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Macromolecules as taxane delivery systems

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Importance of the field: Taxanes have received considerable attention owing to their significant activity against a variety of tumors. Nevertheless, many different approaches have been developed to improve their safety profile and water solubility, in terms of both dosing schedules and delivery strategies.
Areas covered in this review: Among the different taxane delivery systems, macromolecule conjugates have been widely explored; this review collects and summarizes such systems from reports after 1990. Natural and synthetic polymers, proteins and polysaccharides have been covalently coupled with taxanes; immunoconjugates have also been developed for targeted delivery. In-depth descriptions, from synthesis to preclinical or clinical data, are given.
What the reader will gain: The choice of macromolecule, the spacer, the chemistry of the linkage with taxane, as well as other cytotoxic drugs, are key points to obtain effective conjugates with higher activity than that of the free drug, reducing side effects. Critical evaluation of the different approaches may help in comprehending and comparing the results and may elucidate the role of individual components.
Take home message: Taxane covalently-bound to macromolecules shows advanced properties, and although only one compound is in advanced clinical trials, this area deserves attention and seems a promising route to achieve effective new anticancer compounds.

Keywords: anticancer agents, macromolecules, paclitaxel, polymers, polysaccharides, taxanes

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1. Introduction

Taxanes are among the most active cytotoxic agents in use in oncology, and are widely used in adjuvant and advanced treatment settings in multiple tumor types. Paclitaxel (PTX) and docetaxel (DTX) are standard therapies in ovarian and breast cancers [1,2], in combination with cisplatin as primary treatment of non-small cell lung cancer and for second-line treatment of Kaposi's sarcoma associated with AIDS. Both paclitaxel and docetaxel have demonstrated notable activity in patients with hormone-refractory prostate cancer [3]. Important activities have been reported in other malignancies, including head and neck, esophageal, gastric, endometrial, bladder, germ-cell carcinomas, lymphoma and melanoma.

Taxanes cause mitotic arrest in cancer cells by stabilizing the microtubules and preventing depolymerization. As microtubules play a crucial role in regulating mitotic spindle formation, the disruption of cellular microtubule dynamics can have severe effects on cell viability, leading to cell-cycle arrest in the M-phase, followed by apoptosis. Owing to these important effects on cellular processes, microtubules were one of the first targets for tumor chemotherapy, an approach that has been strongly validated and shown to be highly efficacious [4].

Another, more recent but important therapeutic field of PTX regards restenosis following percutaneous coronary intervention. It has been demonstrated that the slow-release, polymer-based, PTX-eluting stent is safe and that it markedly reduces rates of clinical and angiographic restenosis: [5] restenosis is reduced by 35 – 70%

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Article highlights.

- The macromolecular delivery system approach can:
  - improve solubility of taxanes, without the need for solvents/co-formulation
  - increase local drug concentration thanks to high payload delivery
  - significantly reduce systemic toxicity
  - improve retention of the drug delivery system in the tumor thanks to the EPR effect
  - bear targeting moieties.
- Key elements for building efficient conjugates are:
  - carrier size and charge/hydrophilicity
  - structure/function of the drug–polymer linkage
  - choice of appropriate spacer.
- Key elements to achieved a truly effective macromolecular-taxane conjugate
  - use validated *in vivo* models
  - have detailed pharmacokinetic/toxicology analysis
  - define precise methods for assessing compounds' stability/purity/reproducibility.

This box summarizes key points contained in the article.

with drug-eluting stents (with sirolimus or PTX), compared with bare metal stents [6].

Paclitaxel and docetaxel differ in binding affinities and pharmacokinetics [7] but possess the same adverse effects, myelosuppression being the dose-limiting toxicity, followed by neurotoxicity, mucositis, alopecia and mild gastrointestinal toxicity [8]. The taxanes can also cause severe and (rarely) life-threatening hypersensitivity reactions (HSRs) and dose-related pulmonary toxicity [9-12]. Like many other chemotherapeutic agents, their clinical success has been limited by the insurgence of cellular resistance, mainly mediated by expression of the multi-drug resistant (MDR) phenotype or by microtubule alterations [13].

From the pharmaceutical-technology standpoint, the inherent aqueous insolubility of PTX, in particular, has led to formulating the agent in a mixture of the non-ionic surfactant polyoxyethylated castor oil and ethanol. The polyoxyethylated castor oil excipient is probably responsible for most major HSRs, and for the need for protracted and cumbersome administration schedules. A further problem arising from the presence of castor oil and ethanol is the leaching of plasticizers from polyvinyl chloride (PVC) infusion bags and sets in routine clinical use.

Owing to the impressive benefits offered by the taxanes, different strategies are in development with the aim of reducing their toxicity and the need for steroid premedication, while improving antitumor spectra and treatment efficacy. New systems for taxanes could potentially overcome resistance, and are increasingly being used in earlier treatment settings. Some new oral taxane formulations could also potentially facilitate administration and scheduling for patients.

Approaches have been developed in a broad variety of fields. Starting from synthetic analogues, prodrugs and new formulations, the use of medical devices or carriers of different types (polymeric, lipidic) to deliver taxanes more safely and selectively is now being explored [12].

Thousands of new analogues have been synthesized worldwide, but only a few have progressed to clinical development. Development has been along several tracks, for example, improved solubility, oral bioavailability, absence of cross-resistance with marketed taxanes, and, particularly, absence of interaction with P-glycoprotein (P-gp). Taxane analogues, known as Larotaxel, Ortataxel, Milataxel, Simotaxel, TPI-287, and several Bristol-Myers Squibb products such as BMS-188797, BMS-275183 and BMS-184476, are key analogues in clinical trials [14].

Several delivery approaches have been developed in recent years, ranging from pastes and gels, controlled-release depot formulation [15] to emulsions and cyclodextrins complexes. In improved formulation, using lipids, phospholipids, synthetic polymers, polysaccharides or proteins, taxanes have been assembled inside vesicles, micelles [16], micro- and nanoparticles and, more recently, in carbon nanovectors [17] with dimensions ranging from of 20 to 100 nm for micelles and 100 nm to a few micrometers for vesicles (**Figure 1**) [18] (for a comprehensive review, see [19]).

Another approach involves the chemical modification of the structure of taxanes to achieve a prodrug, that is, a bioreversible derivative of an active drug. The prodrug approach is very useful to overcome certain drawbacks in the parent drug's properties, such as low solubility, low permeability, low oral absorption, instability and toxicity [20]. In a prodrug design strategy the group (portion of the molecule) necessary to exploit cytotoxic effect in the taxane molecule must be maintained capped, until a complete *in vivo* release of the parent active drug is obtained. This mechanism can be achieved mainly by hydrolysis of a promoiety, or by site-specific reaction with enzymes or conditions mainly present in tumor tissue. The linkage to the taxane molecule that is exploited to obtain prodrugs principally involves the hydroxyl groups at positions 2′ and 7 (**Figure 2**); however, only the C-2′ free hydroxyl group appears to be required for cytotoxic effects, whereas the C-7 hydroxyl group is not so critical for taxane activity [21].

Prodrugs may comprise a 1:1 drug/promoiety ratio or, using polymeric carriers, there may be an increased ratio. For an extended review of taxane prodrugs of low molecular mass, see [22]. Several compounds have been synthesized following this appproach, and subsequently tested. The review focuses primarily on the role of taxanes' chemical conjugates comprising macromolecules as carrier, from synthesis to clinical data (where available), and also describes the principal competing technologies, such as particulate carriers, so as to stress the advantages of covalently linking taxanes to macromolecules (see Section 2).

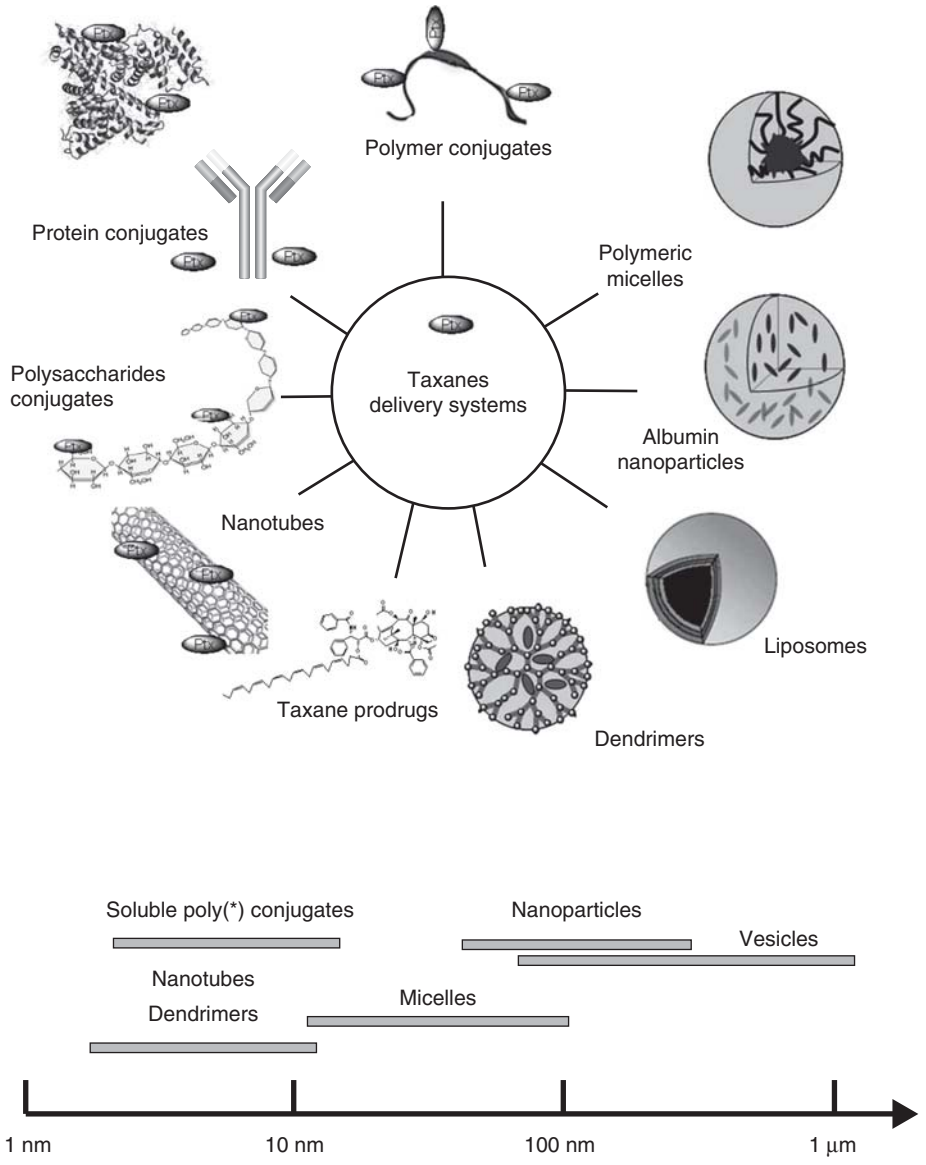

Figure 1. Schematic illustration of various nanomedicine taxane delivery systems.

Although the term 'macromolecular drug' can, in terms of size, also include drug-loaded pharmaceutical nanocarriers, such as liposomes or polymeric micelles, this report evaluates those in which synthetic or natural macromolecules carrying multiple moieties of taxane molecules are covalently linked.

The conjugation of cytotoxic agents to macromolecules may offer several advantages: first, it has been established that high-molecular-mass conjugates passively accumulate in tumor tissue, because of the enhanced permeability of those tissues and the retention (EPR) effect [23,24]. Unlike their low-molecular-mass counterparts, macromolecular drugs often encounter significant permeability barriers in most normal tissues. By contrast, the poorly formed tumor vasculature around solid tumors is more permeable to macromolecules than normal vasculature [25,26]. Furthermore, the small number of lymphatic vessels in tumor tissue allows these macromolecules to be retained in the interstitial space, resulting in a 10 – 100-fold increase in intratumoral drug concentrations compared with that afforded by an equivalent dose of the drug given conventionally [27,28]. To achieve effective tumor targeting through the EPR effect, molecular mass and electric charges of conjugates are critical [29]. Macromolecules with molecular masses above ~ 70 kDa and weak anionic charges are known to circulate in the blood for a long time owing to low hepatic uptake and slow urinary excretion clearance [24,30].

Conjugation of cytotoxic drugs with macromolecules improves the pharmacokinetic profile by decreasing the volume of distribution and prolonging the distribution and

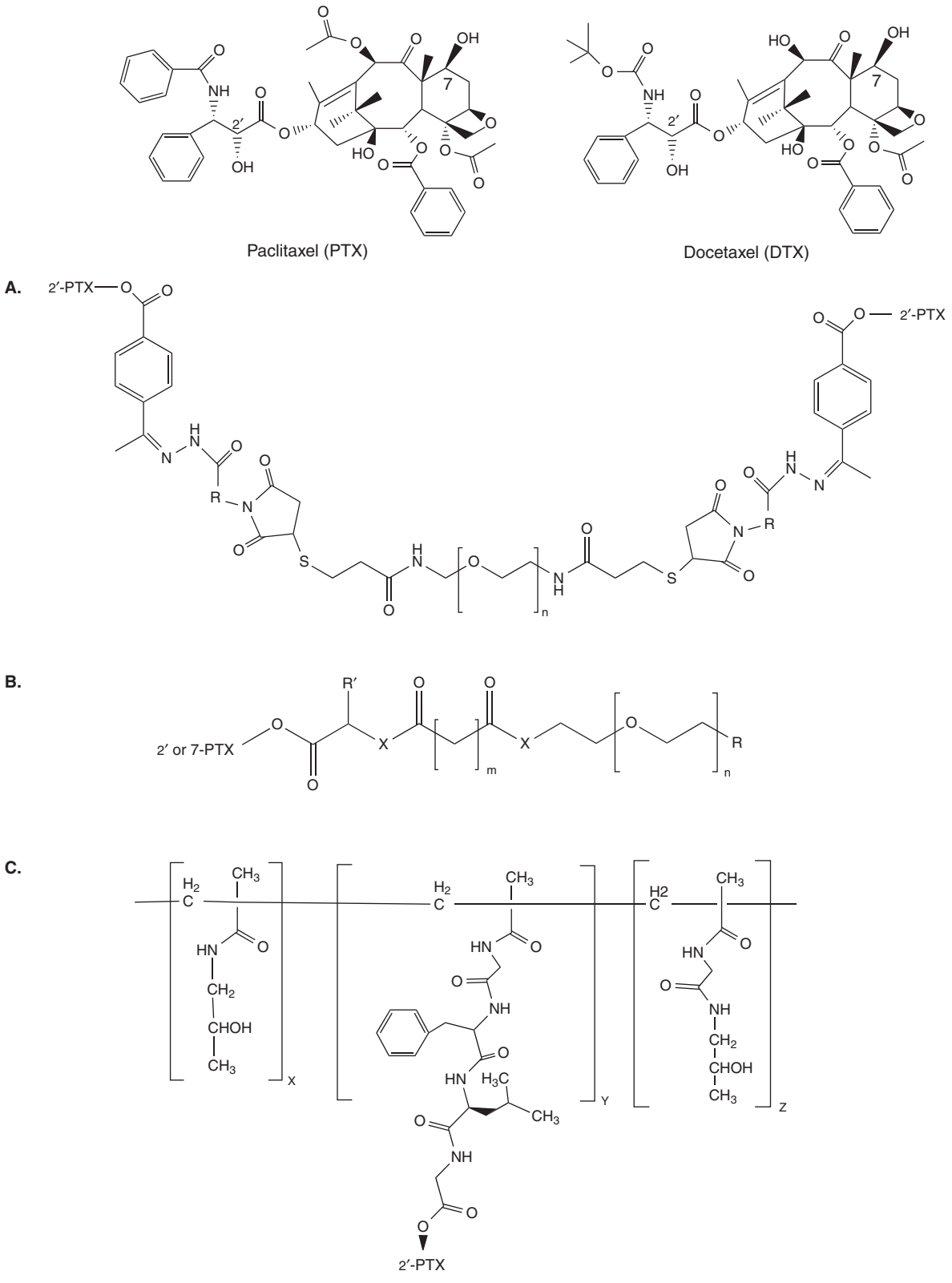

Figure 2. Chemical structures of taxanes and examples of conjugates. A. Maleimide PTX derivatives that incorporate carboxylic hydrazone bonds are coupled to symmetric PEG 20000 (n = 450) bearing two thiol groups. **B.** Example of PEG derivative with different spacer and link: X = O, S, NH; R,R′ = H, CH_3; m = 1 to 6; n = 10 to 1000. **C.** HPMA derivative with amino acid spacer.

elimination phases [31]. Furthermore, the slow release of active drug from the carrier results in sustained high intratumoral drug levels and lower plasma concentrations of the active drug. To accomplish this, the macromolecule–drug conjugate should preferentially release the active drug in the tumor tissue, thus the following the components are essential: a biodegradable linkage, a suitable spacer and a bioactive anticancer agent.

The choice of carrier will be examined below, but particularly aspects related to the role of linkage/spacer and the chemical derivatization of the taxane molecule because, in addition to the delivery efficiency, the stability of the drug–macromolecular linkage is a key factor in determining therapeutic potential. The conjugates, once around or inside the tumor, must be activated (cleaved) to exert their antitumor effect. Theoretically, activation of the free drug can occur intracellularly or extracellularly. Several strategies have been developed to release selectively the therapeutic agent from a prodrug. The principal mechanisms involve the use of spacers cleavable by proteolysis of enzymes overexpressed in the tumor tissue or acid-sensitive linkages cleavable under acidic conditions present in tumors, endosomes and lysosomes [32]. Furthermore, exploiting the tumor hypoxic environment [33], reduction reactions can efficiently release active drug from the non-toxic prodrug. [34] Moreover, self-immolative spacers have been developed comprising drug, linker and trigger. The tumor-specific cleavage reaction takes place between trigger and linker, to form a drug–linker derivative, which then degrades spontaneously by elimination or cyclization to release the free drug [35], preferably inside affected tissues. As a result, exposure of normal tissues is limited, which is potentially associated with a more favorable toxicity profile [36]. This is the major advantage in comparison with nanosized competing strategies (see Section 2). Furthermore, of particular importance for taxanes, polymeric conjugation renders hydrophobic agents water-soluble and eliminates the need for toxic solubilizing agents. Macromolecules can also be designed to include a specific 'active' targeting moiety. Monoclonal antibodies, folic acid, peptides recognized by specific receptors and hyaluronic acid have all been investigated in this connection [34,37]. In addition, polymers might participate in microenvironment-dependent reactions, allowing the polymer itself to have therapeutic applications, and also enabling it to function as a controlled-release vehicle of the conjugated drug.

Thanks to these advantages, the macromolecular approach has been applied to other potent anticancer agents, having significant side effects, such as methotrexate [38], doxorubicin [39], camptothecin [40], platinum complexes [41], maytansinoid [42] and calicheamicins [43].

Macromolecular conjugates can also be used in formulations for oral administration of PTX. This route would offer advantages over intravenous infusion, including better patient compliance and reduced administration costs, and would facilitate the use of chronic treatment regimens. Unfortunately, the oral bioavailability of PTX is extremely low in animals and humans, mainly owing to the effect of the multi-drug efflux transporter P-gp in the intestinal mucosa, as well as the drug's low solubility and its affinity for the intestinal and liver cytochrome P450 metabolic enzymes [44]. To enhance the oral bioavailability of paclitaxel, different strategies have been investigated: taxane analogues, such as BMS-275183 [45], Ortataxel [46], and Milataxel [47], have been developed, but their antitumoral efficacy and toxicological profiles are still to be demonstrated.

2. Principal competing technologies

To achieve an overview of the various approaches to delivering taxanes by means of systems that do not require covalent linkage with a macromolecule, a brief description of competing technologies now in advanced trials follows (**Table 1**). Most of these are based on well-defined 'nano' structures such as liposomes, polymeric micelles, albumin nanoparticles and emulsions. Regarding the prodrug approach, only the docosahexaenoic-PTX derivative (Taxoprexin) can be mentioned (**Figure 1**). The principal advantages lie in the absence of polyoxyethylated castor oil excipient in the formulation and in the potentially increased tumor tissue accumulation, due to the EPR effect. The principal drawback is the potential instability of the formulation that can, hypothetically, give rapid and uncontrolled release of active drug.

Although different liposome formulations have been described, only the preparation developed by NeoPharm, Inc. (IL, USA) [48] is in advanced trials [49]. PTX (LEP-ETU) and DTX (LE-DT) are included in multilamellar liposomes composed of dioleoyphosphatidyl choline and cholesterol, after charge interaction with cardiolipin. This formulation can load PTX into the liposome bilayer at a maximum molar percentage of ~ 3.5%. A dosage as PTX of 275 mg/m^2 may offer an improved therapeutic index versus the free drug.

To overcome the limitations associated with PTX-based chemotherapy, Sonus Pharm. Inc. (WA, USA) designed Tocosol® Paclitaxel (now Bayer Schering Pharma AG (D) compound BAY86-5312), an emulsion using vitamin E and vitamin E derivatives to solubilize, stabilize and formulate PTX. This formulation was designed to reduce toxicity and shorten the infusion period [50], although a recent study found that the relative exposure of unbound PTX at the site of toxicity of Tocosol was double that of a Taxol® formulation [51]. Tocosol is now in clinical development Phase II – III evaluation, but failed its primary end point in Phase III in treatment of metastatic breast cancer (Clinical Trial.org identificator NCT00251095).

Genexol-PM is a PTX-containing biodegradable polymeric micellar system solely comprising a low-molecular-mass, nontoxic and biodegradable amphiphilic diblock copolymer, monomethoxy poly(ethylene glycol)-*b*-poly(DL-lactide) and PTX. A standard formulation loads PTX at 20 wt%. The efficacy and safety of Genexol-PM in patients with

Table 1. Alternative technologies.

Formulation	Drug	Product	Company	Clinical phase
Polymeric micelles	PTX	Genexol	Samyang Pharm	Phase II
Liposome	PTX	LEP-ETU	NeoPharm	Phase II
Liposome	DTX	LE-DT	NeoPharm	Phase II
Emulsion	PTX	Tocosol (BAY86-5312)	Sonus Co	Phase II
Albumin nanoparticles	PTX	Abraxane	Abraxis (Celgene)	Marketed
Albumin nanoparticles	DTX	ABI-008	Abraxis (Celgene)	Phase I, II
Prodrug	PTX	Taxoprexin	Luitpold Pharm.	Phase III

DTX: Docetaxel; PTX: Paclitaxel.

histologically confirmed metastatic breast cancer was reported by Lee *et al.* [16].

Polyunsaturated fatty acids such as linolenic acid and docosahexaenoic acid (DHA) were linked to position C-2′ of PTX and second-generation taxanes, giving very active derivatives [52,53]. In particular, the DHA-PTX (Taxoprexin Injection; Luitpold Pharm., NY, USA) seems a very promising slow-release conjugate and is now in Phase II clinical trials [54]. The results of eight Phase II trials administering single-agent DHA-PTX every 3 weeks revealed a favorable toxicity profile as well as modest activity against a variety of solid tumors [55].

Abraxis BioScience (now Celgene Corporation, NJ, USA) developed a method whereby taxanes and other drugs can be embedded in the nanoparticle albumin bound form (nab technology). The nanoparticles are obtained by emulsion-evaporation followed by high-pressure homogenization. Owing to the high shear conditions during homogenization, a coating layer surrounding the drug particles, including new disulfide bonds, is produced. The nanosuspensions obtained can load 10 wt% of PTX in stable particle with size < 200 nm. The very significant results afforded the launch of the product ABI-007 (Abraxane) on the US and EU markets for treating metastatic breast cancer. Clinical trials on different cancers, using Abraxane as single agent or on combination therapies, demonstrated a lower incidence of adverse effects of PTX and significant antitumor activity [56-59]. Nab formulation with DTX (ABI-008) is now in clinical trials on prostate and metastatic breast cancer.

3. Taxane polymer conjugates

Polymers play an important role in drug delivery systems. These are versatile molecules that can be designed to address specific needs. Although hundreds of different polymers and copolymers have been synthesized to deliver taxanes, most generate micro- or nanoparticle structures in which the effective drug is loaded inside the structure. Synthetic polymers used to conjugate taxanes may chiefly be grouped as: poly(ethylene glycol), polyaminoacids and *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers Macromolecules of natural origin such as proteins and polysaccharides have also been widely reviewed. Moreover, polymers with a highly branched three-dimensional architecture, such as dendrimers, have also been discussed (see **Figure 4** later and **Table 2**).

3.1 Poly(ethylene glycol) conjugates

One of the most popular polymers for prodrug delivery is poly(ethylene glycol) (PEG). PEG is highly soluble in water and in many organic solvents, is non-toxic and non-antigenic, and has been approved by the FDA and EMA for human use. PEGylation has been considered for several antitumor agents, namely proteins, peptides or low-molecular-mass drugs. Most results have demonstrated improved behaviors in terms of biodistribution and *in vivo* stability of the polymer conjugate [60].

A large number of PEG PTX prodrugs, reported in recent years, have provided increased aqueous solubility, enhanced pharmacological activity, lowered systemic toxicity, reduced immunogenicity and prolonged plasma circulation. In general, water-soluble PTX conjugates have been prepared by introducing PEG either at the C-2′ or at the C-7 hydroxyl group [61,62]. Greenwald and co-workers reported several prodrugs produced by modifying PTX with PEGs of various molecular masses [63,64]. The 5 kDa conjugate in the C-2′ position showed high water solubility (660 mg/ml) and maintained *in vitro* activity similar to that of the free drug. Conjugation at the C-7 position was not effective because the derivative produced could not be hydrolyzed. An increase the PEG molecular mass to 40 kDa in C-2′ position showed slightly improved activity over PTX in an *in vivo* experiment, in agreement with the EPR effect [61].

Several research groups have reported studies dealing with the preparation of different efficient linker systems for properly releasing PTX, such as amino acids spacers [65], an acid-sensitive carboxylic hydrazone linker (**Figure 2A**) [66] and a sulfide-containing linker [67]. Self-immolative linkers between PEG and PTX [68] have also been tested for oral delivery, and it has been demonstrated that PEG conjugation at the C-7 position improved oral bioavailability (**Figure 2B**) [69]. A PEG-PTX derivative developed by Enzon (NJ, USA) entered Phase I clinical trials in 2001 [70]. The exact structure of the prodrug is not available, but the importance of conjugation with PEG of molecular mass ≥ 30 kDa to prevent rapid kidney elimination was reported. However, the company discontinued the development of this compound in 2003 [71].

Table 2. Mostly innovative and promising approaches of taxanes carriers in the literature.

Synonyms	Drug	Carrier	Polymer molecular mass (kDa)	Drug loading (% w/w)	Present status (Company)	Ref.	Spacer
NKTR-105 (PEG-DTX)	DTX	Multi-arm PEG	40	9	Phase I (Enzon)	[74,75]	N.A.
Opaxio (PGA-PTX)	PTX	Polyglutamate	48	37	Phase III (CTI)	[82]	Direct ester
PGG-PTX	PTX	Poly(L-*g*-glutamylglutamine)	70	35	Discovery (*vivo*)	[96]	Glutamic (ester)
PNU 166945	PTX	HPMA	30	5	Dismissed after Phase I (Pharmacia)	[107]	G-F-L-G (ester)
	DTX	HPMA	27	8.2	Discovery (*vivo*)	[109]	Levulic acid (hydrazone)
	PTX	Triazine dendrimer	40	25	Discovery (*vivo*)	[121]	Glutarylamido (ester)
	PTX	Heparin	5	68	Discovery (*vitro*)	[129]	Ethylenediammine (carbamate)
	PTX	Heparin	12	16	Discovery (*vitro*)	[130]	V-L-F (ester)
	PTX	Heparin	12	35	Discovery (*vitro*)	[131]	V-L-F (succinyl)
	PTX	Chitosan	10	12	Discovery (*vivo*)	[134]	(Succinyl)
	DTX	Chitosan	10	8	Discovery (*vivo*)	[135]	(Succinyl)
HA-leucyl-PTX	PTX	Hyaluronic acid	10	14	Discovery (*vitro*)	[143]	Leucyl
HYTAD1-p20	PTX	Hyaluronic acid	200	20	Discovery (*vivo*) (Fidia)	[148]	(Succinyl)
AZ10992	PTX	CM-Dextran	150	6	Discovery (*vivo*) (Asahi Kasei)	[150]	G-G-F-G
HSA-PTX	PTX	Albumin	66	8	Discovery (*vitro*)	[160]	(Succinyl)
	DTX	Albumin	66	2	Discovery (*vitro*)	[159]	(Succinyl)
	PTX	mAb	150	7	Discovery (*vitro*)	[170]	PEG arms

DTX: Docetaxel; PTX: Paclitaxel.

PEG has also been used as a hydrophilic and solubilizing spacer in the preparation of targeted molecular conjugates containing PTX and a peptide as vector [72]. The cell surface receptor-directed peptide used was a segment of the bombesin/gastrin-releasing peptide whose receptors are overexpressed in some types of cancer. The *in vitro* cytotoxic assay showed that this conjugate possesses higher activity against target cell lines compared with free or PEGylated PTX. To improve the targeting ability of the conjugates, the same research group recently described the preparation of a 'scorpion' conjugate, in which two segments of peptide were connected to PTX. The resulting conjugate showed low solubility that was improved by the insertion of PEG. The results of the cytotoxicity experiments on randomly chosen human cancer cell lines showed that the divalent conjugate has higher activity than the single-ligand conjugate; and the more soluble PEG conjugates were less toxic. This effect is probably a result of the formation of steric hindrance against ligand receptor binding of the PEG segments [73].

A PEG-DTX derivative (NKTR-105) has just entered Phase I clinical trial on patients with refractory solid tumors in whom other available therapies have failed. This conjugate was obtained by using a multi-arm PEG approach using Nektar's small-molecule PEGylation technology, and showed good preclinical activity against colon and lung cancer xenograft models [74,75] Data suggest that the conjugate could be more efficacious and tolerable than DTX. An evaluation of the role of micellization on PEGylated conjugates was reported by Liu *et al.*, in a study in which 2 kDa PEG and DTX were linked by a direct ester in the 2′ position [76]. Reduced toxicity and maintenance of *in vitro* activity were reported [77].

3.2 Poly amino acid conjugates

Different polymers composed of natural amino acids have been tested as drug delivery systems [78,79], and one of the most promising was polyglutamic acid (PGA), which was used to deliver different anticancer agents [80].

Poly-L-glutamic acid is a highly charged polyanionic peptide, and thus highly water-soluble, with a polymer biodegradable backbone [79]. Several *in vivo* studies have also demonstrated the good biocompatibility and non-immunogenicity of L-PGA. PGA was found to be more susceptible to lysosomal degradation than poly(L-aspartic acid) or poly(D-glutamic acid) [81]. By simple reaction of condensation with PTX hydroxyl groups in C-2′, and to a lesser extent (~ 2%) at the C-7 hydroxyl position, the resulting ester was produced (**Figure 3A**). The drug–polymer linkage is expected to be stable in plasma but inherently chemically labile after cell internalization and to undergo spontaneous release of PTX, through autocatalytic hydrolysis involving nucleophilic attack by the carboxylate group on the ester linkage. Indeed, only after internalization can PGA-PTX be proteolyzed by cathepsin B, ultimately making PTX and PGA-PTX metabolites

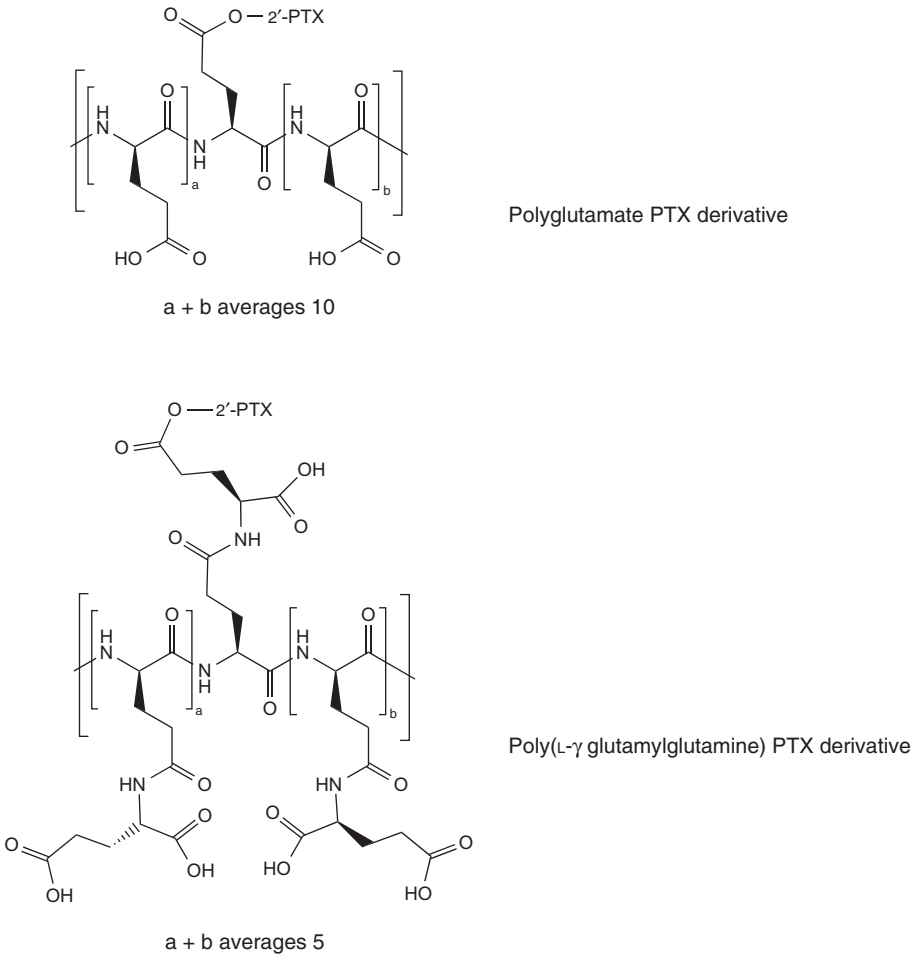

Figure 3. Chemical structure of polyglutamate taxane conjugates.

available intracellularly. Formulations with different molecular masses of PGA and different taxane molar ratios have been explored. The conjugate known as Poliglumex, CT-2103, Xyotax™, now Opaxio™ (PPX, Cell Therapeutics Inc., WA USA) is the most successful macromolecular prodrug, and has reached Phase III clinical trials [82]. The molecular mass of PPX is ~ 48 kDa [83] (80 kDa [82]) and conjugated PTX represents ~ 37 wt%, equivalent to ~ 1 PTX linkage per 11 glutamic acid residues of the polymer [83]. The pharmacokinetic profile (AUC, C_{max} values) was markedly improved, and prolonged circulation allowed for higher tumor exposure to the drug conjugate in treated animals. The chief metabolites were PTX, Glu-2′-PTX and H_2N-Glu-Glu-2′-PTX, although monoglutamyl-2′-PTX is an unstable compound that can degrade to release free PTX [84]. This finding may have biological relevance, as expression of proteases such as cathepsin B is upregulated in malignant cells, particularly during tumor progression [85]. These data support a model in which PPX accumulates in tumor tissue through the EPR effect, followed by the cathepsin B-mediated release of active drug [84].

PPX has shown antitumor activity against various human tumor xenografts as well as in Phase I trials (**Table 2**) [86-88]. Clinical experiences thus far have confirmed several advantages of PPX in the management of cancer patients. This product is easier to administer than Taxol®, as it can be delivered more rapidly and the treatment results only in infrequent and mild hypersensitivity reactions. Therefore, unlike Taxol® therapy, no premedication is necessary. Furthermore, patients undergoing PPX therapy show better compliance.

The PPX formulation seems not to protect against the development of side effects [88]. Dose-limiting toxicities, consisting of grade 3 neutropenia, neuropathy and fatigue, were the commonest toxicities. For this reason the recommended dose for Phase II studies was reduced to 70 mg/m^2 weekly (210 mg/m^2 for a 3-week cycle). Neuropathy, in particular, has also been related to a paclitaxel exposure threshold of 0.05 μM [89]. In the pharmacokinetic study by Boddy *et al.* [90] the released PTX, using high PPX dosage (> 170 mg/m^2), passed the threshold concentration and maintained the levels achieved. Nevertheless, because the population of patients in these studies was heavily pretreated with neurotoxic

chemotherapies, it was not clear whether the schedule of PPX (70 mg/m^2) administration helps to prevent neuropathy.

At the time of publication, 22 clinical Phase I/II trials have been reported (http://www.clinicaltrials.org, with the search for CT-2103) spanning different cancers: breast, advanced non-small cell lung, advanced head and neck, esophageal, prostate and ovarian cancers, where PPX was tested both as a single agent and in combination with chemotherapeutics and/or radiotherapy.

When PPX was administered at 175 mg/m^2 every 21 days as second- or third-line therapy in patients with epithelial ovarian or primary peritoneal cancer, only modest activity of limited duration was observed. The incidence of neuropathy using this dose in recurrent ovarian cancer was higher than predicted from studies in other tumors with PPX. The Gynecology Oncology Group is now exploring its use at 135 mg/m^2 every 28 days in a randomized trial evaluating maintenance chemotherapy in first remission [91] (clinical trials.org id NCT00017017, NCT00045682).

Phase III trials on patients with non-small cell lung cancer (NSCLC) were recently completed and the results reported in three articles. The first was a comparison with DTX, in patients who previously received platinum-based chemotherapy, where PPX was administered at 75 or 210 mg/m^2 or DTX at 75 mg/m^2 (NCT00054184). PPX and DTX produced similar survival results but had different toxicity profiles: PPX had less febrile neutropenia and less alopecia [92]. In the other two studies, naive patients randomly received single-agent PPX or a comparator (single-agent vinorelbine or gemcitabine) and PPX/carboplatin or PTX/carboplatin. In both studies overall survival was similar between treatment arms, but single-agent PPX or in combination chemotherapy was active and well tolerated in patients with poor performance status owing to advanced NSCLC (NCT00054210) [93,94]. Interestingly, there was a greater increase in survival for women treated with PPX than for men, most marked in the case of premenopausal women. On this basis Cell Therapeutics, Inc. (CTI) presented a marketing authorization application for Opaxio to the Committee for Medicinal Products for Human Use (CHMP) for first-line treatment of patients with NSCLC who have a low performance status. Unfortunately, concerns about the risk/benefit ratio (in particular side effects such as neuropathy) and efficacy, not higher than comparators, in late 2009, caused CTI to withdraw its application. Another study aimed to detect the maximum tolerated dose of PPX with concurrent radiotherapy in patients with esophageal and gastric cancers (NCT00522795) [95]. More recently, patients with metastatic adenocarcinoma of the prostate, which had progressed despite standard hormonal therapy and after DTX-containing chemotherapy, were treated with transdermal estradiol for 4 weeks followed by the same dose of transdermal estradiol and PPX. This regimen of low-dose transdermal estradiol induction followed by PPX had no activity in taxane-pretreated patients with castration-resistant prostate cancer [96] (NCT00459810). CTI plans to meet the FDA in the second half of 2010 to explore a potential Phase III registration study based on these results of this last study on esophageal cancer [97].

In an attempt to improve the therapeutic potency of the PTX conjugated with PGA, Wang *et al.* [98] very recently reported an application of poly(L-γ-glutamylglutamine) (PGG) in which an extra glutamine side chain was added to each glutamyl monomer (**Figure 3B**). Using PGG of 70 kDa and a PTX loading extent of 35 wt%, in aqueous solution the conjugate spontaneously forms micellar nanoparticles with a median diameter of 20 nm. The presence of a carboxyl group in a closer position on the ester linkage modified the release of the drug. This new formulation has activity superior to that of Abraxane in the B16 murine melanoma, NCI-H460 non-small cell lung cancer, and 2008 ovarian cancer models [98,99].

The importance of the role of the amino acidic spacer was also reported by Zhang *et al.*, who used alanine in a similar approach to PGG-PTX [100]. The presence of an alanine spacer markedly increased the release of PTX (20% released in 15 h, versus 15% in 140 h for PGG-PTX), enhancing the clearance value of the conjugate versus PTX.

α,β-Poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA), a freely water-soluble, non-toxic, non-antigenic and non-immunogenic multifunctional macromolecule, proposed as plasma substitute, has been investigated as a PTX carrier [101]. PTX was conjugated with PHEA by means of a succinyl spacer, and a drug content of ~ 19.8 w% was obtained. The pharmacokinetic results showed the polymeric conjugate to be cleared from the bloodstream more rapidly than free PTX, with massive disposition in the liver. Successively, this conjugate was also tested in a targeting approach with oxytocin and an improved *in vitro* activity was reported [102].

3.3 *N*-(2-Hydroxypropyl)methacrylamide conjugates

One of the polymers most widely explored as an anticancer agent carrier is the HPMA copolymer [103]. The HPMA copolymer is biocompatible, almost non-immunogenic and non-toxic, but the copolymer backbone has the disadvantage that it is not inherently biodegradable. Therefore, as for all non-degradable polymeric carriers, there is a risk of cellular accumulation, especially if chronic administration or high doses are used. For this reason, to ensure elimination by glomerular filtration after parenteral administration, the molecular mass of drug–polymer conjugates was kept below 30 kDa.

HPMA copolymer conjugates have also been produced not only as linear, but also as branched, grafted and dendritic star-structured forms [104]. Furthermore, linear HPMA conjugates can be receptor-mediated targeted [105], and the simultaneous delivery of two drugs on the same polymer chain is also possible [106].

HPMA has been widely tested with a wide array of polymer–drug linkers. The best results have been obtained using the oligopeptide spacer Gly-Phe-Leu-Gly, susceptible to enzymatic (proteolytic) degradation. This spacer was

examined as a promising candidate for drug carrier systems for a variety of drug conjugates. Linkage with the drug has primarily evolved with the goal of exploiting the chemical functionality available in the bound bioactive agent (e.g., ester linkages for paclitaxel, pH-sensitive hydrazone bond for doxorubicin).

Doxorubicin, platinum complexes, camptothecin and PTX have been tested. All these conjugates reached clinical trials, but with different outcomes. Polymer-conjugated prodrug of PTX (PNU 166945) [107] demonstrated higher solubility than the free drug, but unfortunately the highest drug content did not exceed 5 wt%. It was evaluated in a Phase I clinical trial and, in contrast to Taxol formulations, hypersensitivity reactions were not observed. In a clinical study with 12 patients with advanced breast cancer, one partial remission after a dose of 100 mg/m^2 was observed. However, at the same time the results showed drug-related toxicities, including bladder toxicity, which could be explained by instability of the ester bond of the conjugate during its blood circulation and urine-excretion phases. Clinical trials were discontinued mainly because of significant neurotoxicity (grade 3) seen in preclinical testing in rats and also in a proportion of patients. Summarizing, the reasons for the failure of HPMA copolymer–PTX largely depend on its very low, impractical, drug loading rate, and also on the fast release of PTX from the ester *in vivo* [108].

Nevertheless, very recently this approach was renewed by Etrych *et al.* [109], who produced conjugates of PTX and DTX with HPMA, exploiting an acid-sensitive hydrazone linkage instead of the peptide spacer. The resulting HPMA copolymer contained 5.7 mol% of hydrazide groups, and the drugs were esterified in the C-2′ position with suitable molecules containing a keto group. The drug content ratio was ~ 8 wt%. On mouse models only conjugates induced a complete cure and only the DTX conjugate efficiently reduced EL4 lymphoma tumor growth. Treatment with the polymer conjugates was devoid of side toxicity.

For an in-depth critical review of the last 30 years of research on HPMA and anticancer conjugates, please see the commentary article by Duncan *et al.* [110].

3.4 Dendrimeric conjugates

Dendrimers are a class of well-defined artificial macromolecules with a tree-like three-dimensional structure, in which highly branched units radiate out from a common central core. Dendrimers are organized in layers of branching (i.e., generations) that provide a high degree of surface functionality [111]. Besides multivalency, another advantage of using a dendritic structure instead of a linear polymer to obtain drug conjugates lies in their highly monodisperse size and shape, which derives from a precisely controlled iterative synthesis. Even if dendrimers may also carry molecules through the encapsulation into their interior void spaces, here only the covalent linkage of dendrimer functional groups with taxanes (in particular PTX) is reviewed.

A first example of a PTX-dendrimer conjugate was obtained starting from nitrodiol to obtain a branched structure linked to two PTX molecules through the 2′-hydroxy function of the drug [112]. In particular, 2-(4-nitrobenzylidene)propane-1,3-diol was activated using 4-nitrophenyl chloroformate to give the corresponding bis (4-nitrophenyl carbonate), which was subsequently linked with two equivalents of PTX to yield the conjugate. Chemical reduction under mild conditions of the nitro group to the amine induces simultaneous release of both endgroups (in particular of the drug molecules) from the double-release linker. Similarly, in the same work a more complex dendritic structure was obtained, where two generations of double-release linkers were connected through carbamate linkages. After activation, four equivalents of PTX were coupled. Chemical reduction determined the simultaneous release of all PTX molecules. These compounds, in which a single triggering event in the dendritic core simultaneously frees all endgroup molecules, are called 'cascade-release dendrimers' (**Figure 4**) [112]. These dendrimers are characterized not only by an amplification of the effect of a single activating reaction with a consequent increase of activity, but also by a complete and rapid degradation that facilitates their clearance from the body, which is often a limiting factor for macromolecules used in the design of drug delivery systems.

Another example of a PTX-conjugated dendrimer, which disassembles into its building blocks in a self-immolative manner to release the drug after fragmentation initiated by a triggering reaction, is the compound obtained by conjugating the HPMA copolymer to PTX through a self-immolative dendritic linker [113]. The HPMA, linked to the dendrimer by means of its glycine-phenylalanine-leucine-glycine moiety and an enzyme-cleavable linker (the dipeptide phenylalanine-lysine-*p*-aminobenzyl), enhances the water solubility of the entire compound. The six-amino-acid peptide was used to provide convenient conjugation chemistry, a longer spacer and higher probability of cleavage. This structure releases three molecules of PTX from a single dendritic unit, as the result of cleavage by the endogenous enzyme cathepsin B. The dendrimer-based structure showed enhanced cytotoxicity on murine prostate adenocarcinoma (TRAMP C2) cells in comparison with a classic monomeric drug–polymer conjugate because the dendrimer acts as an amplifier.

After activating its 2′-hydroxy function as the *N*-hydroxy-succinimidic ester of PTX-hemisuccinate, PTX was also conjugated to generation-five (G5) poly(amidoamine) (PAMAM) dendrimers by means of their primary amino groups after their full glycidylation to yield an ester linkage [114,115]. Other functional molecules, such as fluorescein isothiocyanate (FITC) and folic acid, were also conjugated to the same G5 PAMAM dendrimer structure, in order to obtain a trifunctional dendrimer with a covalently linked fluorescent probe and an active targeting. The average number of PTX, FITC and folic acid molecules attached to the dendrimer

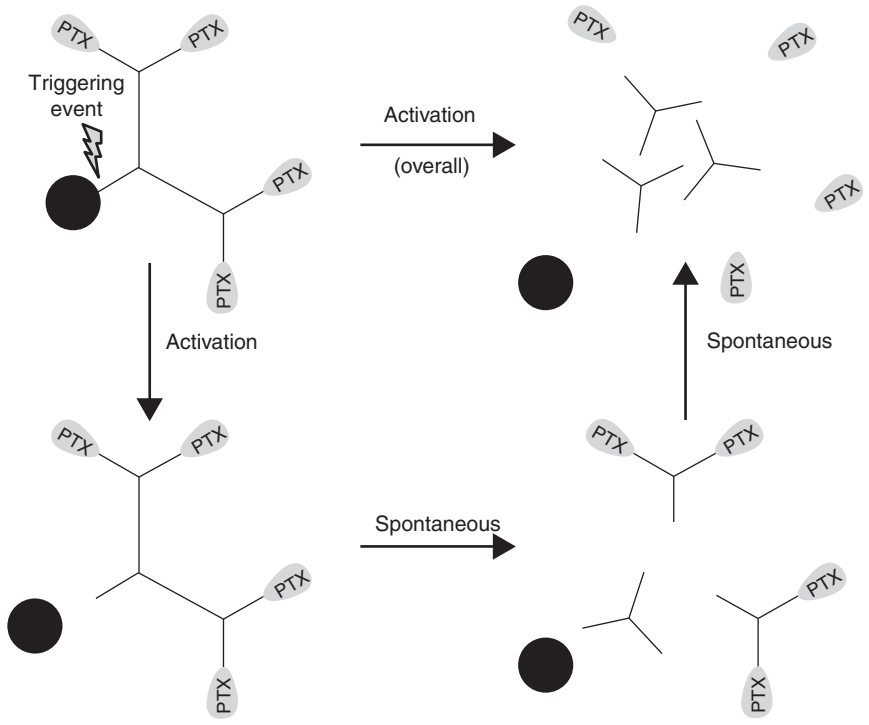

Figure 4. Liberation of all PTX endgroup molecules (in gray) by a single activation of a second-generation cascade-release dendrimer (in black) that triggers a cascade of self-eliminations.

Adapted from [112].

PTX: Paclitaxel.

was 3, 5 and 5, respectively. These conjugates were tested *in vitro* on KB cells and showed a higher uptake and cytotoxic effect of these multifunctional dendrimer conjugates in comparison with non-targeted dendrimers.

Another PTX-dendrimer conjugate, PTX-G4 PAMAM, was designed and compared, in terms of drug release, delivery and anticancer effect, with a linear derivative of the drug, the α,ω-bis((2-carboxyethyl)-PEG)-PTX conjugate [116]. Paclitaxel was covalently linked with bis(carboxyethyl) PEG and with the G4 PAMAM hydroxyl-terminated dendrimer by the condensation method. In particular, for PAMAM-PTX the anticancer drug was previously linked to succinic acid and then coupled to the G4 PAMAM structure (ratio of PTX molecules per dendrimer: 0.857), whereas for the PEG-PTX conjugate the drug was directly reacted with PEG (conjugation ratio PEG/PTX: 0.99). The release of PTX from the conjugates after enzymatic hydrolysis by an esterase at pH 7.4 after 24 h was higher for the PEG-PTX conjugate (30%) than for the PTX-PAMAM derivative (20%), although the dendrimer conjugate has two ester sites for hydrolysis and the PEG-PTX only one. This may be the consequence of the dendrimer architecture, which shows higher steric hindrance for esterase degradation when compared with linear PEG derivative. Analysis of the two conjugates showed that they both greatly enhance the drug's solubility, although the increase is better for the dendrimer conjugate than for the PEG-PTX one. Moreover, whereas conjugation to the PEG polymer significantly decreased the cytotoxicity of PTX, its conjugation to the G4 PAMAM dendrimer substantially increased its cytotoxicity, leading to a decrease in the IC_{50} dose of > 10 times versus the free drug.

The physicochemical characteristics and anticancer activity of a similar G4 PAMAM dendrimer conjugated with PTX and an imaging agent (near-infrared cyanine Cy5.5) were also compared not only with linear polymers (PEG-PTX), but also with other nanocarriers, such as PEGylated liposomes [117]. A synthetic analogue of luteinizing hormone-releasing hormone (LHRH) peptide, targeted to receptors overexpressed on the membrane of cancer cells, was attached to all nanocarriers as a tumor targeting moiety by means of a non-biodegradable amide bond, as for Cy5.5. PTX was attached to the dendrimer G4 PAMAM structure by means of a succinic spacer, whereas for liposomes it was incorporated in the phospholipid bilayer of the membrane. The average size of dendrimers, PEG polymers and liposomes was ~ 5, 30 and 100 nm, respectively. The study found significant differences between non-targeted and peptide-conjugated nanocarriers both *in vitro* and *in vivo*, because the presence of the targeting moiety enhanced the anticancer efficacy of all the delivery systems considered. Nevertheless, for targeted systems, the architecture, composition and size of nanocarriers have a negligible influence on their efficacy as imaging and anticancer treatment.

The same research group has recently modified the LHRH-targeted G4 PAMAM dendrimer to obtain an internally quaternized and surface neutral structure [118]. The internal cationic charges may enable small-interfering RNA or antisense nucleotides to be complexed and the dendrimer architecture could limit their *in vivo* degradation.

Paclitaxel was also conjugated to triazine dendrimers and the compound was then PEGylated to confer biocompatibility and solubility to the hydrophobic triazine structure [119]. The chemistry of triazine enables the chlorine atoms on triazine rings to be substituted with amine nucleophiles for covalent linkage with the drug [120]. Thus, a dichlorotriazine-modified PTX was obtained in four steps. First, PTX was reacted with glutaric anhydride and then the product was activated as the *N*-hydroxysuccinimidic ester, which was treated with 1,3-diaminopropane to obtain an amino group able to be linked to triazine ring. The reaction of the dichlorotriazine-modified PTX with an amino group-bearing dendrimer gave a generation-three dendrimer with 16 paclitaxel groups attached by labile ester linkages. Then, PEGylation with 2 or 5 kDa PEG was performed in two steps and the final products (2 and 5 kDa PEGylated derivatives) were 30 and 18 wt% PTX, respectively. Biological characterization of a similar PEGylated generation-three triazine dendrimer bearing 12 ester-linked PTX molecules (25 wt% PTX) revealed that PTX release occurs in the plasma and is nonlinear. Moreover, triazine dendrimer-PTX showed cytotoxicity and *in vivo* toxicity comparable to that of Abraxane. Anticancer efficacy was observed in a PC-3 prostate tumor model [121].

PEGylated paclitaxel-triazine dendrimer conjugates were further developed by introducing an ester-disulfide linkage between the drug and the dendrimer by reaction of *N*-hydroxysuccinimidic ester of 2′-glutaryl-PTX with cystamine and subsequent conjugation to triazine ring. These disulfide-containing derivatives were compared with an ester-linked PTX-triazine dendrimer [122]. In these compounds 12 PTX groups were linked to the dendrimer (~ 26 – 30 wt% PTX). Two kilodalton PEG was linked to the dendritic structure by an ester or an ether bond. Cytotoxicity of these constructs on a human prostate cancer cell line PC-3 reveals IC_{50} values in the low nanomolar range, with dithiothreitol and glutathione enhancing the toxicity of the disulfide-containing constructs. *In vivo* these compounds appear to be well tolerated at doses equal to 200%, the maximum tolerated dose of PTX. The analysis showed tumor localization at low levels for all the synthesized dendrimers.

To the authors' knowledge, DTX has not yet been conjugated with dendrimers, but only associated to them through inclusion into glycodendrimer-conjugated cyclodextrins [123].

3.5 Heparin conjugates

Heparin is a natural highly sulfated glycosaminoglycan well known for its anticoagulant activity, but having an effect on tumors when associated with the binding of growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [124]. Heparin is also associated with the inhibition of heparanases, enzymes that are thought to be required by tumor cells for invasion of the vascular basement membrane [125,126]. These features make heparin an interesting candidate as a carrier, with its own antitumoral activity, for targeted drug delivery. Furthermore, heparin contains different reactive functionalities such as sulfate, hydroxyl and carboxyl, some of them responsible for its anticoagulant activity [127,128], thus multiple synthetic approaches are possible.

Park and co-workers [129] introduced a carbamate linkage on the 2′-hydroxyl group and reacted the ethylenediammine derivative with the carboxyl group of heparin (**Figure 5A**). The polymer had a molecular mass of 5 kDa with an amount of PTX conjugated ranging from 0.8 to 4.1 mol per mole of heparin. As with many other PTX conjugates, after freeze-drying the derivative once added/dispersed in water formed nanoparticles (size 200 – 400 nm) with highly negative charge. The reduction of the number of carboxyl groups reduced the anticoagulant activity, although because the sulfate group was intact, PTX-conjugated heparin retained its ability to bind with angiogenic factors as well as its anti-heparanase activity. The activity on tumor cells of the most heavily loaded conjugate was similar to PTX.

Wang and co-workers [130] described two different synthetic approaches on 12 kDa heparin. O-acetylated heparin was directly condensed to the carboxyl groups of PTX, or using the amino acid spacers Val, Leu, Phe, and ester linkage. In this case, although 16 – 25 wt% of PTX was linked, the resulting conjugates were soluble. Therefore, the hydrolysis rate and *in vitro* efficiency for heparin conjugates were investigated. The same group, using a succinyl linker and the same amino acids as spacers, obtained conjugates with improved PTX incorporation (35 – 39 wt%). These compounds self-assembled in water, giving nanoparticles of 140 – 180 nm with charge ranging from -21 to -31 mV [131]. The conjugates were tested on the ovarian carcinoma SKOV3 model, demonstrating an activity that was not better than PTX, although the systemic toxicity was lower. Although the heparin approach might be promising, the authors of this study did not clearly discriminate, in the *in vivo* models, the role of anticancer activity resulting from released drug from that resulting from the intrinsic antiangiogenetic activity of heparin.

3.6 Chitosan conjugates

Chitosan is a natural linear polysaccharide composed of β-(1→4)-2-amido-2-deoxy-D-glucan (glucosamine) and β-(1→4)-2-acetamido-2-deoxy-D-glucan (acetyl glucosamine) units, and is obtained by deacetylation of chitin. Chitosan has excellent biocompatibility, low toxicity, immunostimulatory activities, antibacterial and antifungal action, and anticoagulant properties [132,133]. Furthermore, the degradation products of chitosan (i.e., aminosugars) are non-toxic, non-immunogenic and non-carcinogenic.

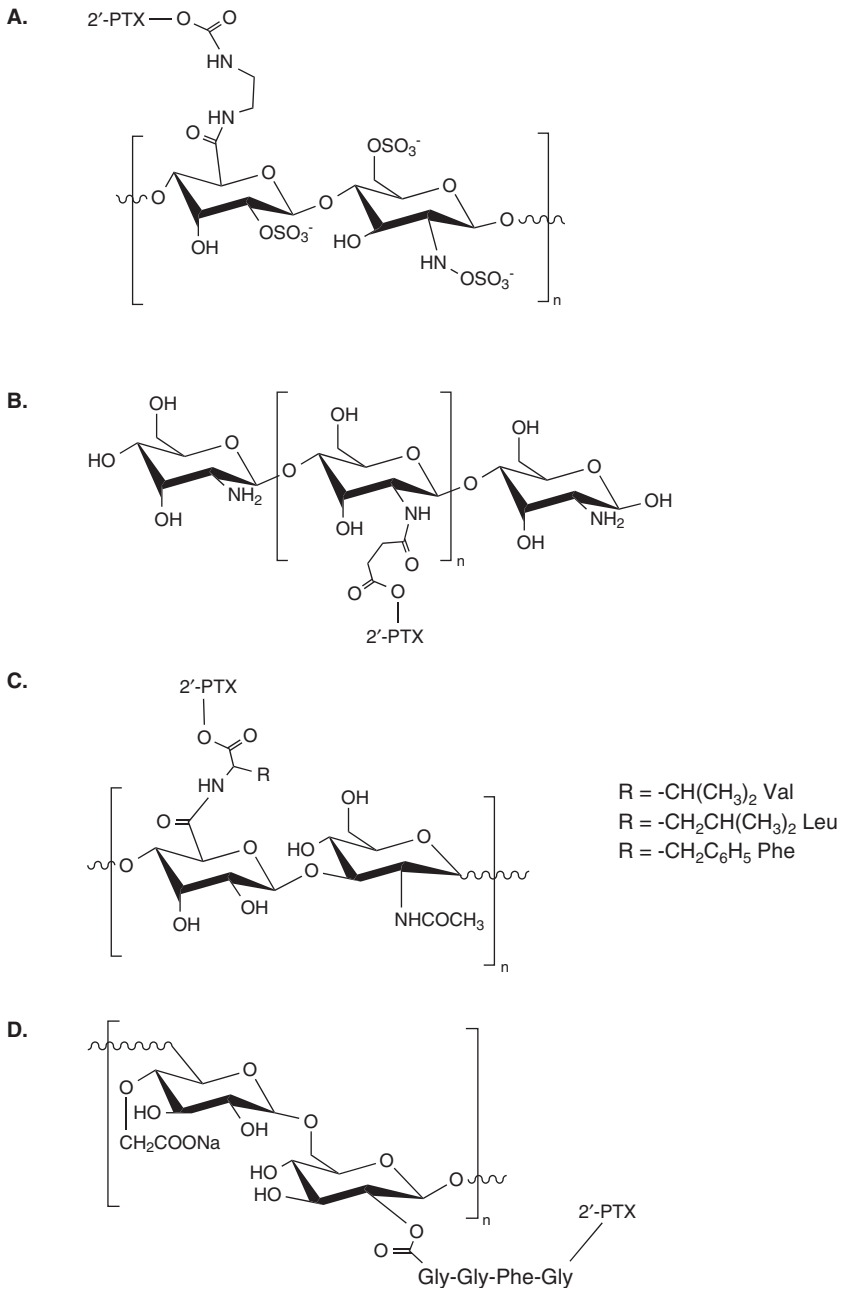

Figure 5. Examples of polysaccharide PTX conjugates with different components. A. Heparin with ethylenediamine spacer and carbamate linkage. **B.** Chitosan linked through a succcinate spacer. **C.** Hyaluronic acid linked through an amino acid spacer. **D.** Carboxymethyl dextran linked through peptide spacer.

In addition, low-molecular-mass chitosan (LMMC) has recently emerged, with interesting characteristics such as opening the tight junctions between intestinal epithelial cells in a Caco-2 cell model [134]. Furthermore, LMMC (molecular mass < 10 kDa) showed more favorable characteristics than high-molecular-mass chitosan, such as lower toxicity and higher water solubility. For this reason, with the aim of increasing the oral bioavailability of PTX to clinically useful values, low-molecular-mass chitosan was conjugated with PTX (LMMC-PTX) (**Figure 5B**) [135].

The PTX was coupled through a succinate linker and yielded 12 wt% drug content [136]. The *in vivo* antitumor efficacy was estimated on murine melanoma and on xenografted human non-small cell lung carcinomas after oral administration. The results showed strong antitumor activity of LMMC-PTX that was attributable to the greater water

solubility, prolonged retention in the gastrointestinal tract, and ability to bypass P-gp efflux pumps in the gastrointestinal tract, as well as to CYP 450-dependent metabolism in the intestine and liver.

The same group more recently reported [137] an analogous approach with DTX, obtaining a conjugate with 8 wt% drug loading. LMMC-DTX conjugate (by mouth) showed comparable *in vivo* antitumor efficacy to DTX (intravenous) even at the same dosage, but with a reduction of the subacute toxicity.

3.7 Hyaluronic acid conjugates

Hyaluronic acid (HA) is a linear polysaccharide that can be found throughout the connective, epithelial and neural tissues. Hyaluronic acid plays important roles in biological function, such as cell adhesion, growth and migration [138], and also acts as signaling molecule in cell motility, inflammation and cancer metastasis [139]. Moreover, as some specific HA receptors (CD44 and RHAMM) are overexpressed in various malignant cell types, linking an antitumor drug such as PTX to HA might improve the targeting activity of the conjugate and overcome the problem of low drug solubility.

Prestwich and co-workers reported the preparation of an HA-PTX conjugate using low-molecular-mass HA (molecular mass 11 kDa) and adipid hydrazide as linker to PTX-succinate [140,141]. It was shown that the conjugate was internalized into cancer cells through receptor-mediated endocytosis, followed by intracellular release of active drug. The study also showed that the activity of the conjugates depends on PTX loading: high loading reduces solubility and causes modification in the HA structure, reducing its targeting ability and hence the conjugate's cytotoxicity. More recently, Auzenne and co-workers obtained interesting *in vivo* results on this HA-PTX conjugate, which showed better antitumor efficacy and lower toxicity against $CD44^+$ human ovarian carcinoma xenografts compared with free PTX [142].

The role of the amino acid spacer in releasing PTX was recently investigated by Xin *et al.* (**Figure 5C**) [143]. Hyaluronic acid 10 kDa derivatized with valine, leucine, or phenylalanine was able to load PTX up to 14 wt% and the HA-leucyl-PTX was more effective than other conjugates, owing to increased release of the parent drug. This phenomenon had been demonstrated by Nicolau *et al.* [144] in PTX prodrugs, where amino acid spacers and strong electron-withdrawing substituent in the α-position of the PTX ester may accelerate the hydrolytic cleavage of PTX [145,146]. Nevertheless, the high loading entails the conjugates' self-assembling into small uncharged nanoparticles.

Fidia Farmaceutici SpA (Italy) reported the preparation and physicochemical and biological characterization of HYTAD1-p20, a conjugate obtained by carboxyl esterification of HA with PTX [147]. The results showed that HYTAD1-p20 is a significant improvement over PTX in terms of water solubility, higher *in vitro* activity against human bladder cancer cells, and *in vivo* biocompatibility. The same group recently evaluated the conjugate's activity against human ovarian xenograft following intraperitoneal administration. They found that this conjugate gives better results than PTX in terms of *in vivo* tolerability and therapeutic efficacy in the intraperitoneal chemotherapy approach against ovarian cancer [148]. Recently, exploiting the binding to CD44 receptor, efficient activity of HA-PTX was demonstrated in intraperitoneal treatment of ovarian tumor model [142].

3.8 Dextran conjugates

In an attempt to overcome the side effects and improve the pharmacological profile of PTX, Asahi Kasei Corp (Japan). has designed a series of PTX conjugates with high-molecular-mass carboxymethyl (CM) dextran This type of polymer was chosen as candidate carrier for several reasons: dextran is generally recognized to be safe; CM dextran contains a sufficient number of carboxyl groups for drug attachment, which provides sufficient carrying capacity for the drug; and the resulting CM dextran–drug conjugate has a high probability of being water-soluble. The conjugates were linked at the 2′ position of PTX through several amino acid spacers (**Figure 5D**) [149]. The compound AZ10992, characterized by a Gly-Gly-Phe-Gly linker, a molecular mass 150 kDa and with a drug content of 5.5 – 6.5 wt%, demonstrated improved solubility (> 100 mg/ml in saline) and important activity on Colon26 carcinoma mouse model. The strongest antitumor activity, combined with no body weight loss and reduced neurotoxicity, was observed in the treatment model [150].

More recently, a dextran of 70 kDa was derivatized in mild conditions with carbonylimidazole and ethylenediamine, to generate stable derivative with PTX through a carbamate linkage. Furthermore, the dextran conjugate was targeted with folic acid using the same synthetic procedure. The degree of incorporation of amino groups was ~ 10% of dextran sugar units, whereas linked PTX and folate were 1.2 and 1% of dextran sugar units (4.8 wt% PTX and 2.0 wt% folate). The macromolecules were described as water-soluble and with a higher *in vitro* activity than free drug, when observed on folate receptor-expressing cell lines [151].

3.9 Protein and peptide conjugates

Not only synthetic polymers or polysaccharides, but also proteins and polypeptides have been used in the delivery of taxane prodrugs. Serum proteins offer the promise of selective delivery of anticancer agents, because of their accumulation in tumor tissues. In particular, tranferrin and albumin have been evaluated.

Transferrin is a serum glycoprotein involved in iron transport that also acts in cell growth regulation through a membrane receptor. A targeting prodrug strategy could be interesting because the number of transferrin receptors is increased in tumor cells [152]. The only conjugate that has been reported utilized a 2′-glutaryl hexanediamine linkage.

The cytotoxicity data on H69 cells showed a 5.4 times reduction in the activity of the conjugate versus PTX [152].

Albumin (intended as human serum albumin [HSA]) has several characteristics that make it an attractive drug vehicle in oncology [153]. It is a natural carrier of endogenous hydrophobic molecules (such as vitamins, hormones, and other water-insoluble plasma substances) that are bound in a reversible non-covalent manner. Moreover, albumin appears to help endothelial transcytosis of protein-bound constituents, principally by binding to a cell surface 60-kDa glycoprotein (gp60) receptor [154].

Taxanes have been shown to bind HSA tightly, inducing a conformational change in the protein, although in different ratio complexes; 1.9-3.9 to 1 for PTX [155] and 1 to 1 for DTX [156]. As a versatile protein carrier for taxane delivery, HSA was used following two approaches: chemical conjugation as for the other macromolecules reported above, or 'entrapment' using a nanoparticle-forming formulation.

Soluble albumin conjugates were obtained using a succinyl spacer, and an optimized loading capacity of PTX of 8 wt% was reported [157]. To increase the favorable pharmacokinetic profile, increasing solubility and adding a shielding moiety PEGylate HSA-PTX have also been carried out. The *in vivo* behavior confirmed the favorable properties of PEG, reducing localization in the liver and spleen and increasing the permanence in the bloodstream, mean residence time being increased by a factor of 10 over that of the free drug [158]. The same approach was also attempted for DTX, but in this case nanometric structures (90 – 110 nm) were also reported [159]. The conjugate was more active than Taxotere on cell lines and an improved biodistribution profile with higher levels of DTX in organs including the lung and spleen were observed.

Recently, the targeting capacity was combined to an HSA-PTX conjugate [160]. Folate-PEG derivative was linked through the partially reduced disulfides of HSA, which had previously been derivatized with PTX. The cytotoxicity test on an overexpressing folate receptor cell line confirmed the importance of targeting, with a 10-fold increase versus the untargeted conjugate.

3.10 Monoclonal antibody derivatives and targeted prodrugs

The role that monoclonal antibodies (mAbs) play in therapy is a promising one and continues to expand, thanks to the production of highly specific engineered mAbs, well tolerated and thus having valuable clinical activity. Nowadays > 20 mAbs have been approved for use in many indications, including cancer [161,162].

Even though mAbs exert therapeutic efficacy, their activity is often not sufficient to produce a lasting benefit. Conjugation with drugs or toxins combining the ability to harness mAb specificity and target the delivery of a cytotoxic agent to the tumor may significantly enhance both activities. Also for the preparation of mAb-drug conjugates there are three key components: the mAb, the drug and the linker, as for other macromolecular conjugates. In particular, the linkage must be stable but releasable inside/around the tumor; the loss of specificity resulting from an increased level of drug conjugation and the intrinsic low water solubility are great challenges [163].

PTX was conjugated with intact mAb, or with fragments such as anti-EGF or anti-Herb-2 mAb, usually through a succinic or glutaric spacer [164-167]. Improved cytotoxicity was observed versus unconjugated PTX [168]. The degree of derivatization was normally very low in comparison with other macromolecular conjugates (2:1, 1:1), thus the mAb must be very active, because only a small fraction of PTX can enter the tumor cells. Highly potent second-generation taxanes have been conjugated with high derivatization degree (4 – 5:1) using a disulfide linkage [169]. Only very recently was the drug molar loading of PTX increased to 10 – 12, using a branched PEG as spacer-solubilizer of molecular mass ~ 3 kDa and succinic or glutaric acid as linker [170]. On the basis of drug release and cytotoxicity results, the use of glutaric linker for PTX conjugates intended for systemic (*in vivo*) applications was proposed, whereas succinic acid was suggested for *in vitro* studies.

The drug targeting approach with mAb must resolve two crucial problems because delivery is limited by the antigen copy number and the limited drug loading [171]; only highly potent drugs, such as the auristatins, maytansines and calicheamicin, now appear promising in preclinical and clinical trials. The gemtuzumab conjugate with a calicheamicin derivative (ozogamicin) has reached the market as Mylotarg® (produced by Pfizer), although very recently the firm voluntarily withdrew it at the request of the US FDA owing to doubts over its safety and effectiveness.

4. Expert opinion

The high activity of taxanes against a variety of malignancies makes them attractive drugs for anticancer agents. Increased solubility, improved drug concentration in the tumor and reduction of side effects are needed, and the high versatility of macromolecular drug conjugates allows effective treatments to be designed and developed.

The key elements in a macromolecular approach that must be borne in mind concern the size and charge/hydrophilicity of the polymer, and the nature of the linkage and spacer. For polymer backbones that are not inherently biodegradable (PEG, HPMA), a molecular mass up to 40 kDa could ensure elimination by glomerular filtration, while a wide range of molecular masses, from very low (5 – 12 kDa) to high (150 kDa), are now in use for biodegradable polymers, such as polysaccharides, protein conjugates and polyaminoacids [18].

The water solubility of PTX is always greatly improved after conjugation with macromolecules (from 0.4 μg/ml to > 100 mg/ml), and this depends on the intrinsic charge

ability of the polymer. Indeed, high-molecular-mass and weak anionic charged polymers are known to circulate in the blood for a long time owing to low hepatic uptake and urinary excretion clearance [30]. In the choice of weight/charge characteristics, the balance between the tissue diffusivity and longer circulation is of critical importance

As has been clearly demonstrated with HPMA or mAbs conjugates, another point of crucial importance is loading capacity. The PTX conjugate had a very low, sometimes impractical, drug loading, to which conjugation was unable to impart any pharmacokinetic benefit. Nevertheless, from the reported data it is evident that in many cases the increased solubility was also obtained by stable colloidal dispersions (size 50 – 400 nm) formation. Indeed, owing to their high lipophilicity, especially when linked to long hydrophilic polymer, the taxanes tend to micellize into nanoparticles. In this way, the difference with taxane loaded in micelles (such as in Genexol-PM) is mainly owing to the kind of interaction (covalent link in conjugates, hydrophobic interaction in micelles). This is a key point because in micelles the full potency of the drug is guaranteed, rather than an active process being required to release the drug, as in the case of conjugates (by proteolysis, hydrolysis in acidic microenvironment, etc.). This active process requires more time but may occur only in selected conditions, increasing the selectivity and reducing the side effects resulting from a rapid release of the drug. It is a delicate balance that has been observed using drug-polymer conjugates with amino acid linkers, where *in vivo* release is governed by the circulatory retention of the high-molecular-mass polymeric drug and its gradual dissociation. The rate of the conjugate's tissue uptake and/or circulatory dissociation must therefore be faster than its rate of circulatory elimination, to allow for optimal activity of the bound drug. Hence, the rate of *in vivo* conjugate breakdown affects the drug's biodistribution and may ultimately affect both safety and efficacy.

From the toxicity standpoint, the presence of a covalent linkage allows the intrinsic side effects of taxanes (hematologic toxicity, neurotoxicity, myalgias) to be greatly reduced, and controlled taxane release is required to achieve antitumor effects. Furthermore, release may result in amino acid-taxane metabolites being produced, which would maintain the bioavailability.

The linkage on taxane molecules occurred essentially on the C-2′ hydroxyl position, and because restoration of the hydroxyl group is required for tubulin interaction, the ester linkage is the most selected bond. Indeed, this coupling linkage gave excellent results in the only PTX prodrug in advanced clinical trials, Taxoprexin. A direct ester linkage with the polymer is easy to obtain, and allows an efficient release of the drug, as demonstrated by the significant results of PPX (Opaxio). Nevertheless, increased exposure of the ester linkage to the medium, through a branched polymer, seems to increase the conjugate potency (PGG). Certainly, a more detailed evaluation of the upcoming *in vivo* results (risk/benefit ratio) of PGG is necessary before any conclusions may be drawn. In most conjugates, the most used spacer is the succinic ester, otherwise, when cleavage by lysosomal thiol-dependent proteases is chosen, the tetrapeptide sequences GFLG or GFGG show better results [172].

The combined characteristics of solubility, stability and efficient release must be properly tested in preclinical tests. Indeed, although still widely used, *in vitro* cell screening is often misleading, as is the determination of release of the linked drug by incubation in different media (plasma, buffers and tumor homogenate). In the cell line tests, the conjugates that contain lowest levels of contaminating free drug, and have a polymer–drug linkage that is stable in the culture medium but degraded following endocytic uptake, are generally markedly less cytotoxic *in vitro* compared with the parent drug. By contrast, conjugates that are unstable, contain even small amounts of free cytotoxic drug that will enter cells rapidly, and polymers that are inherently toxic, often perform best in *in vitro* screening assays, but are usually toxic and show poor benefits in terms of *in vivo* activity.

Biodistribution estimation in animal models can also be important to evaluate the exposure toxicity in the kidney and bladder, which may be very much higher than normally anticipated for the free drug. Indeed, one of the major side effects that were encountered with HPMA-PTX was a significant bladder side effect [107].

Furthermore, before an investigational drug program can be obtained, important regulatory considerations concerning macromolecular conjugates must be addressed [108]. This point must be considered in particular when very complex, multicomponent systems are proposed. Their complexity, both in terms of quality and reproducibility of manufacture, make them extremely challenging.

The combination of all these aspects and particularly the lack of validated *in vivo* models, and the absence of detailed pharmacokinetic analysis and of precise methods for assessing a compound's stability/purity/reproducibility may explain why at present no macromolecular-taxane conjugate is capable of providing all the expected improvements, although very considerable effort has been made. Finally, as demonstrated recently by Opaxio, only Phase II/III clinical trials are able to clarify the true risk/benefit balance of the macromolecular approach.

Achievement of the full therapeutic potential requires a rational approach based on comparison and in-depth analysis of the data on existing conjugates, combined with a multidisciplinary view, with the improved biological understanding on development and progression of tumor and metastasis. For example, future projects should take into account the recently suggested role of NO in facilitating the EPR effect and the possibility of using drugs such as nitroglycerine to enhance the EPR-mediated penetration of macromolecular drugs in tumors [173].

To hypothesize effective drug delivery systems, the choice of a selective, tunable or site-controlled trigger to release the free drug from a soluble conjugate is far from having been determined, but by exploiting the promising results of supramolecular devices one can conceive (in the authors' opinion) an improved concentration through the 'external' targeting approach. This approach may be achieved by either direct or indirect methods. In the first case the conjugate can be properly addressed, with mAb fragments, peptides, and so on, and this increases both the selectivity and the production complexity, and regulatory requirements, but new tools are offered by nanotechnology. Ultra-small gold or magnetoresponsive nanoparticles can be designed to be embedded in taxane conjugates and target the delivery of macromolecules. In this case some sort of theranostic (therapeutic plus diagnostic components) device can be obtained. Nanomaterials such as carbon nanotubes, which have effectively been used to deliver taxanes inside cells [174,175], are attracting particular attention as new carriers owing to their unique physicochemical properties and their ability to cross cell membranes. These new nano-structured materials may have great potential in molecular diagnosis and targeted therapy of tumors, although their possible toxic effects remain of concern [176,177].

A pretargeting approach involves separating the targeting agent (mAb) from the subsequent delivery of an imaging or therapeutic agent that binds to the tumor-localized agent, which then accumulates in the area. Using radiolabeled compounds, this approach has been shown to enhance target-to-background tissue ratios. A renewed approach not yet explored for conjugates could involve this strategy using a tried and tested approach such as biotin-avidin [178], or a new method such as inverse-electron-demand Diels–Alder [179]. In this way, coupling therapeutics to synthetic polymers should provide protection for the drug during transport, followed by concentration and subsequent release at the targeted site.

A multidisciplinary collaboration on such a variety of different preclinical and clinical skills, including knowledge of molecular targets, polymer and linker chemistry, and improved animal models, is needed to focus on simpler but innovative systems in order to achieve medical treatments with highly enhanced therapeutic value.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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• **A report on a new approach using PEG and mAbs for paclitaxel delivery.**

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• **First report of a new pretargeting approach based on inverse-electron-demand Diels–Alder reaction.**

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