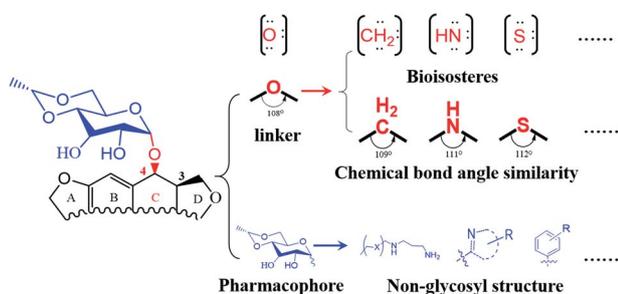
Fig. 6 Molecular structure modification of podophyllotoxin at the C-4 position. The linker replacement was based on the principle of bio-electronics. Various non-glycosidic structures replace the glycosidic structures so as to improve the activity, reduce the toxicity, improve the water solubility, and avoid drug resistance.
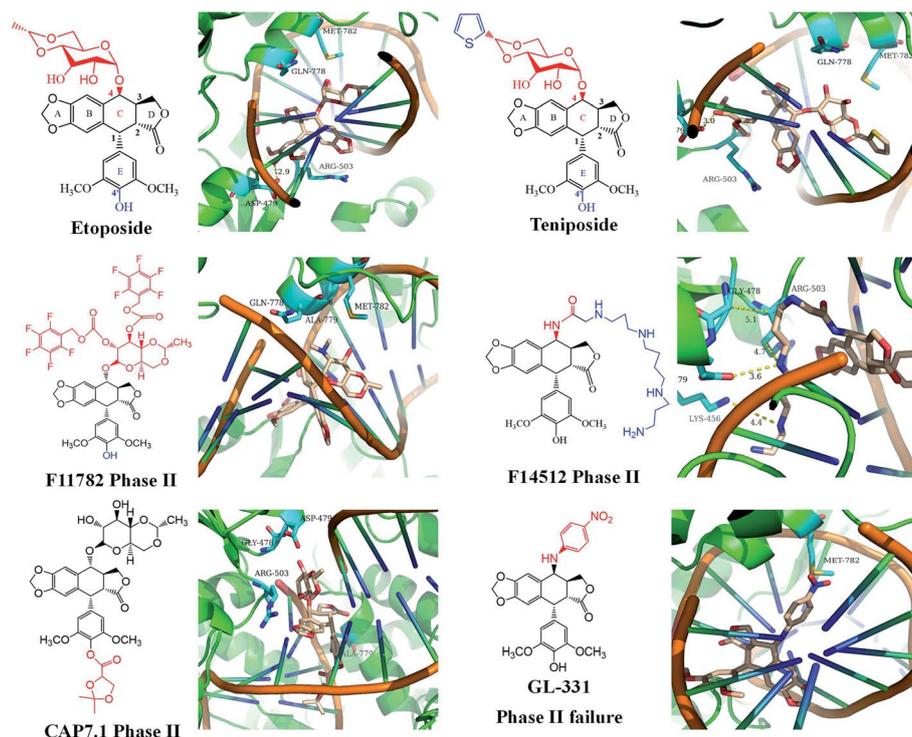
Fig. 7 Molecular structure mechanism of interaction between podophyllotoxin-derived marketing and clinical trial drugs and topoisomerase II.

for reversing the process of drug resistance.[88] Considering the two-pronged approach of inhibiting tumor cell proliferation and destroying the tumor microenvironment for antitumor therapy has also attracted much attention in recent years. Dithiocarbamate has a strong chelating ability to metal ions, which can inactivate the matrix metalloproteinases that are overexpressed in the tumor microenvironment, and has certain application prospects in cancer treatment. When it is hybridized with podophyllotoxin, the new molecules formed could inhibit the growth of tumors through dual pathways and inhibit the transformation of epithelial mesenchyme, thus preventing tumor progression and metastasis.[89] The click reaction is a common way of covalently linking the modifying group to the drug core and also generates a nitrogen-containing module triazole. Compared with etoposide, the triazole derivative of DMEP has stronger anticancer activity and better Topo II binding. Therefore, by using a click reaction, a series of PTOX dimers were synthesized and screened for compound **29** with a perbutylated glucose residue on the glycerol linker, and 4-*O*-methylation on the E ring. The inhibitory effect on cells such as A549 and MCF-7 was increased by about 5–20 times, which is significantly lower than that of etoposide. Compared with the normal BEAS-2B lung cell line, compound **29** had a higher selectivity, with a selectivity index in the range of 4.4–35.7.[90] When things come to PTOX and PPP, it seems equally effective. Bromobenzene was connected to PTOX with an amide–triazole bond to obtain the leading compounds, of which the antitumor activity was significantly improved (*i.e.*, IC$_{50}$ values from the micromolar to the nanomolar range). In addition to causing increased levels of caspase-3 protein, they could also induce G$_2$/

M phase arrest and subsequent apoptosis. Molecular docking analysis showed strong hydrogen bonding (*i.e.*, Ala333, Val177, and Pro175) and pi–pi conjugation (*i.e.*, Val177, Asp179, Pro348, and Pro175) between the compounds and tubulin.[91] In addition, the compound obtained by modifying the disaccharide to PPP with a triazole bond also showed a lower IC$_{50}$ value than that of etoposide.[92] 4α-Triazole acetate-substituted PTOX derivatives were found to have certain tumor cell selectivity, whose selection index (*i.e.*, normal cell IC$_{50}$ value/tumor cell IC$_{50}$ value) varied in the range of 18–27.[93] The improvement in the antitumor effect of nitrogen-containing molecules is already a matter of course. In addition, there have been some modifications to the PTOX glycosides. For example, on introducing an isopropyl group at the second position of the furanosyl group, the obtained derivatives have an IC$_{50}$ value of 41.6–95.2 nM for tumor cells such as MCF-7 and A549. The results of molecular docking showed that the furanosyl group could form a strong hydrogen bond with Ser178, which provided higher binding affinity for the tubulin–PTOX derivative complex and higher cytotoxicity.[94]

In a previous study, C-4 modified podophyllotoxin derivatives could achieve the purpose of reversing the drug resistance of multidrug resistant cell lines by down-regulating the expression level of P-glycoprotein. In addition to the previously mentioned nitric oxide effect *in vitro*, artemisinin and aspirin hybrid podophyllotoxin achieved good results.[95,96]

Tang *et al.* found that the addition of benzodiazepines can greatly reduce the toxicity of DMEP to normal cells at the millimolar level while maintaining antitumor activity. For normal cells, the damage of Topo II is almost minimized but

unfortunately, we still do not know the pharmacological mechanism.[97,98] A series of podophyllotoxin piperazine acetate derivatives were synthesized and the compound C5 was screened, which showed significant cytotoxicity to cancer cells without causing damage to normal cells by inhibiting the tubulin assembly. Also, it shows high selective damage to the MCF-7 cell line (IC_{50} value of 2.78 μM). The treatment of MCF-7 cells with C5 resulted in cell cycle arrest at the G_2/M phase and the destruction of the microtubule network.[99]

Recent developments on podophyllotoxin have afforded structure–activity relationships (SAR), which have assisted in the design and synthesis of new podophyllotoxin derivatives with potential antitumor activity. The modification of the A ring gave compounds that showed significant activity but less than that of etoposide, whereas the modification of the B ring resulted in a loss of activity. The modification of the C ring by aromatization or expansion gave compounds that were less potent than podophyllotoxin. Reports have been made of *cis*- and *trans*-lactone isomers, either natural or synthesized by transformations and interconversions, although from the point of view of activity, the most interesting were the *trans*-lactone isomers. In general, the derivatives that lack a lactone ring are less potent as antitumor agents. E ring oxygenation did not affect DNA cleavage. It has also been observed that the free rotation of the E ring is necessary for antitumor activity. The C-7 substituted aglycones and the aza analogues have a significant place in these recent developments. The substitution of a glycosidic moiety with aryl or alkyl amines produced enhanced activity, for *e.g.*, TOP-53. The high selectivity of TOP-53 has been attributed to its distribution in the lung and its persistence. The modification of the sugar ring resulted in the development of the NK-611 agent, which is currently undergoing clinical trials.

4.3 Drug delivery system for improving the drug targeting and bioavailability

The dosage of podophyllotoxin derivatives is often forcibly increased due to their poor water solubility and low bioavailability. However, marketed drugs such as etoposide also have problems of rejection by the tumor cells due to the presence of P-glycoprotein and rapid hepatic clearance through the metabolism of cytochrome P450 enzymes.[100] In order to improve the therapeutic effect without producing side effects, scientists have put forward the strategy of nanodrug delivery systems. There are two main types of drug delivery systems. Firstly, the surface of nanoparticles can also be modified with some targeting groups by loading the payload into hollow nanoparticles directly in an encapsulated form without covalent bonds so as to achieve the purpose of delivery (Fig. 8A). Secondly, by using covalent bonds to link the hydrophilic biological macromolecules with fat-soluble payloads to form amphiphilic compounds, they could self-assemble in the aqueous solution to form micelle particles with the fat-soluble parts inwards and the water-soluble parts outwards so as to achieve the protection of drugs. In such systems, covalent incorporation of the active molecule in the skeleton of the nanometric carrier enables high drug content and prevents early release of the therapeutic agent (Fig. 8B).

The tumor microenvironment is different from that of a normal tissue.[101] The development of a prodrug that retains its efficacy in the tumor microenvironment can be useful for enhancing the anticancer properties of podophyllotoxin.
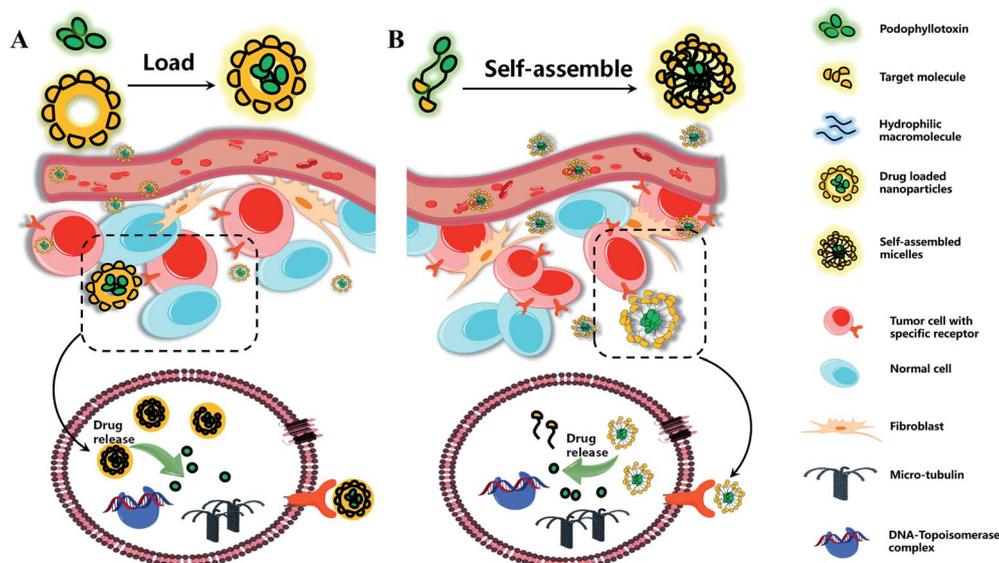
Fig. 8 Drug delivery system to improve the drug targeting and bioavailability. (A) The surface of nanoparticles could also be modified with some targeting groups by loading the payload in hollow nanoparticles directly in an encapsulated form without covalent bonds so as to achieve the purpose of delivery. (B) Hydrophilic biological macromolecules were linked with fat-soluble payloads by using covalent bonds to form amphiphilic compounds, which could self-assemble in aqueous solution to form micelle particles with fat-soluble parts inwards and water-soluble parts outwards so as to achieve the protection of drugs.

Polyethylene glycol (PEG) is a well-known biocompatible polymer that can extend the blood circulation time of nanoparticles. The PEGylated prodrug nano-delivery system could extend the blood circulation time and reduce the cytotoxic effect on normal healthy tissues during chemotherapy. An innovative podophyllotoxin prodrug (PTOX–PEG) was designed by linking podophyllotoxin with poly(ethylene glycol)$_{(n)}$ monomethacrylate with a H_2O_2-responsive oxalate ester bond.[102] PTOX–PEG can self-assemble into stable nanoparticles (PTOX–PEG NPs). PTOX–PEG NPs can be activated by hydrogen peroxide, resulting in podophyllotoxin release and high toxicity against colon carcinoma CT26 cells *in vitro* but no toxic effect on NIH3T3 cells. In contrast to free PTOX, the same dose of PTOX–PEG NPs has superior antitumor activity and no systemic side effects when it is used to treat CT6 tumors *in vivo*. Moreover, prolonged PTOX–PEG NP circulation in the blood improved the antitumor efficacy for effective accumulation at the site of the tumor due to the PEGylated prodrug strategy. The integration of H_2O_2-responsive release of the parent drug and PEGylated prodrugs is expected to provide an alternative strategy, which minimizes systemic toxicity of anticancer drugs and efficiently delivers the drug agents *in vivo*.

PEG was connected with other macromolecules for forming a block polymer. The block polymers have different excellent properties of several macromolecules. The molecular weights of PEG are controllable and their molecular structures are easier to design. PEG with more superior properties have been commercialized, such as poly(L-glutamic acid)-*g*-methoxy poly (ethylene glycol) (PLG-*g*-mPEG), which is a representative class of nano-drug carriers. NC-6004 is a cisplatin compound micelle drug that uses PLG-mPEG as a carrier material, which has good application prospect because of the well-tolerated and superior antitumor activity.[103] PLG-*g*-mPEG–PTOX was prepared by conjugating PTOX with PLG-*g*-mPEG using ester bonds. Compared with free PTOX, the maximum tolerated dose (MTD) of PLG-*g*-mPEG–PTOX is greatly increased by about 13.3 times than that of PTOX. *In vivo* studies have shown that the tumor suppression rate (TSR) is 82.5%, compared with that of free PTOX, and anticancer efficacy was significantly improved. The PTOX conjugate self-assembled into nano-micelles with an average particle size of about 100 nm. The experimental results prove that PLG-*g*-mPEG–PTOX can effectively inhibit the expression of P-gp in drug-resistant MCF-7/ADR cells. The resistance index (RI) of PLG-*g*-mPEG–PTOX to different drug-resistant cell lines was 57–270 times lower than that of the traditional microtubule inhibitor chemotherapy drugs PTX and DTX.[104] In addition, the polymer could also carry deoxypodophyllotoxin to form micelles. The micelles of mPEG–PLA micelles could be taken up by HeLa cells, which reached saturation after 12 h, and could stay in the tumor tissues for 8 h *in vivo*, effectively exerting antitumor activity.[105]

Another common partner of PEG is acetylated carboxymethyl cellulose (Ac-Mc). PEG and the payload is usually covalently coupled to the main chain of Ac-Mc through an ester bond. The polymer self-assembles into nanoparticles of various sizes according to the ratio of the payload to PEG. The PTOX/PEG molar ratio is positively correlated with the size of the nanoparticles, and the smaller the size of the nanoparticles, the stronger is its penetration ability and lethality to tumor cells. Compared with larger NPs, the cell lethality of the 20 nm nanoparticles increased by about 2 to 5 times, and tumor transmission increased by about 5 to 20 times. From the periphery of the blood vessels, the biodistribution of 20 nm nanoparticles is highly selective for tumors, which is 8 times higher than that of all the other examined tissues. Compared with free PTOX and standard taxane chemotherapy, 20 nm PTOX-NPs showed significantly improved efficacy in MDR tumors in mice and low toxicity.[106] Another research also confirmed the above results, showing the great prospects of lower-size PEG–Ac-Mc–PTOX nanoparticles for the treatment of MDR tumors.[107] In 2017, the nanoparticle Ac-Mc "Celludo" was trialed on the mouse MT6-AR1 lung metastasis model.[108] The results showed that Celludo could selectively localize in the metastatic nodules and increased the median survival to 20 days compared to 6–8 days with docetaxel and PTOX treatment. In the intraperitoneal metastatic model of human ovarian NCI-ADR/RES tumor, Celludo prolonged the median survival from 50 to 70 days, whereas the standard therapy involving PEGylated liposomal doxorubicin showed no effect. No major toxicity was detected with the Celludo treatment. These results demonstrate that Celludo is effective against metastatic and MDR tumors.

In order to enhance the aggregation degree of the micelle hydrophobic core and to reduce the size of the particles, the stearyl chain was substituted at the E ring-4′ of DMEP as the more hydrophobic core component and PEG was substituted at the C ring-4. The C18-DMEP–PEG micelles formed by the self-assembly of the polymer are only 8 nm in size; *in vitro* experiments showed that the micelles have an IC_{50} value of about 21 μM, which could effectively aggregate around the tumor *in vivo* through their enhanced permeability and EPR effect.[109]

GSH-responsive disulfide bond connection is one of the typical methods for drug delivery systems targeting the tumor microenvironment. In addition to linking macromolecular polymers such as PEG, peptides have shown huge drug delivery potential because of their biocompatibility, easy synthesis, controllable molecular weight, chemical diversity, and intracellular penetration. PRA is an octapeptide that strongly binds to the exchange GTP/GDP binding site of tubulin, which may inhibit tubulin polymerization and induce tumor cell apoptosis. PRA and PTOX were combined by a disulfide bond. The resulting polymer could spontaneously form a vesicle structure in an aqueous solution and could be reloaded with doxorubicin. The vesicles could improve the poor water solubility and reduce toxic and side effects of PPT by folic acid modification. The physical mixture of PRA, PTOX, and DOX inhibits HepG-2 cells similar to the vesicle system but the toxicity was two times than that against HL-7702 cells.[110] The functional podophyllotoxin derivatives linked by GSH-responsive disulfide bonds have also been researched: the authors linked PTOX with NIR fluorescent groups through disulfide bonds and encapsulated them in PEG nanoparticles, which could be successfully internalized by the tumor cells and played a role in tracking while resisting the tumors.[111]

Most of the above self-assembled nanoparticles rely on the EPR effect caused by the tiny-sized nanoparticles. With the hydrophilicity, biocompatibility, and "stealth strategy" of PEG, nanoparticles could achieve better delivery effect but this is still passive targeting. In contrast, podophyllotoxin modified with active targeting groups seems to show better results.

The surface of tumor cells has a large content of the polyamine transport system (PTS), which can be used as a tool for tumor cells to take up polyamines from the extracellular matrix so as to maintain their own rapid growth, or as a special channel for antitumor drugs to enter the tumor cells.[112] The covalent conjugate conjugated with polyamine can be targeted into the tumor cells *via* PTS under the guidance of polyamine, thereby increasing the anticancer effect of the drug and reducing the toxic and side effects of the drug. The spermine ligand was linked with DMEP by a hydrophobic stearin chain for synthesizing a polyamine functional amphiphilic drug conjugate with micelle formation properties to deliver a payload to the target DNA–Topo II. The polyamine functionalized nano-micelles could combine tissue-specific passive accumulation (EPR effect) and active cell-specific targeting (mediated by spermine–PTS interaction), which becomes a valuable choice for the treatment of solid tumors.[113] Similarly, there are a large number of biotin receptors on the surface of tumor cells. The target group biotin was linked to PTOX. The IC_{50} value of the screened compounds reached 130 nM. *In vivo* experiments show that the dose of 15 mg kg^{-1} could significantly inhibit the growth of transplanted tumor of S180 cells in ICR mice.[114] Using the ligand of transferrin receptor as the targeting group, modifying it on PEG, and then connecting the PTOX through a disulfide bond, which could self-assemble to form micelles, the maximum tolerated dose (MTD) increased by 21 times compared to that of free PTOX.[115] The bifunctional targeting compound obtained by linking the methotrexate molecule, which could act on the over-expressed dihydrofolate reductase and PTOX through disulfide bonds, also achieved good results.[116] In addition, we could also use the low pH of the tumor microenvironment to achieve the targeted function of the drug delivery system. Chitosan's property of being easily soluble in acid was used to prepare nanoparticles encapsulating PTOX for tumor delivery. Compared with free PTOX, there was no significant difference in the equivalent concentration of CS-Nps within 24 h but when the treatment time was extended to 48 and 72 h, the nanoparticle group showed significantly better anticancer activity than that of free PTOX.[117]

Many types of drug delivery systems are not connected by covalent bonds but are by encapsulated payloads, including silica nanoparticles, polymer nanoparticles or nanogels, liposomes, and nano-lipid carriers. The main component of liposomes is phospholipids, and the nanolipid carrier is mainly composed of solid lecithin and other components.

Nano-lipid carriers combine the advantages of fat emulsions, polymer nanoparticles, and liposomes, and are of great benefit to cancer chemotherapy. A nano-lipid carrier (NLC) containing etoposide (VP-16) was developed in 2015. The IC_{50} values of ETP-NLC and VP-16 were 6.3 and 56.5 μg mL^{-1}, respectively; the IC_{50} value of ETP-NLC was 9 times than that of the VP-16

solutions. Compared with the free VP-16 solution, the inhibition rate of ETP-NLCs was significantly higher and the inhibition efficiency was also significantly higher.[118] Later, folic acid (FA) could target the over-expressed folate receptor protein on the surface of the tumor cells to NLCs, which carried ETP for the antitumor activity. The study found that FA–ETP-NLCs could exist stably in serum and the IC_{50} value of FA–ETP-NLCs is 4–5 times higher than that of ETP-NLCs, which significantly reduces the tumor volume *in vivo*. Based on the previously optimized formulation, the FA-decorated ETP-NLCs formulation containing lipid/drug ratio were optimized, the amount of surfactant and FA–PEG–DSPE was designed by Box–Behnken, then their cytotoxicity was investigated *in vitro*, and their therapeutic effect on mouse colon cancer xenograft model was evaluated. The optimized FA–ETP-NLCs could effectively target the tumor cells that overexpress the FA receptors. Similarly, FA–ETP-NLCs could fully transfer ETP to the cancer cells and enhance the antitumor ability. Therefore, FA–ETP-NLCs may prove to be an excellent nanomedicine for tumor treatment.[119] The delivery of drugs to the posterior segment is still a challenging task due to the anatomic and physiologic barriers of the eye. Multiple intravitreal injections are very risky, which could easily lead to inflammation or even retinal detachment. Etoposide solid lipid nanoparticles were used for ocular administration and no obvious vitreous damage was found in the rat experiment, which obtained similar results when rabbits were used as the model animals.[120,121]

However, lipid nanoparticle carriers with poor loading capacity (less than 10%) can only encapsulate hydrophobic drugs and are prone to drug leakage in the blood, leading to drug loss before reaching the tumor site, which makes the clinical application of lipid nanoparticles very limited. Therefore, similar to PLG–mPEG with a covalent bond linked to a payload antitumor drug, the researchers also designed such liposomes. Phosphocholine (PC) is a very effective hydrophilic head group, which can form a very good balance with two hydrophobic fatty acid tails on the phospholipid molecule, thus making it an extremely hydrophilic and biocompatible lipid amphiphile. Ling and others imitated the structure of phospholipids and combined two PTOX molecules with succinic glycerophosphocholine (GPC) to design di-podophyllotoxin succinic glycerophosphocholine (Di-PTOX–GPC), which could self-assemble into liposomes with particle sizes of about 160 nm in an aqueous solution and can continue to dissociate and release the effective load PTOX in a weakly acidic environment. The liposomes had a drug loading of up to 66.2% *in vivo* and *in vitro*.[122]

The liposome delivery of PTOX has not only been used in antitumor research but also in antiviral research. Because of the severe stimulating side effects of PTOX, nano-lipid carriers (NLCs) were loaded with PTOX. PTOX–NLCs with 0.5% PTOX provided sustained drug delivery for 72 h *in vitro* and 10 h in the mucosa *in vitro* and *in vivo*. Compared with a tincture formulation of free PTOX, PTOX–NLC induced less inflammatory cytokine production in the cervical mucous and led to a decreased histopathological score. In addition, the cytotoxicity assay demonstrated that the inhibition of PTOX–NLCs was

98.4% at 24 h and remained >98% up to 72 h.[123] In 2019, this PTOX–NLC preparation successfully cured a patient with condyloma acuminatum who was still positive for HPV after ALA-PDT treatment. Surprisingly, the HPV DNA test result was negative after the second course of treatment and the patient received a total of three courses of treatment. After 28 weeks, the review results were still negative, indicating that the PTOX–NLCs preparation shows good efficacy and safety but large-scale high-quality clinical trials have not been conducted.[124]

The safe and effective treatment of solid tumors remains a challenge. Conventional treatments, *i.e.*, oral and injection chemotherapy, have shown serious systemic side effects and insufficient efficacy due to dose limitations. Implantable drug delivery systems are a tumor-targeted therapy that can replace traditional treatment methods. The Gliadel wafer is the first and only chemotherapeutic implant approved by the FDA, which has drawn attention to implantable systems for solid tumor treatment. Silk fibroin with great biocompatibility and degradability is widely used in drug delivery and has a higher drug loading capacity; thus, it could be made into various dosage forms such as fiber, films, and tubes. By using silk fibroin, an implantable and degradable silk wafer delivery system was developed for prolonging the release time of etoposide for the treatment of neuroblastoma. Compared with etoposide, no obvious cytotoxicity was found; etoposide killed 50% of the cells at 1 μg mL^{-1} concentration and the wafer formulations demonstrated significant cytotoxicity up to 22 days when compared to the untreated cells. They could reach a continuous sustained release time of 45 days. *In vivo* experiments show that the silk wafer effectively inhibited the tumor growth in the mouse *in situ* neuroblastoma model, whereas histological examination revealed tumor cell necrosis adjacent to the drug-loaded silk wafer.[125]

In order to solve the problem of differential solubility for incorporation of etoposide and platinum drug combination in nanoparticle systems, a polymeric micelle system based on amphiphilic block copolymer poly(2-oxazoline)s poly(2-methyl-2-oxazoline-*block*-2-butyl-2-oxazoline-*block*-2-methyl-2-oxazoline) was used along with an alkylated cisplatin prodrug to enable co-formulation of etoposide and platinum in a single high-capacity vehicle. Kabanov and Sokolsky-Papkov *et al.* demonstrated that this approach could greatly improve the treatment outcomes of etoposide and platinum drug combination therapy that two-drug loading of over 50% wt for small cell lung cancer treatment. A superior antitumor activity of co-loaded etoposide and platinum drug micelles compared to single drug micelles or their combination as well as free drug combination was demonstrated using several animal models of small cell lung cancer and non-small cell lung cancer.[126] It has been demonstrated that surfactants/excipients could increase drug absorption by inhibiting P-glycoprotein (P-gp). Ping *et al.* evaluated the effect of *N*-octyl-*O*-sulfate chitosan (*N*OSC) on the absorption of VP-16, a substrate of P-gp with low water solubility. Compared with some P-gp inhibitors, *N*OSC was confirmed to have stronger ability using rat intestinal circulating perfusion *in situ* and Caco-2 cell uptake and monolayer membrane penetration *in vitro*. The results indicated that

various concentrations of *N*OSC increased the intestinal absorption of VP-16 in rat jejunum and ileum significantly and there was no significant difference in the ileum between the enhancing effects of *N*OSC and other P-gp inhibitors. The VP-16 uptake of the Caco-2 cell was increased by the *N*OSC solution with different concentrations. As the *N*OSC concentration was close to its critical micelle concentration (CMC), the cell uptake of VP-16 reached a maximum value. Both *N*OSC and verapamil (Ver) dramatically enhanced the transport of VP-16 from the apical side to the basolateral side in the Caco-2 cell monolayers. Transepithelial electrical resistance (TEER) of Caco-2 cell monolayers showed no significant change during the study. These studies demonstrated that *N*OSC had the potential of inhibiting P-gp so as to improve the absorption of oral drugs, which were P-gp substrates.[127]

Small cell lung carcinoma (SCLC) is a highly aggressive form of malignancy with rapid recurrence and poor prognosis. Dual peptide-modified nanoparticles (NPs) for improving chemotherapy against drug-resistant small cell lung carcinoma cells have been developed. In this study, the SCLC targeting ligand, antagonist G peptide (AG), and cell-penetrating peptide, TAT, modified NPs were used to encapsulate both the anticancer drugs VP-16 and PIK3CA small-interfering RNA (siPIK3CA).[128]

Local chemotherapy with VP-16-loaded drug delivery systems could provide continuous release of the drug at the target site, while minimizing systemic toxicity. In this study, we prepared the poly-L-lactic acid (PLLA) based VP-16-loaded implants (VP-16 implants) by the direct compression method. The VP-16 implants were characterized with regard to the drug content, micromorphology, drug release profiles, differential scanning calorimetry (DSC), and Fourier transform infrared spectroscopy (FTIR) analyses. Furthermore, the biodistribution of VP-16 *via* intratumoral chemotherapy with VP-16 implants was investigated using the murine Lewis lung carcinoma model. Our results showed that VP-16 was homogenously dispersed in the polymeric matrix. Both *in vitro* and *in vivo* drug release profiles of the implants were characterized by high initial burst release, followed by sustained release of VP-16. The VP-16 implants showed good compatibility between VP-16 and the excipients. Intratumoral chemotherapy with VP-16 implants resulted in significantly higher concentration and longer duration of VP-16 in the tumor tissues compared with a single intraperitoneal injection of the VP-16 solution. Moreover, we found a low level of VP-16 in the plasma and normal organ tissues. These results suggested that intratumoral chemotherapy with VP-16 implants enabled high drug concentration at the target site and has the potential to be used as a novel method to treat cancer.[129]

5 Conclusions

Podophyllotoxin have proved to be a classical natural effective leading compound with antitumor, antiviral, and antibacterial activities since ancient times. Podophyllotoxin and its derivatives could arrest the cell cycle, block the transitorily generated DNA/RNA breaks, and blunt the growth-stimulatory by targeting topoisomerase II, tubulin, or insulin-like growth factor 1 receptor. Not only did the podophyllotoxin derivatives exhibit

superior antitumor activity but also antiviral activity against multiple viruses, including human papillomavirus and human immunodeficiency virus. However, the initial version of podophyllotoxin was far from perfect. Almost all the podophyllotoxin derivatives, including the FDA-approved drugs etoposide (VP-16) and teniposide, were seriously limited in clinical therapy due to systemic toxicity, drug resistance, and low bioavailability. This issue has been appreciably overcome in the past decade and it will be eradicated completely with continuous efforts in future. The paucity of summary information on druggability such as the clinical limitations of podophyllotoxin and its derivatives as a lignan family has severely limited our understanding of new podophyllotoxin-derived drug discovery. The future perspectives on how to apply cutting-edge technology and strategies, such as drug combination in clinical therapeutics, deep learning for drug design based on AI, new drug-target discovery, and personalized precise chemotherapy have been developed for next-generation superior podophyllotoxin-derived antitumor and antiviral drugs.

Podophyllotoxin molecules actually possess many features of bioactivity that make them attractive subjects for intensive analysis of the druggability of natural products. A multidisciplinary approach for unveiling the physiological functions of podophyllotoxin will continue to provide valuable information for other natural products and medical studies. Such knowledge will improve the likelihood of successful and efficient drug discovery from natural products for controlling various disease conditions.

6 Conflicts of interest

There are no conflicts to declare.

7 Acknowledgements

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