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Perspective

Paclitaxel Prodrugs: Toward Smarter Delivery of Anticancer Agents

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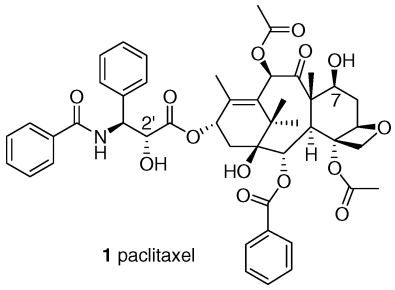
Introduction

The introduction of paclitaxel (**1**,[1] Figure 1) in clinical cancer chemotherapy has markedly improved patient survival time.[2−4] Paclitaxel is a taxoid that promotes tubulin polymerization leading to abnormally stable and nonfunctional microtubules. Cells are arrested at the G2-M phase of the cell cycle, resulting in apoptotic death.[5] These days, paclitaxel is in clinical use for treating a variety of malignancies, including ovarian, breast, and non-small-cell lung cancers, as well as AIDS-related Kaposi's sarcoma. Although protein kinase inhibitors (PKIs[a]) that are very potent against a wide range of cancers have recently been developed,[6] paclitaxel cancer therapy is still of significance. Combination therapies, including both PKI and paclitaxel, are very promising.[7−11] A novel application of paclitaxel has recently been considered. The therapeutic potential for paclitaxel against Alzheimer's disease was suggested, with limitations due to low drug bioavailability in the brain.[12]

Despite the hope and promises that paclitaxel has engendered, drug-resistant cells of certain cancer types as well as dose-limiting toxicity corresponding to paclitaxel's side effects are significant drawbacks. Many investigations have identified a variety of resistance mechanisms. The action of efflux pumps, such as P-glycoprotein (Pgp), which actively pumps out paclitaxel, is known to cause subtherapeutic intracellular drug concentrations. Resistance is also caused by changes in β-tubulin or apoptotic regulatory and mitosis checkpoint proteins.[13] This phenomenon, known as multidrug resistance (MDR), causes tumor resistance against chemotherapy because of the demand for higher toxicity-limiting doses of paclitaxel. Additionally, paclitaxel's low water solubility is a real problem in intravenous administration. Because paclitaxel is solubilized with a detergent, Cremophor EL, a prolonged intravenous administration time is required to inject paclitaxel at low concentrations. Significant side effects associated with hypersensitivity to Cremophor EL have also been observed, and premedication with corticosteroids and antihistamines is often required.[14,15] In addition, it was recently reported that Cremophor EL used in the paclitaxel formulation reduces the antitumor efficacy of the drug.[16] To overcome these problems, four strategies have been proposed: the design of new paclitaxel derivatives, changes in the paclitaxel formulation, coadministration with other antitumor agents, and the temporary modification of paclitaxel to a prodrug form. In this Perspective, we will emphasize the last strategy, i.e., prodrug design.



1 paclitaxel

Figure 1. Structure of paclitaxel **1**.

A prodrug is a bioreversible derivative of an active drug. The prodrug is used to overcome certain barriers in the parent drug's properties such as low solubility, low permeability, low oral

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[a] Abbreviations: PKI, protein kinase inhibitor; Pgp, P-glycoprotein; MDR, multidrug resistance; BMS, Bristol-Myers Squibb; MTD, maximum tolerated dose; ADEPT, antibody-directed enzyme prodrug therapy; MAb, monoclonal antibody; PEG, poly(ethylene glycol); BBN[7-13], QWAVGHLN-NH_2; FR, folate receptor; DHA, docosahexaenoic acid; EPR, enhanced permeability and retention; HA, hyaluronic acid; HPMA, *N*-(2-hydroxypropyl)methacrylamide; PG, poly(L-glutamic acid); EGFR, epidermal growth factor receptor.

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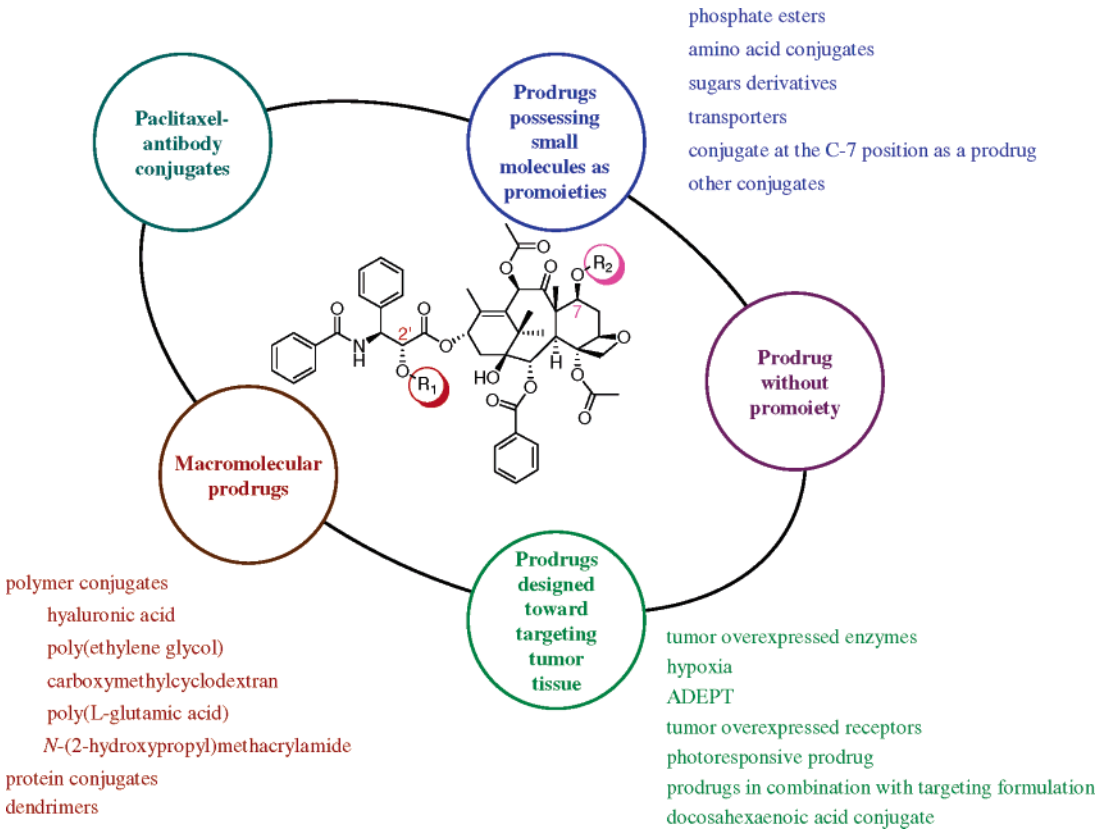
Chart 1. Prodrugs of Paclitaxel



absorption, instability, toxicity, and nontargeting.[17] The term "prodrug" is also defined as "a chemical with little or no pharmacological activity, undergoing transformation to a therapeutically active agent after absorption or distribution in the body."[18]

In 2001 and 2002, at least 14% of all new approved worldwide drugs were prodrugs.[19] This trend marked a dramatic increase in the number of submitted prodrug patents (over 20-fold increase in 2002 compared with 1993), with claims for cancer treatment comprising 37%.[19] This clearly shows that a large number of research groups are focusing on prodrugs to develop promising anticancer agents.[20] Patents for prodrugs and other derivatives of paclitaxel are one of the most popular groups among anticancer patents.[21−23] Although a large number of reviews deal with paclitaxel, only a few deal briefly with its prodrugs.[21−24] Reviews that have generally focused on prodrugs provide only incomplete information on paclitaxel conjugates.[25−28]

Paclitaxel (**1**) prodrugs (Chart 1) are generally designed by introducing hydrophilic or targeting moieties (promoieties) to the C-2′ and/or C-7 positions. A promoiety is a chemical "cap" that alters the physical and biological properties of the parent drug molecule and is subsequently cleaved from the prodrug with the release of the parent drug in the host body. However, only the C-2′ moiety is ideal for derivatization in the prodrug design, as a free hydroxyl group at this position seems to be required for cytotoxic effects.[30,31] Furthermore, esters at C-2′ are more labile and can be synthesized selectively without protecting the C-7 hydroxyl group. Therefore, "inactive" chemicals (prodrugs) at the C-2′ position could be unmasked in vivo to release active paclitaxel. In contrast, the C-7 hydroxyl group is not so critical for paclitaxel activity.[30,31] Thus, most paclitaxel derivatives at the C-7 position were as potent as the parent drug, or these derivatives were so stable under physiological conditions that they did not release the parent drug. Therefore, in this Perspective, derivatives of **1** at the C-7 position will be named only as "conjugates" unless there is clear evidence that a particular compound is a real prodrug.

Among reported paclitaxel prodrugs, two main approaches can be identified: prodrugs with auxiliary moieties that are enzymatically cleavable and prodrugs transformed into the parent drug by chemical reactions in the body. Biocleavage can be used for specific activation of targeting prodrugs. However, targeting is often limited to cases with highly overexpressed tumor-specific enzymes or to strategies that employ specific delivery of the appropriate enzyme to the tumor site. In a strategy using chemical cleavage, it is generally not necessary to consider the level of enzymes in different tissues (with the exception of prodrug stability against undesired enzymatic cleavage). However, the classification of prodrugs, based on their enzymatic or chemical mechanism of activation, is sometimes difficult. It is not easy to discriminate between chemical cleavage and nonspecific enzymatic hydrolysis during in vivo experiments. Therefore, in this Perspective, prodrugs are grouped according to the chemical classes of the derivatives:[17,18] (1) carrier-linked prodrugs that are mainly activated by hydrolysis of the promoiety, (2) bioprecursors that do not include any promoiety, (3) site-specific chemical delivery system that is composed of prodrugs designed toward targeting tumor tissue, (4) macromolecular prodrugs consisting mainly of polymer derivatives, and (5) drug−antibody conjugates, where an antibody that is specific to tumor tissue is attached to paclitaxel. However, it should be noted that this classification is arbitrary and some prodrugs could be included in more than one group.

Although this Perspective focuses on the prodrugs of paclitaxel, most of the strategies used in their design can be applied to other taxoids. Moreover, a similar or identical design can be observed in prodrug development for other anticancer agents.

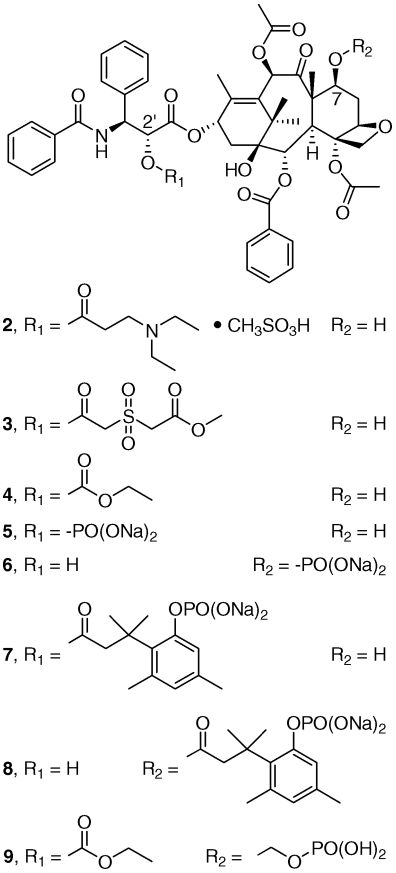


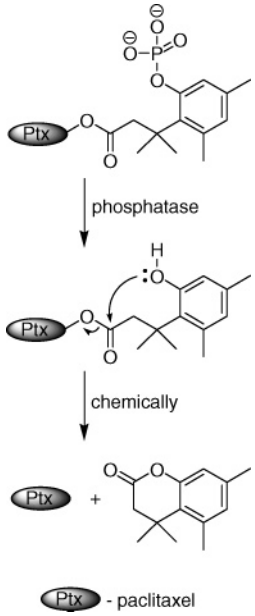
Figure 2. Early designs of paclitaxel prodrugs.

Thus, this Perspective also shows general advances in medicinal chemistry.

Prodrugs of Paclitaxel Possessing Small Molecules as Promoieties

Early Development. The compounds discussed in this section mainly consist of water-soluble derivatives of paclitaxel (**1**). Targeting prodrugs that are not necessarily water-soluble are discussed separately. Because the water solubility of paclitaxel is very low (0.000 25 mg mL^{-1}),[32,33] it is co-injected with Cremophor EL, which causes undesired side effects.[14−16] Early attempts in paclitaxel prodrug design were focused on improving its water solubility to avoid the use of detergents. The first group of such derivatives was synthesized as paclitaxel C-2′ or C-7 esters including succinate, glutarate, sulfonate, and amino acid derivatives.[34−36] Although water solubility of the prodrugs was improved in most cases, serious problems exist with high instability in aqueous media or human plasma for C-2′ derivatives and inability to release the parent drug in C-7 conjugates, along with reduced activity in vivo compared with paclitaxel.[29] The most promising prodrug among these derivatives was 2′-[3-(*N*,*N*-diethylamino)propionyl]paclitaxel **2** (Figure 2), prepared by Stella and co-workers.[36] Compound **2** showed antitumor activity similar to that of **1** against mammary carcinoma xenograft in mice and much higher solubility (>10 mg mL^{-1}). In 1993, Nicolaou and co-workers reported prodrugs, paclitaxel 2′-monoester of dicarboxylic acid, incorporating heteroatom derivatives, exhibiting high stability at neutral pH, and readily releasing paclitaxel under basic conditions.[37] The prodrug design took into consideration the hypothetical basic microenvironment around certain tumor cells. As the most promising agent, they suggested derivative **3** with 1.2 mg mL^{-1} water solubility.

Scheme 1. Activation Mechanism of Derivatives **7** and **8** Based on the *gem* Effect



However, these days, it has been well documented that the tendency is quite the opposite.[38] To be precise, extracellular pH is usually lower in the tumor than normal tissue. The high stability of **3** at pH 7.5 ($t_{1/2}$ > 500 min) may also cause problems in that the time necessary for the release of the parent drug should be sufficient for systemic distribution but not so long as to avoid metabolism, resulting in excretion of the prodrug. Indeed, the authors subsequently reported that these prodrugs were rapidly excreted through the kidneys.[39] Therefore, further studies on **3** were abandoned. Another example of a simple paclitaxel prodrug is carbonate **4**.[40] This compound demonstrated low cytotoxicity in vitro. Indeed, **4** was 10 times less cytotoxic than **1** against human colon cancer cells HCT116. In contrast, upon incubation in plasma, **4** released paclitaxel and demonstrated higher efficacy than **1** in mice when administered at higher doses. However, prodrug **4** was extremely insoluble in water.[29,40] More detailed information about the early designs of paclitaxel prodrugs can be found in the book "The Chemistry and Pharmacology of Taxol and Its Derivatives".[29]

Phosphate Esters. The use of phosphate ester derivatives is one of the most popular strategies in water-soluble prodrug design because phosphate salts can increase the water solubility of prodrugs, and the parent drugs can be released by the action of phosphatases in vivo. Thus, the use of phosphates was one of the earliest approaches by Bristol-Myers Squibb (BMS) toward water-soluble paclitaxel prodrugs.[32] A phosphate group was introduced at the C-2′ or C-7 hydroxyl group in **5** or **6**, respectively. Although these paclitaxel derivatives were more water-soluble (>10 mg mL^{-1}) than **1** in vitro, they remained unconverted both in plasma and against alkaline phosphatases and exhibited no efficacy in vivo. It was suggested that the anionic phosphate moiety was shielded by paclitaxel and could not be accepted as a substrate by phosphatases. To avoid such steric hindrance, BMS researchers proposed the use of a self-immolating linker between paclitaxel and the phosphate moiety. Of interest for future prodrug design, a linker is commonly used to avoid steric interference between enzymes that activated prodrugs and the bulky taxane ring. The BMS prodrug strategy is based on a "trimethyl lock" linker as shown in Scheme 1. Interlocking of the geminal methyl groups (*gem* effect)[41] produces severe conformational restrictions and accelerates

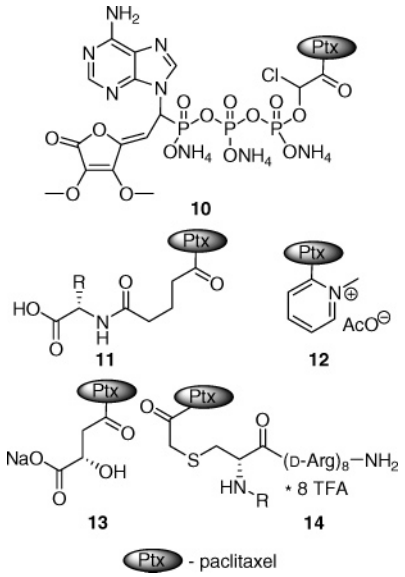


Figure 3. Prodrugs of paclitaxel with auxiliary moiety attached at the 2′ position.

intramolecular cyclization. Thus, the spacer can be released quickly and efficiently. Two potential prodrugs **7** and **8** showed the ability to release the parent drug upon phosphatase activation in vitro.[42] Prodrug **7** showed low in vivo activity, and the activity of conjugate **8** was similar to that of **1** only when it was administered with a 2- to 4-fold higher dose than **1**. The reduced activity of the prodrugs was probably caused by extensive binding to plasma proteins and thereby lacked sufficient activation by phosphatase in vivo. Further studies were conducted by modifying the linker along with combining the phosphate approach and previously reported prodrug **4**.[43,44] Compound **9** was synthesized and showed 1000-fold higher water solubility than **1** with similar in vivo efficacies in mice.[44] Later investigations of paclitaxel 2′-ethylcarbonate (**4**) and related compounds demonstrated that rat serum carboxylesterase was capable of hydrolyzing the carbonate moiety of prodrug **4** in a mouse model of human cancer, and much more of compound **4** remained unconverted in human serum than in rat and mouse sera. These results suggest that the in vivo activities obtained in rodent studies would not reflect the outcome in humans.[40,43−45] Therefore, predicting the antitumor efficacy of prodrugs based only on in vivo mouse or rat models could be inappropriate in regard to the enzymatic mechanism of drug release.[46]

Recently, a new approach also involving the action of phosphatase was reported.[47] The "pro-dual-drug" **10** (Figure 3) contained a potent anticancer agent with very low lipophilicity, triphosphono-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide, using glyoxylic acid as a linker attached to paclitaxel at the 2′ position. Thus, this bifunctional prodrug **10** exhibited about 1000-fold higher lipophilicity than the triphosphonate agent alone and higher water solubility (500-fold) than **1** and therefore possessed a better hydrophilic−lipophilic balance, resulting in superior bioavailability and greater anticancer activity against murine leukemias and breast carcinoma cell lines than both its components separately as well as mixed. The authors also presented data that suggested a lack of involvement of phosphatase in prodrug activation. They proposed a pure chemical release mechanism via hydrolysis or partial involvement of nonspecific esterases during cleavage of the linker, only at the 2′ position of paclitaxel and not between the triphosphonate agent and linker.

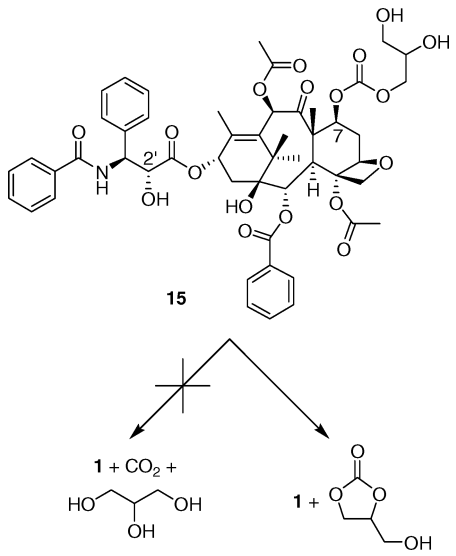
Amino Acids, Sugars, and Other Conjugates. Continued interest in amino acid prodrugs of paclitaxel, despite the aqueous instability problem previously mentioned, led Michel Page and Renée Paradis to apply a linker for the derivatization of paclitaxel at the 2′ position, **11**.[48] Aspartate, glutamate, and lysine were connected to paclitaxel by a glutaryl linker. However, the prodrugs were again too unstable to perform cytotoxicity assays. In contrast, asparagine- and glutamine-derived prodrugs were stable for months in aqueous solution. Thus, these prodrugs were tested against several cancer cell lines and showed potent cytotoxic activity in nanomolar range, although their IC_{50} values were higher than that of the parent drug. In vivo testing was not performed.

Nicolaou and co-workers proposed another modification at the 2′ position. Paclitaxel-2′-methylpyridium acetate (**12**) showed high stability under several aqueous conditions, high water solubility, and fast and complete conversion to the parent drug in plasma.[39] It was not clarified whether the rapid conversion in human plasma is enzyme-assisted. The cytotoxicity of the prodrug was evaluated against a series of cell lines such as ovarian, lung, and breast cancers, leukemia carcinoma, and melanoma cells. The respective IC_{50} values were similar to that of **1**. Moreover, an in vivo study with a prostate tumor xenograft nude mouse model revealed that **12** exhibited tumor growth inhibition equal to that of **1** at the same dose. Further investigations also demonstrated that although **12** did not inhibit tubulin depolymerization in vitro, **12** maintained high activity in vivo.[49] These results were contrary to those shown in paclitaxel-7-methylpyridium acetate, thus clearly suggesting that modification at the 2′ position of paclitaxel led to the prodrug's properties. Moreover, **12** was better tolerated than **1**, with no detectable toxic side effects and no significant weight loss in tumor-bearing nude mice.[49] Surprisingly, the mesylate salt (instead of the acetate salt) of **12**, studied by a group from The University of Kentucky, did not show any prodrug behavior and was not efficiently converted to the parent drug in plasma.[50] A possible explanation for this discrepancy could be the difference in the self-assembled nanostructure between both salts in aqueous media. Self-aggregation of both paclitaxel derivatives was reported.[39,50]

Another approach involved the use of a sugar moiety as a water-soluble auxiliary unit. The derivatization of paclitaxel via a linker with glucose, galactose, mannose, or xylose at the 2′ or 7 position resulted in increased water solubility, but evidence for parent drug release was not presented.[51,52] In 2000, Damen et al. reported a prodrug of paclitaxel with malic acid at the C-2′ position as a water-soluble moiety (**13**).[53] Prodrug **13** demonstrated improved water solubility (0.6 mg mL^{-1}), was stable at pH 7.4, released the parent drug in plasma with a $t_{1/2}$ value of 4 h, and showed in vitro toxicity similar to that of **1**. The maximum tolerated dose (MTD) of **13** was higher than that of **1**. At this MTD, the prodrug had a higher therapeutic index than **1** in p388 tumor model in vivo, and long-term survivors were present. Again, as in most cases with conjugation at the C-7 position, paclitaxel was not released in vivo.

Transporters. Wender and co-workers reported prodrugs of paclitaxel with the application of oligoarginine-based molecular transporters (**14**).[54] Extensive structure−function studies of an HIV-tat transporter sequence (RKKRRQRRR) demonstrated that short oligomers of arginine often exhibit superior membrane translocation activity.[55−57] Thus, instead of simple solubilizing functionalities, a promoiety that can both enhance water solubility and facilitate uptake through the lipophilic bilayer of a cell was introduced. Prodrugs **14** with high water solubility

Scheme 2. Intramolecular Cyclization Mechanism of Paclitaxel (**1**) Released from Prodrug **15**



were synthesized. The prodrugs released the parent drug in a pH-dependent manner; i.e., the stability of the prodrugs decreased with increasing pH. However, experimental proof of improvement in bioavailability of such prodrugs was not demonstrated.[54] Related approaches with polyamine-based transporters have recently been reported in which spermine and penetratin peptides were conjugated to paclitaxel.[58,59] Once again, proof of increased bioavailability of such derivatives over that of **1** was not demonstrated.

A Conjugate at the C-7 Position as a Prodrug. Protaxel (**15**), is a carbonate conjugate of paclitaxel at the C-7 position.[60−62] Its success was unpredicted, as derivatives of paclitaxel at the C-7 position do not usually exhibit prodrug properties. The main problematic points for conjugates at this position have been prolonged half-life for parent drug release and cytotoxicity of the prodrug itself. However, **15** readily released paclitaxel in human serum at 37 °C ($t_{1/2}$ = 10 min). This was explained by a pH-dependent intramolecular cyclization mechanism for parent drug release (Scheme 2). On the basis of this mechanism, a stable stock solution was prepared in acidic pH while the shift to physiological pH resulted in a prompt release of the parent drug. Moreover, prodrug **15** was 2.5−3 times better tolerated than its parent paclitaxel (i.e., higher MTD), which also indicated that the prodrug is much less toxic than **1**. Water solubility of protaxel was 0.05 mg mL^{-1} (200-fold higher than that of paclitaxel). Additionally, **15** showed improved anticancer activity in athymic mice bearing human cell line xenografts of prostate (PC-3, DU-145), ovary (OVCAR-3), and colon (HT-29) cancers over **1** when administered under MTD conditions. Thus, **15** was also effective against multidrug resistant (MDR) xenograft of ovarian and prostate tumors.[60,61] This phenomenon is probably due to the absence of a high peak concentration of the active parent drug, as a result of a slow release from a hydrolytically activated prodrug after intravenous injection that greatly reduced systemic toxicity and allowed administration of substantially higher doses. Prodrug **15** was under pilot clinical trials in Europe and was effective in patients with end-stage cancer, including two complete reemissions and three partial responses in a group of 10 patients treated. Although the study indicated decreased nonselective toxicity of **15** similar to that observed in animal studies, several toxicities involved in this prodrug therapy were observed.[62] Clinical evaluation of **15** seems to have been discontinued because there has been no information about new trials since 2002. The concept of **15** is a good indication that the strategy used for the design of paclitaxel prodrugs can find application for other anticancer agents. An etoposide prodrug with the same auxiliary moiety showed efficacy against multidrug-resistant T-cell leukemia in vitro and in vivo.[63] Therefore, this approach may be a promising strategy for the treatment of MDR-1 malignances.

In general, the prodrugs of paclitaxel described in this section were designed to improve water solubility of the parent drug and to avoid toxic Cremophor EL in the drug formulation or at least to reduce the amount of this detergent in the formulation. However, according to the general nature of prodrugs, the pharmacokinetic profile of paclitaxel can also be improved while reducing side effects. An example of such advantageous application is the aforementioned prodrug **15**. Another advantage of prodrugs found in this group is the relatively low cost of production. However, the release of auxiliary units (promoieties) used in all strategies in this section may cause some undesired side effects.[17] Therefore, potential side effects of the auxiliary units should also be evaluated. Alternatively, safe and nontoxic auxiliary units already approved by registration authorities should be used. It should, however, be noted that even the commonly used pivalic acid showed some toxicity associated with changes in carnitine homeostasis.[64] Moreover, phosphate groups were reported to cause hypocalcemic effects, and special cautions are implemented, for example, for patients with renal impairment.[65] Also, formaldehyde that is released upon hydrolysis of some linkers can be a toxicological concern.[17]

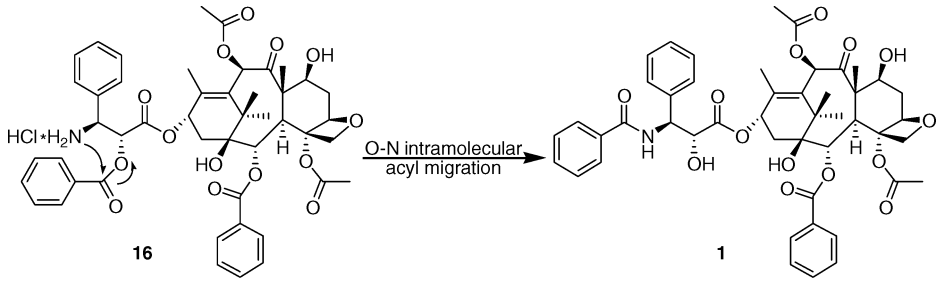
According to the historical development of prodrug **15** and despite over 20 years of research that has been carried out on prodrugs of paclitaxel possessing small molecules as promoieties, there is not a single prodrug in advanced clinical trials. Advantages coming from simple water-soluble paclitaxel prodrugs seem to be insufficient to prompt further development of these compounds for clinical use.

Prodrugs of Paclitaxel without a Promoiety

Bioprecursors are defined as prodrugs that do not contain a promoiety and are activated by a biosynthetic reaction.[17,18] In contrast, auxiliary units are used in all other prodrug strategies, and the release of these moieties may cause some undesired side effects.[17,18] Hence, when the use of additional moieties is avoided, prodrug design becomes more promising. This would be an advantage in toxicology and general pharmacology, and the cost for the evaluation of auxiliary units could be saved during prodrug development.

Among the large number of paclitaxel prodrugs, there is no apparent bioprecursor. However, although it is not activated via biosynthetic reaction, one compound can be classified as a precursor that is activated under physiological conditions by a simple chemical mechanism. Kiso and co-workers developed isotaxel **16**, a 2′-*O*-acyl isoform of paclitaxel **1**.[33,66] This prodrug showed 1800-fold higher water solubility (0.45 mg mL^{-1}) over **1** and released the parent drug via an O−N intramolecular acyl migration reaction without any side reactions under physiological conditions (Scheme 3). The production of the parent drug was pH-dependent (pH 7.4, 37 °C, $t_{1/2}$ = 15 min), while the prodrug was stable in solid form and under acidic conditions, which suggested a practical injection condition for clinical use. Generally, the pH-dependent action mechanisms and solubility and kinetics of parent drug release from isotaxel were similar to those of protaxel (**15**). The major difference between **15** and **16**, and the advantage for **16**, was the lack of any auxiliary unit for solubilization. On the basis of these promising results, the

Scheme 3. O−N Intramolecular Acyl Migration Mechanism of Paclitaxel (**1**) Released from the Prodrug Isotaxel (**16**)



O−N intramolecular acyl migration strategy was successfully expanded to a number of taxoids that are active against MDR tumors, as they all possess the α-hydroxy-β-amino acid moiety necessary for migration.[67,68] In addition, it demonstrated for the first time the successful application of the O−N intramolecular alkoxycarbonyl migration reaction in prodrug design.[67,68] However, an in vivo study has not yet been performed to confirm the advantage of O−N intramolecular migration reaction for prodrug strategy.

Prodrugs Designed toward Targeting Tumor Tissue

This section includes prodrugs designed to be specifically delivered to tumor tissues. Although both polymer- and antibody-conjugated derivatives of paclitaxel have general targeting properties, they will be discussed separately. Tumor-targeting prodrugs are designed to achieve a high local concentration of the antitumor drugs to decrease undesired side effects caused by non-tissue selectivity of the drugs. In fact, lack of paclitaxel selectivity is one of the serious drawbacks in conventional cancer chemotherapy. To activate the prodrugs specifically at the tumor tissues, the enzyme involved in prodrug activation must be selectively present in or near the target tissue or the tumor should selectively take up the prodrugs.[27,46] A problem with tumor-specific prodrugs is that cancer cells do not contain any molecular targets (enzyme, receptor, etc.) that are completely discrete from the host. Fortunately, there are several phenotypic differences between tumor and normal tissues such as the overexpression of certain enzymes that can be used for prodrug targeting.[46,69]

Targeting Tumor Overexpressed Enzymes. One of the possible target enzymes is neuraminidase (sialidase), which is overexpressed in cancer cells.[70] Takahashi and co-workers proposed a water-soluble conjugate of sialic acid via a self-immolative linker (1,6-elimination spacer) to paclitaxel at the C-7 position as a potential neuraminidase cleavable prodrug. However, proof of enzyme-dependent parent drug release was not provided. The conjugate induced microtubule assembly without activation and showed 10-fold lower IC_{50} value in vitro than paclitaxel.[71] Another protease, plasmin, plays a key role in tumor invasion and is overproduced at the surface of cancer cells. Active plasmin is rapidly inhibited in blood circulation. Therefore, this serine protease is a tumor-specific target for prodrug chemotherapy.[72] Scheeren's group designed several prodrugs, including lead derivative **17** (Figure 4), which exhibited plasmin-dependent parent drug release, whereas the prodrugs showed reduced cytotoxicity in various tumor cell lines without activation by plasmin.[73] Prodrug **17** showed high stability in Tris buffer (pH 7.3) and readily released paclitaxel once exposed to plasmin ($t_{1/2}$ = 42 min). Interestingly, in the case of another prodrug **18**, plasmin promptly hydrolyzed a tripeptide moiety in the prodrug ($t_{1/2}$ = 3.5 min) probably because of a less bulky and more flexible spacer.[73] However, after enzymatic hydrolysis of the tripeptide, the spacer was still attached to paclitaxel and its elimination time to release paclitaxel was very long, with a half-life of 23 h. Thus, the drug−linker intermediate might diffuse from the target tissues before the parent drug could be released.[74] Therefore, proper selection of a spacer is also very important in tumor-specific prodrug design.

Targeting Hypoxia. Hypoxia, a phenomenon of very low oxygen concentration in tissue, is a specific feature observed in most solid tumors. While hypoxia is increasingly recognized as a limiting factor in successful treatment of solid tumors by conventional radio- and chemotherapy, its unique occurrence in such tumors is also recognized as a target for possible specific prodrug activation.[72,75−78] Bioreductive paclitaxel prodrugs have been investigated to preferentially target hypoxic cells in tumors.[79,80] These prodrugs were constructed in a manner in which reductive activation, catalyzed by reductive endogenous enzymes, led to paclitaxel release. Scheeren's group reported such prodrugs employing aromatic nitro groups as bioreductive triggers.[79] Paclitaxel was released upon reduction of the nitro group to an amino or hydroxylamino group and upon subsequent 1,6-elimination of a 4-amino or 4-hydroxylamino benzyloxycarbonyl moiety, respectively. The most promising prodrug (**19**) demonstrated significant reduced cytotoxicity against several human tumor cell lines. Although cytotoxicity after hypoxic activation was not demonstrated, the release of paclitaxel after chemical reduction was confirmed.[79] Because the cleavage of a disulfide bond is a facile biochemical transformation under a reductive environment, unsymmetrical disulfide prodrugs consisting of both 2,2-dimethyl-4-mercaptobutyric acid and a hydrophilic unit were reported by Vrudhula et al.[80] The α-geminal dimethyl group in the linker was introduced to improve stability toward serum esterases, as well as facilitate cyclization of the intermediate thiol leading to paclitaxel formation due to the *gem* effect.[41] The captopril moiety, known to exhibit antiangiogenic effects,[81] was utilized as a hydrophilic unit in the design of lead prodrug **20**. The prodrug showed 650-fold lower toxicity than **1** and released paclitaxel after reductive activation by dithiothreitol. In an in vivo study with L2987 lung carcinoma implanted in nude mice, compound **20** at a dose of 125 mg kg^{-1} (its MTD) demonstrated marked tumor regression or even complete cure, whereas paclitaxel did not regress tumors at its MTD (30 mg kg^{-1}). The observed antitumor effect might be attributed to the antiangiogenic effects of captopril combined with the cytotoxic effects of paclitaxel because **20** served as a prodrug of both paclitaxel and captopril.

Antibody-Directed Enzyme Prodrug Therapy (ADEPT). Tumor targeting focused on native human enzymes overexpressed in tumor tissues has potential disadvantages. These enzymes are also present at lower concentrations in normal tissues. The substrate link to the parent drug might also be prematurely cleaved by nonspecific enzymes. The concentrations of targeting enzymes in tumor tissue might be insufficient to activate the prodrug. One potential strategy to

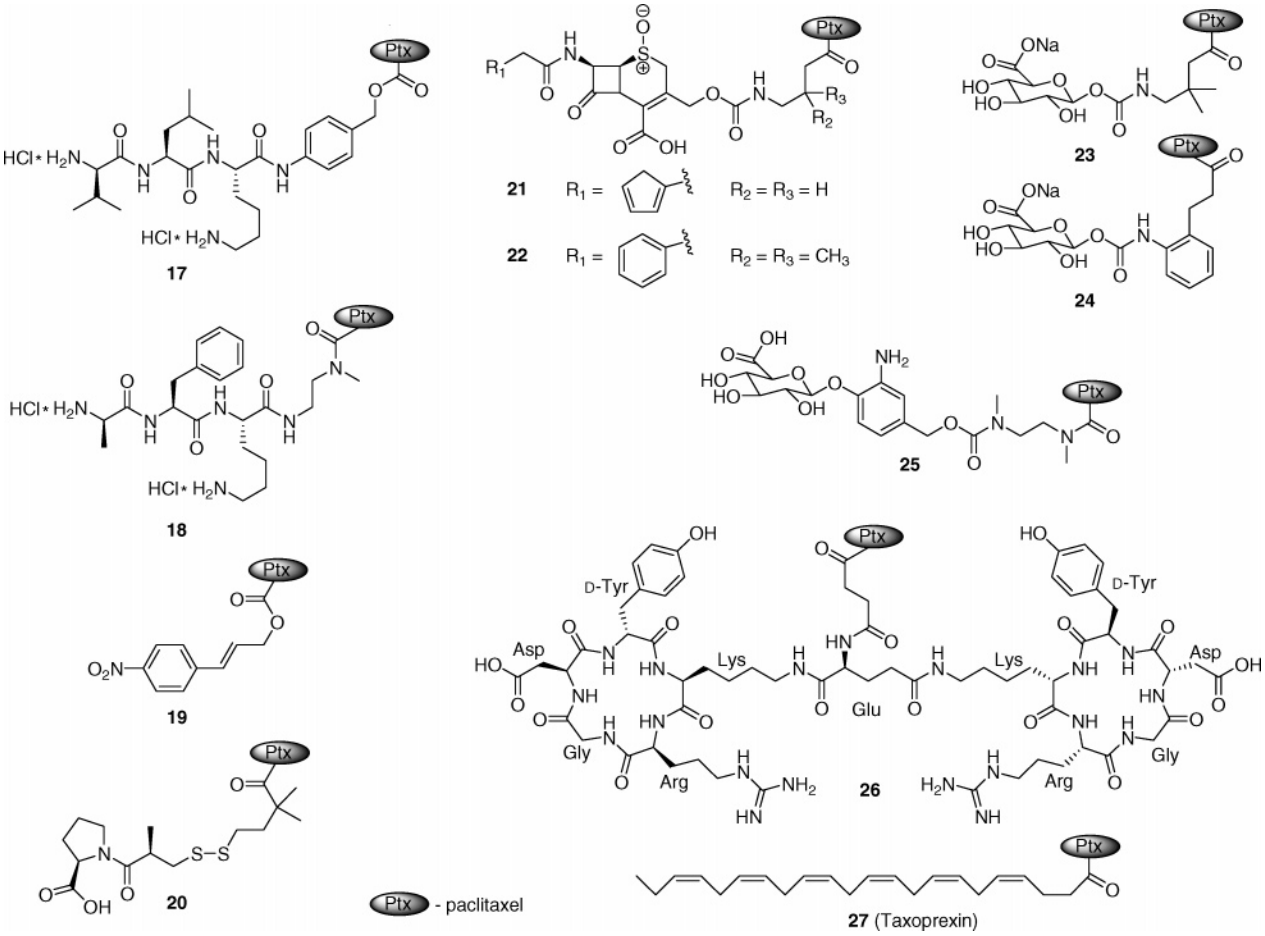


Figure 4. Tumor targeting prodrugs of paclitaxel. An auxiliary moiety is attached to paclitaxel at the 2′ position.

overcome these problems is antibody-directed enzyme prodrug therapy (ADEPT).[82,83] A two-step approach is embodied in ADEPT. In the first step, antibody conjugated with a prodrug-activating enzyme is directed against a tumor-specific antigen located on the surface of tumor cells. In the second step, a nontoxic prodrug, a substrate of the antibody-conjugated enzyme delivered to the tumor tissue in the first step, is administered systemically. Both enzyme and prodrug should fulfill certain requirements in this strategy. In the ideal case, the enzyme either should be of nonhuman origin or is inaccessible to the prodrug if the same enzyme is present in normal human tissue. The prodrug should be a good substrate for such an antibody−enzyme conjugate and not be activated by endogenous enzymes in normal tissues. Also, a high concentration of tumor-specific antigen expressed at the tumor tissue is crucial for a successful application of this strategy. The first paclitaxel prodrug designed for the ADEPT strategy (**21**) used a cephalosporin linked via an aminobutyryl linker to paclitaxel at the C-2′ position.[84] Prodrug **21** is activated by β-lactamase, which is delivered as an enzyme−antibody construct to the tumor tissues, followed by self-elimination of the linker. Although toxicity was clearly reduced before activation of the prodrug and restored after activation, the drug release rate was inadequate. In spite of very fast β-lactamase-dependent activation of the prodrug, over 16 h was needed for complete paclitaxel release because the linker−paclitaxel intermediate was not readily severed. During this process, the intermediate could have diffused away from the tumor site. Therefore, the goal of targeting would not be achieved. To overcome this problem, researchers from Bristol-Myers Squibb designed a modified linker. The use of the *gem* effect (Scheme 1) led them to the development of a promising β-lactamase dependent prodrug **22**.[85] This prodrug was stable for a long time in human serum ($t_{1/2}$ = 350 min), and paclitaxel was released 7-fold faster in the presence of the enzyme ($t_{1/2}$ = 50 min), which is comparatively several-fold faster than in prodrug **21**. However, the rate-limiting step for parent drug release from the cephalosporin conjugate was still in the linker cyclization reaction. In vitro, **22** was found to be 12-fold less toxic than **1** on 3667 melanoma cells while maintaining similar activity to **1** when melanoma cells were first saturated with monoclonal-antibody(MAb)−β-lactamase fusion protein L-49-sFv-bL. In addition, the use of nonhuman enzyme, such as β-lactamase, has a potential risk of immune response in vivo. On the other hand, human β-glucuronidase may be a good candidate for tumor-specific prodrug conversion. Increased activity of this enzyme in tumor tissue as opposed to normal tissue has been reported.[86] In addition, the ADEPT strategy can be employed to further increase the levels of β-glucuronidase in tumors. Scheeren and co-workers designed and synthesized paclitaxel prodrugs that were expected to be activated by human β-glucoronidase in the ADEPT strategy.[87] These prodrugs (**23** and **24**) showed higher water solubility and stability under aqueous conditions. The cytotoxicity of the prodrugs was 2 orders of magnitude less than that of paclitaxel, and upon activation by human β-glucuronidase, cytotoxicity was restored almost to paclitaxel's level. The enzyme-catalyzed half-lives for prodrugs **23** and **24** were 2 h and 45 min, respectively. Intermediates bearing a spacer moiety attached to paclitaxel were not detected. However, these glucuronide prodrugs of paclitaxel might not be suitable even for ADEPT because the enzyme concentration required for activation had to be high (10 μg mL^{-1}). Scheeren and co-workers suggested that longer spacers

may solve the problem associated with inefficient hydrolysis.[74,86] The same problem of high β-glucuronidase concentration requirement for prodrug activation was noted by Monneret and co-workers.[88] They designed a prodrug of paclitaxel with a linker that allowed movement of the glucuronic moiety away from the taxane core for easier accessibility to the enzyme.[89] Prodrug **25** was stable in phosphate buffer at 37 °C (pH 7.2). During enzymatic assay, paclitaxel was released from **25**, via a briefly stable intermediate (paclitaxel−linker conjugate), with a short half-life (10 min) at an enzyme concentration of 2.5 μg mL^{-1}. Recently, further modifications of the linker in **25** to develop more efficient β-glucuronidase activated prodrug was studied.[90] The relatively small number of purpose-oriented prodrugs tested so far leaves the ADEPT strategy ready for further development.[82]

Targeting Tumor Overexpressed Receptors. In rapidly dividing cells such as cancer cells, membrane receptors are often overexpressed and thus can be differentially targeted.[91] Numerous investigations have examined the use of paclitaxel conjugated directly to receptor ligands, as a targeting prodrug strategy. Safavy et al. reported paclitaxel conjugated via 2′-succinate−poly(ethylene glycol) (PEG) to a synthetic heptapeptide BBN-[7-13] (QWAVGHLN-NH_2): **1**−PEGBBN[7-13].[92] BBN[7-13] binds to the cell surface bombebsin/gastrin-releasing peptide receptor, which was found to be associated with tumor growth.[93,94] This water-soluble prodrug (250 mg mL^{-1}) showed high binding affinity to the receptor and released paclitaxel in human plasma ($t_{1/2}$ = 113 min). The prodrug showed higher potency against NCI-H1299 human non-small-cell lung cancer (IC_{50} = 6 nM) compared to **1** (IC_{50} = 15 nM) under the same conditions. The fact that **1**−PEGBBN[7-13] showed improved cytotoxicity suggested a specific receptor-mediated delivery of paclitaxel to the tumor cells. This anticipation was recently confirmed.[95] Despite these promising results, the linear peptide may be vulnerable to endogenous proteases in vivo. Another overexpressed receptor by tumor cells is the receptor for the neuropeptide somatostatin.[93,94] A prodrug targeting for this receptor included an analogue of somatostatin that had improved metabolic stability (H_2N-D-Phe-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-Thr-ol) that was linked to paclitaxel at the 2′ position via a succinate spacer.[96] Using a fluorescein-labeled analogue of somatostatin, the authors first demonstrated that this fluorescein−peptide analogue was selectively recognized by the receptor and internalized via endocytosis into tumor cells. The same result was later observed in the case of the prodrug. Moreover, the authors demonstrated that the prodrug, but not free paclitaxel, was exclusively toxic to somatostatin receptor-expressing tumor cells. A constitutive peptide receptor (α_v integrin receptor), which is highly overexpressed in metastatic cancer cells, was recently chosen as a target for specific delivery of the prodrug by Chen and co-workers.[97] Designed integrin-targeting prodrug **26** improved tumor specificity and the cytotoxic effect of paclitaxel in experiments with nude mice bearing MDA-MB-435 breast carcinoma, which is known to overexpress integrin $\alpha_v\beta_3$. This resulted in a lower systemic dosage and toxicity to obtain the same anitumor efficacy as paclitaxel. The prodrug also induced tumor cell apoptosis greater than that of paclitaxel. The authors claimed that further evaluations of **26** in a preclinical study were in progress. About a decade ago, researchers identified a folate receptor (FR) as an antigen that was significantly overexpressed in a broad spectrum of human cancers and served as a target for tumor selective therapy.[98,99] Several prodrugs were designed with folic acid, a natural vitamin B that exhibits high affinity for FR, conjugated to paclitaxel at the C-2′ and/or C-7 position via a short PEG linker. Although the lead paclitaxel−folate conjugates retained most of the receptor-binding affinity of the folate ligand and were less toxic than **1** in normal mice, they failed to demonstrate selective killing of folate receptor-expressing tumor cells in vitro and could not reach similar in vivo antitumor activity as **1**, when administered in an equimolar quantity formulated in the same injection vehicle.[100] One of the possible reasons explaining the ineffectiveness of the prodrug could be the slow release of **1** under acidic pH ($t_{1/2}$ = 197 h at pH 5) because upon binding to the cell surface receptor, the prodrug would most likely be internalized into endosomes where the pH is around 5. Similar unpromising results were reported in paclitaxel conjugates designed to target breast tumor-related estrogen receptor.[101]

A Photoresponsive Prodrug. Another approach using the concept of photodynamic therapy and caged compound chemistry was demonstrated by Kiso and co-workers. They propose a photoresponsive prodrug, (7-*N*,*N*-diethylamino-4-hydroxymethylcoumarin) linked via a 3′-carbamate to **16**.[102] The prodrug was activated by visible light laser irradiation (430 nm), resulting in the cleavage of the coumarin derivative and liberating intermediate **16**. Paclitaxel was released by subsequent spontaneous O−N intramolecular acyl migration. Tumor tissue targeting for this prodrug after its administration could be achieved by selective light delivery, for example, by utilizing an endoscope.

Prodrugs in Combination with Targeting Formulation. In addition to prodrug strategies used to improve paclitaxel properties, the formulation of paclitaxel has also been studied. Emulsification, micellization, liposome formation, and the like have been used not only for improving the solubility of paclitaxel but also for tumor targeting; for example, polymer micelles are convenient passive targeting carrier systems of anticancer drugs.[14] Prodrugs with high lipophilicity are favored for incorporating paclitaxel into lipid carriers, leading to improved pharmacokinetic profiles in such a drug delivery system. On the basis of this fact, a formulation−prodrug system targeting folate receptors was recently investigated.[103] Paclitaxel−2′-carbonylcholesterol was synthesized and incorporated into lipid nanoparticles that included folate−PEG−cholesterol as a receptor-targeting moiety.[104] This prodrug formulation showed promising targeting properties in vitro as well as in vivo. The same formulation loaded with **1** did not show any therapeutic advantage. The enhanced antitumor efficacy of the formulation−prodrug system might be a consequence of increased prodrug lipophilicity and stability of prodrug inclusion in the formulation. These would provide more time for nanoparticles to reach their target sites prior to releasing paclitaxel from the prodrug−formulation. Similar results were recently reported for fullerene conjugated to paclitaxel in a liposome formulation.[105] Mayhew and co-workers reported 2′-α-bromohexadecanoyl paclitaxel as a hydrolyzable prodrug. Hydrolysis of the prodrug was facilitated by the bromine atom at the α position of the acyl chain.[106−108] This prodrug showed improved activity in vivo against subcutaneously (sc) grown OVCAR-3 human ovarian cancer over paclitaxel when administered with Cremophor EL. Improved therapeutic effect could be explained by the selective accumulation of bromohexadecanoyl prodrug in tumor tissues. Alternatively, because of the slow release mechanism of paclitaxel from the prodrug, sustained levels of paclitaxel may be available to tumor cells for prolonged periods.[106] In further investigations, Mayhew and co-workers abandoned toxic Cremophor EL and proposed a new formulation.[107,108] The liposomal formulation of this prodrug demon-

strated higher efficacy than paclitaxel in the treatment of human ovarian tumor xenograft in mice.[108] Another lipophilic paclitaxel derivative reported was 2′-oleate paclitaxel incorporated into a lipid emulsion.[109,110] This prodrug−lipid emulsion, which was further covered with a hydrophilic polymer, improved the pharmacokinetic parameters of paclitaxel.[109] When the derivative is incorporated into a microemulsion targeted against a tumor-overexpressed low-density lipoprotein receptor, the stability of this formulation was improved when compared with that of paclitaxel alone.[110]

A Docosahexaenoic Acid−Paclitaxel Conjugate. A unique example of a lipophilic prodrug is the docosahexaenoic acid (DHA)−paclitaxel conjugate (**27**).[111−115] DHA is found in human milk, added to infant formula, and classified as a nutritional additive by the FDA. It has been suggested that some fatty acids are taken up by tumors from arterial blood, presumably for use as biochemical precursors and energy sources. Conjugation with DHA was expected to change the pharmacokinetic profile of paclitaxel, allowing prolonged exposure of tumor cells to paclitaxel and reduced peak drug concentrations, thereby allowing administration of the prodrug at higher concentrations. Protarga Inc. chose DHA for the design of paclitaxel targeting prodrug **27**.[111−114] The prodrug was formulated with 80% less Cremophor EL than the paclitaxel formulation. Compound **27** demonstrated significant enhanced tumor distribution and antitumor activity in various tumor models compared with paclitaxel in equitoxic or equimolar doses.[112] Prodrug **27** had no microtubule assembly activity in cell-free solution and no cytotoxic activity until metabolized to the active molecule of paclitaxel. Compound **27** was converted to paclitaxel at an apparent higher level in tumor than in plasma. In the Madison 109 tumor model, **27** caused a complete regression of the tumor at a dose of 120 mg kg^{-1}, whereas tumor regression was not observed after administration of paclitaxel at an equitoxic dose of 20 mg kg^{-1}. In addition, **27** was a 4-fold weaker substrate for P-glycoprotein than **1** and thus may be active in some drug-resistant tumors that overexpress P-glycoprotein. In vitro, 99.6% of **27** was found to be bound to human plasma, which might explain in part the unique pharmacokinetic profile of the prodrug.[113] Prodrug **27** also showed promising results in a phase I clinical study alone[114] and in combination with alkylating anticancer agent carboplatin.[115] Generally, the prodrug has been shown to have mild side effects (no hypersensitivity, no hair loss, and mild nausea). Recently, **27** reached phase II clinical investigation for advanced skin melanoma and advanced eye melanoma and reached phase III for advanced non-small-cell lung cancer (http://www.clinicaltrials.gov/). Closed clinical trials included phase II for metastatic hormone-refractory prostate cancer, metastatic marcinoma of the pancreas, advanced metastatic or unresectable renal cell cancer, and metastatic colorectal cancer (http://www.cancer.gov). Taking into account the number of clinical trials against wide ranges of tumors, there are hopes and expectations that this prodrug would finally reach clinical use.

To improve current chemotherapeutic treatments and diminish severe side effects, the concept of targeting prodrugs aimed at achieving site-specific delivery of paclitaxel is of special interest. This section of the Perspective concentrates on the development of small-molecule promoieties, carrying paclitaxel prodrugs, designed for direct recognition of tumor-associated factors, such as hypoxia, tumor-overexpressed enzymes, and receptors. Although the detailed mechanism of tumor targeting of DHA is not clear,[27] fatty acid-conjugated prodrugs such as **27** seem to be very promising. The ADEPT strategy, the most complicated approach in this Perspective, is highly promising. However, ADEPT is costly and has so far shown low effectiveness in human.[46] The development of targeting prodrugs of paclitaxel is a relatively new approach for improving the antitumor efficacy of the parent drug with the majority of results just published during the past few years. Further investigations are needed to improve the targeting properties of such prodrugs, including optimization of the substrates for tumor-associated enzymes or of the ligands for tumor-overexpressed receptors, amelioration of the effectiveness of linkers, choice of appropriate enzymes for ADEPT therapy, and so on. Therefore, access to clinically available targeting prodrugs of paclitaxel is not likely to occur in the very near future, with maybe the exception of promising prodrug **27**. Nevertheless, expected advantages for the applications of targeting prodrugs in antitumor therapy, especially in the case of successfully overcoming existing problems, are numerous.

Macromolecular Prodrugs

Macromolecules have been traditionally employed as drug carriers in cancer chemotherapy.[116] The most common feature of macromolecular prodrugs is their ability to accumulate in tumor tissues because of an enhanced permeability and retention (EPR) effect.[117,118] This selective accumulation is related to the nature of tumor tissues: large molecules are easily transported into tumor tissues; tumor vascular permeability is high; lack of effective lymphatic drainage prevents the macromolecules or macromolecular prodrugs from being removed.[119] For such passive targeting, polymeric auxiliary units are usually used.

1. Polymer Conjugates. 1.1. Poly(ethylene glycol). One of the most popular conjugation polymers for prodrug delivery is poly(ethylene glycol) (PEG).[25] PEG is a highly water-soluble nontoxic polymer that is eliminated from the body by renal and hepatic pathways and has been approved for human application by the FDA.[25] The first PEG−prodrugs of paclitaxel were published by Greenwald et al.[120] The 5 kDa polymer prodrug **28** (Figure 5) showed high water solubility (660 mg mL^{-1}) and maintained in vitro activity similar to that of paclitaxel. This prodrug was further evaluated in an in vivo study against P388 leukemia but demonstrated lower activity than paclitaxel.[121] Even in in vitro studies, the conjugation of PEG with paclitaxel at the C-7 position was not effective because the produced derivative could not be hydrolyzed. In contrast, a 40 kDa PEG prodrug conjugated at the C-2′ position showed slightly improved activity over paclitaxel in an in vivo experiment.[121,122] Similar results were reported by Wallace and co-workers.[123] These results clearly indicated the necessity of in vivo experiments to verify in vitro cytotoxicity results. The data presented were also in agreement with the fact that the higher molecular weight polymers (∼40 kDa and above) enhanced circulation time and improved entrapment of the prodrugs in tumor tissues by EPR.[119] In a similar manner as described for lipophilic prodrugs, the PEG−prodrug of paclitaxel was also used to improve the pharmacokinetic parameters of liposomal formulation of paclitaxel.[124] Further studies on PEG−paclitaxel conjugates mainly concentrated on the development of efficient linker systems for properly releasing paclitaxel from the prodrug. Yuan and co-workers tested several amino acids as spacers.[125] They claimed that their prodrugs demonstrated remarkable reduced side effects, enhanced in vitro cytotoxicity, and comparable in vivo antitumor activity to the native paclitaxel. However, it is difficult to evaluate these results because information on the size of these PEG conjugates was not disclosed.

Maleimide derivatives of paclitaxel, in which an acid-sensitive carboxylic hydrazone linker was incorporated, were prepared

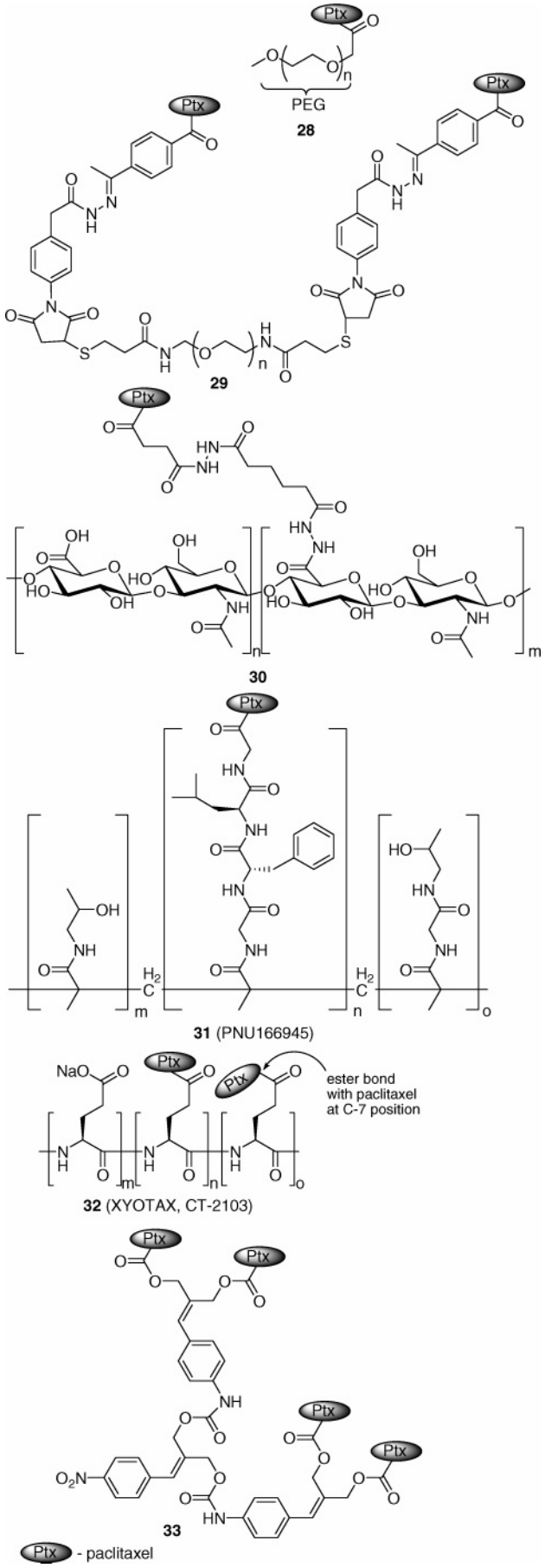


Figure 5. Macromolecular paclitaxel prodrugs. An auxiliary moiety is attached to paclitaxel at the 2′ position.

and coupled to bifunctional PEGs (20 kDa).[126] The incorporation of an acid-sensitive spacer between the drug and polymer enabled the release of an active drug from the carrier in tumor tissues, either in slightly acidic extracellular fluid or, after endocytosis, in the endosomes or lysosomes of cancer cells.[127] The authors expected that 20 kDa would be a sufficiently high molecular weight for the PEG−prodrug to target tumor cells by the EPR effect. Although representative conjugate **29** demonstrated pH-dependent stability and showed a 5- to 20-fold decrease in activity compared with that of the free drug against three human tumor cell lines, in the cell-conditioned medium paclitaxel was not released because the linker was not completely hydrolyzed. Therefore, it was not clear whether free paclitaxel was the cytotoxic agent in the in vitro experiment.[126] Schoenmakers et al. reported a study on the effect of the linker on the hydrolysis rate of PEG−linker−phenylisoserine conjugates. However, only model compounds were presented without any evidence that their model pharmokinetics would be maintained for the real prodrug.[128] Another group performed similar studies using polylactic acid as a linker.[129] Choi and co-workers investigated new methods to enhance paclitaxel bioavailability. Paclitaxel has low oral absorption because of its low solubility and high elimination by the multidrug efflux transporter P-glycoprotein, which is abundant in the gastrointestinal tract. They demonstrated that PEG conjugation with paclitaxel at the C-7 position improved oral bioavailability.[130−132]

1.2. Hyaluronic Acid. Hyaluronic acid (HA), a human polysaccharide, is elevated in various tumor tissues. This biopolymer is recognized by specific receptors that are often overexpressed by cancer cells. HA is immunoneutral, and its structure allows multiconjugation with a therapeutic agent via free carboxylic groups instead of only two attachment sites as in the case with PEG. This makes HA a promising polymer for tumor targeting drug conjugation.[27] Prestwich and co-workers designed a conjugation of 11 kDa HA−paclitaxel (**30**) with a paclitaxel loading rate of 1−15%.[133,134] It was demonstrated that **30** was internalized into cancer cells via receptor-mediated endocytosis, and then paclitaxel was released intracellularly. The prodrug showed specific and effective toxicity against breast, ovarian, and colon cancer cells that overexpressed HA receptors, and higher cytotoxicity was observed as paclitaxel loading was increased. However, at 15% loading, the cytotoxicity of the highly modified HA with paclitaxel was decreased. This is probably caused by blockade of the HA receptor recognition sites by paclitaxel moieties. Recently, an interesting possible application of an HA prodrug for biomedical implants was demonstrated.[135] The prodrug was prepared by linking paclitaxel to HA via a labile succinate−ethylenediamine spacer and incorporated in a layer-by-layer assembled polyelectrolyte multilayers system with chitosan. The authors reported that this delivery platform is expected to show improved properties, such as stability, when compared with inclusions of free paclitaxel into the multilayers. The release of paclitaxel from the paclitaxel-loaded multilayers upon hydrolysis of the ester linkage resulted in drastic cell death.

1.3. *N*-(2-Hydroxypropyl)methacrylamide. Another drug carrier widely employed as an anticancer agent conjugate is an *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer. The HPMA copolymer is a biocompatible, almost nonimmunogenic, nontoxic, and targetable carrier, to which drugs are usually bound via a peptidyl spacer, Gly-Phe-Leu-Gly. The parent drug is released by the action of thiol-dependent lysosomal proteases, particularly cathepsins.[136,137] Such a polymer-conjugated prodrug of paclitaxel, **31** (PNU 166945), was evaluated in a phase I

clinical trial.[138] In contrast to a free paclitaxel formulation, hypersensitivity reaction was not observed when this water-soluble prodrug was administered without Cremophor EL. Prodrug **31** showed a high MTD (in dogs 250 mg m^{-2}). In a human study with 12 patients, one partial remission after a dose of 100 mg mL^{-1} was observed in a case of advanced breast cancer. However, at the same time, during the course of the clinical study, the result of a 13-week toxicity study in rats indicated severe neurotoxicity. Moreover, one patient developed grade 3 neurotoxicity, and therefore, any further study was discontinued.

1.4. Carboxymethylcyclodextran and Others. Asahi Kasei Corporation reported a series of paclitaxel conjugates at the 2′ position with a high molecular weight carboxymethylcyclodextran (150 kDa) attached via several amino acid spacers.[139] A lead prodrug, with glycine as a linker, exhibited high water solubility (>100 mg mL^{-1} as a paclitaxel equivalent), slow conversion to the parent drug in mouse plasma ($t_{1/2} \approx 24$ h), higher MTD than free paclitaxel, and accumulation in tumor tissues. A paclitaxel-resistant tumor mouse model (colon 26) was chosen for an in vivo study. In comparison with **1**, the prodrug caused significant tumor growth delay and did not decrease mouse body weight. These results demonstrated that passive targeting is advantageous probably because of the EPR effect, and even MDR tumors can be successfully treated with a polymeric prodrug. Other similar polymers have been employed as auxiliary moieties in paclitaxel prodrug design.[140−142]

1.5. Poly(L-glutamic acid). One of the most successful prodrugs has been designed by conjugating paclitaxel with poly(L-glutamic acid) (PG). This polymer is water-soluble, nonimmunogenic, and biodegradable to its nontoxic amino acid component (L-Glu) and contains a large number of side chain carboxylic groups for drug attachment.[143] Li et al. reported paclitaxel prodrug **32** in which paclitaxel was directly conjugated to 36 kDa PG (without any spacer) at mixed positions C-2′(mostly) and C-7.[144,145] Prodrug **32** contained 20% paclitaxel by weight, demonstrated high water solubility (100 mg mL^{-1}), had a very low release of paclitaxel in plasma (<0.1% in 144 h), was better tolerated than paclitaxel, and could therefore be administered with higher MTD. A single dose of the prodrug produced complete regression of well-established murine ovarian OCa-1 and rat breast 13762F tumors.[144] Prodrug **32** possessed therapeutic potential against a variety of others solid tumors, including paclitaxel-resistant tumors.[145] Further investigations revealed slow release of paclitaxel from the prodrug in tumor tissues and increased the therapeutic ratio as a result of enhanced biodistribution to tumor tissues via the EPR effect.[146−148] Poliglumex (CT-2103), which evolved from **32**, is an approximately 80 kDa polymeric conjugate at the 2′ position of paclitaxel with an *m*/*n* ratio of around 10 (Figure 5), corresponding to 37% paclitaxel content by weight. Other prodrugs with deviations in formula, i.e., paclitaxel content and size of polymer, were also reported to have excellent antitumor activity.[148−150] Poliglumex also demonstrated high efficiency in clinical investigations[151−156] and has been recently evaluated or is under evaluation in clinical trials from phase I up to III, alone or in combination therapy, against several different cancer types including ovarian, non-small-cell lung, and breast cancer (http://www.clinicaltrials.gov/). Moreover, combined treatment of **32** and radiation therapy produced greater tumor growth delay without any effect on normal tissue injury, compared with treatment with radiotherapy and **1** or compared with only chemotherapy with prodrug.[157−160] Overall, clinical trials indicated that **32** has antitumor efficacy comparable to that of **1** with reduced side effects, such as neutropenia, hair loss, and alopecia and allowed a more convenient administration schedule without the need for routine premedications. In June 2006, Cell Therapeutic, Inc. announced its plans to file a marketing authorization application in Europe in the first half of 2007 for **32** as a single agent for first-line treatment of non-small-cell lung cancer with equivalent effectiveness (noninferiority) and superior safety using existing phase III clinical trials data (http://www.cticseattle.com/).

2. Protein Conjugates. Not only polymers but also proteins can be used in targeted delivery of macromolecular prodrugs. Serum proteins offer promises of selective delivery of anticancer agents because of their accumulation in tumor tissues.[161,162] Transferrin is a serum glycoprotein involved in iron transport and also acts in cell growth regulation through a membrane receptor. Transferrin can be used in a targeting prodrug strategy because the number of transferrin receptors is increased in tumor cells.[91] Indeed, the first-reported membrane receptor targeting prodrug of paclitaxel was a paclitaxel conjugate via 2′-glutarylhexanediamine to transferrin. However, only cytotoxicity tests were reported. Antiproliferation activity in H69 cells of this conjugation was 5.4-fold lower than that of paclitaxel.[163] Human serum albumin has also been applied for the design of targeting paclitaxel prodrugs.[164,165] However, clear advantages for this strategy have not been demonstrated.

3. Dendrimers. Another type of macromolecular prodrug is a conjugate of paclitaxel and a dendrimer. Dendrimers are versatile, well-defined, treelike chemical polymers with sizes and physicochemical properties resembling those of biomolecules, e.g., proteins.[166] Scheeren et al. reported prodrugs of paclitaxel, "cascade-release dendrimers", that have been built to completely and rapidly dissociate into separate building blocks (including a parent drug) upon a single triggering event in the dendritic core.[167] Therefore, by a single action of a specific factor in the tumor tissue, a multiple number of drug molecules could be released, which is advantageous for targeting prodrugs. Although this strategy was demonstrated in small dendrimers, it was expected that such a design could extend to dendrimers of sufficient sizes for passive targeting to tumor tissues by the EPR effect. Representative prodrug **33**, a modified compound from previously described prodrug **19**, was subjected to reduction of its nitro group with Zn in AcOH to initiate a chain reaction for releasing paclitaxel. Chromatographical analysis indicated a rapid and complete disappearance of the starting compound and formation of paclitaxel under reductive conditions. The potential for **33** in tumor targeting has not yet been exploited.

The utilization of macromolecules as a targetable drug delivery platform has been the focus of extensive studies. The EPR effect provided an excellent targeting phenomenon for such prodrugs. Conjugations can be produced at relatively low cost by using of biocompatible, nonimmunogenic, and toxic polymers or other "friendly" macromolecules. In this context, the use of high molecular weight polymers was particularly advantageous, while small molecular weight polymers conjugated to paclitaxel only led to simple water-soluble prodrugs without EPR targetable properties. The pharmacokinetic profile of the parent drug released from the prodrug is an important factor. Conjugation must be stable so that liberation of the parent drug is predominantly or specifically only in tumor cells. In addition, the slow-release mechanism of the drug inside tumor cells should allow long-lasting cytotoxic action upon a single dose of the prodrug, thereby improving the therapeutic effects of the anticancer agent.

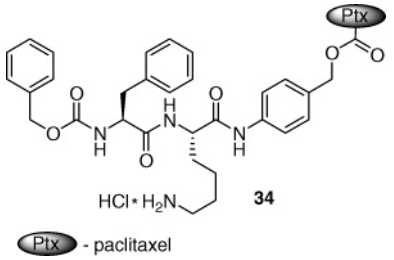


Figure 6. Prodrug designed for antibody conjugation. An auxiliary moiety is attached to paclitaxel at the 2′ position.

Paclitaxel−Antibody Conjugates

The discovery of antigens that are selectively overexpressed on the surface of cancer cells suggested that the targeted delivery of a prodrug with an auxiliary unit that can distinguish malignant tissues from normal tissues could be possible. Strategies using receptor-targeted prodrug and antibody-directed enzyme prodrug therapy (ADEPT) were discussed previously. However, monoclonal antibodies (mAbs) could also be used as vehicles to selectively deliver cytotoxic agents to tumor cells by a simple cross-linking method. The mAb moiety can specifically recognize and bind to its corresponding antigen on the tumor cell surface, and then the mAb−drug conjugate can be internalized via endocytosis followed by the release of the parent drug. However, it should be noted that not all antibodies are internalizing proteins. The efficacy of immunoconjugates as chemotherapeutic agents is strictly dependent on the tumor specificity of mAb; the potency of the cytotoxic agent, which is related to the therapeutic dose; and the efficiency of the linker that connects mAb to the drug.[26−28] The linker must be both stable in circulation and efficiently cleaved inside cancer cells.

In one approach, lysosomal cathepsin B was chosen as a parent drug-releasing trigger.[168] Prodrug **34** (Figure 6) was designed with the conjugation of a cathepsin B-sensitive dipeptide Phe-Lys derivative via a self-immolating spacer[169] to paclitaxel.[170] Prodrug **34** was stable in human plasma, while the parent drug was released specifically in the presence of cathepsin B but with an excessively long half-life ($t_{1/2}$ = 9.0 h). In contrast, paclitaxel was released much more quickly in rat liver lysosomes ($t_{1/2}$ = 19 min). Experiments with prodrug **34** linked to mAb have not been performed. Saragovi and co-workers reported the conjugation of paclitaxel at the 2′ position with a mAb MC192 via a labile glutaryl group in equimolar ratio. This antibody has been developed against a receptor for nerve growth factor, TrkA.[171] The TrkA receptor is expressed on normal cells in low density, and overexpressed in many types of cancer cells.[172] The paclitaxel−MC192 prodrug afforded selective toxicity toward cells expressing the TrkA receptor and was more cytotoxic in vitro than equimolar concentrations of free paclitaxel, or free paclitaxel plus free mAb. In an in vivo model of xenografted tumors, although the prodrug prevented tumor growth and prolonged survival time of mice over the free drug, systemic administration of the prodrug showed limited efficacy in reducing tumor growth. Safavy and co-workers reported the synthesis and biological evaluation of paclitaxel conjugates to the antiepidermal growth factor receptor (EGFR) mAb C225, in which succinic acid was used as the drug−mAb linker.[173] EGFR is known to be overexpressed in several tumors, and the antibody C225 itself can be used as an antitumor agent.[174] Improved cytotoxicity was observed for the prodrug when compared with **1**. Although antibody C225 and the prodrug showed high antitumor activity, there was no significant advantage of the conjugate over C225 alone. The authors hypothesized that the reason could be nonselective in vivo paclitaxel release, as a result of the low stability of the succinate linker. Indeed, experiments performed in DU-145 tumor-implanted nude mice demonstrated that the time needed for paclitaxel cleavage from the succinic acid-linked prodrug was shorter than that for C225 tumor localization. A glutaric acid-linked conjugate showed a significantly longer drug release mechanism, and compared with previously performed tests on the succinate conjugate, noticeable improved antitumor activity was observed with the glutarate in an in vivo experiment.[175] A prodrug reported by Correa and Page in which paclitaxel was conjugated to an antibody via a PEG linker has not demonstrated significant advantages of such conjugation over paclitaxel itself.[176] The unexpected failure of these paclitaxel−antibody prodrugs could have been caused by nonspecific activation of the linker outside the tumor cells or the insufficiently low inherent cytotoxicity of paclitaxel itself. The cytotoxic agents used in the immunoconjugates should be exceedingly cytotoxic because only a limited number of molecules can be loaded on each mAb without diminishing the binding affinity of the antibody, and a limited amount of antigens is overexpressed on the tumor cell surface.[27,28] Indeed, Ojima and co-workers have reported extremely potent antibody conjugates using highly cytotoxic paclitaxel derivatives that were linked to mAbs via a disulfide linker that was specifically cleaved by tumors.[27,28,177]

Although the required cytotoxicity of a drug used in the immunoconjugate is believed to be in the 10−100 pM range (∼IC_{50}) and paclitaxel is unfortunately at least 10-fold less active,[28] there is still hope for paclitaxel−antibody conjugation. Improved pharmacokinetics of paclitaxel released from the prodrug by using an efficient linker and the use of highly loaded paclitaxel polymers conjugated to the antibody can drastically improve the properties of the prodrugs. Moreover, such conjugates may also demonstrate targeting by the EPR effect and be safer than the conjugates of an antibody and an extremely cytotoxic agent. A very toxic agent with imperfect targeting properties may cause severe side effects.

Conclusion

This Perspective presents various advantages and achievements for paclitaxel prodrug strategies including carrier linked prodrugs, bioprecursors, site-specific chemical delivery systems, macromolecular prodrugs, and paclitaxel antibody conjugates. Paclitaxel prodrug approaches have shown a bright future for the development of anticancer therapy for several reasons: the use of toxic Cremophor EL can be omitted, and selectivity against tumors can be improved while side effects are reduced. This would allow higher equivalent doses of paclitaxel in cancer treatment (higher MTD), and the prodrugs can be effective in cases of paclitaxel-resistant tumors. Because the therapeutic index of an established antitumor agent, paclitaxel, is improved, the prodrug strategy avoids some substantial risks associated with the development of completely new drugs. A few prodrugs of paclitaxel have already reached clinical trials, and **27** and **32**, both in phase III clinical trials, are currently the most promising. However, as it stood in June 2006, there are almost 800 phase III clinical trials reported by the U.S. National Institutes of Health on the development of new anticancer drugs (http://www.cancer.gov). Only 11 oncology drugs were approved by the FDA in 2005. Therefore, prodrugs **27** and **32**, which did not show a higher therapeutic effect than **1**, may have obstacles to surmount prior to FDA approval. On the other hand, these compounds showed considerably reduced side effects compared to the parent drug and thus can be much more attractive for patients in terms of quality of life.

Despite the advantages of the prodrug strategies, they can be criticized in the same manner as the application of new formulations to known drugs.[178] Prodrugs are generally more complex, more expensive, and often less stable than parent drugs. Problems with resistance against paclitaxel in some tumor types may not be so easily overcome by the prodrug strategy. Improved efficacy of the prodrugs over paclitaxel, as demonstrated in preclinical study, has not been confirmed in clinical trials. However, detailed investigations in this field can triumph over the drawbacks. Studies on targeting moieties and linker behavior can improve the stability as well as efficiency of the paclitaxel prodrugs. In spite of the cost of prodrug treatment, which can be higher than that of the free drug, the overall cost for the cure can be lower. As examples, prodrug dosing could be less frequent than that of paclitaxel and premedication could be omitted. Potential therapeutic benefits and improvements in quality of life of the patients can also be sufficiently worthy of the higher expenses. Moreover, a promising prodrug strategy developed once for paclitaxel may quickly be applied for other anticancer agents, maybe with even better results.

As demonstrated in this Perspective, many challenging problems in the design and development of paclitaxel prodrugs were successfully overcome. However, still more research is needed to be done to develop really effective and clinically acceptable prodrugs of paclitaxel. However, there is hope that prodrugs with more advanced properties, such as those with receptor-specific peptides, those that are activated by tumor overexpressed enzymes, and those with paclitaxel conjugated to polymers, should make significant contributions to anticancer chemotherapy in the near future.

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Biographies

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Yoshio Hayashi received his Ph.D. in Peptide Chemistry in 1990 from Kyoto University under the supervision of Prof. Haruaki Yajima and Prof. Nobutaka Fujii. From 1986, he worked for Calpis Corporation, and from 1988, he worked for Life Science Research Center, Nippon Steel Corporation. In 1999, he moved to Kyoto Pharmaceutical University as a lecturer and in 2001 was promoted to Associate Professor. He has been working on peptidomimetic-related medicinal chemistry based on antiplatelet, anticancer agents, and protease inhibitors as well as chemical pharmaceutics for water-soluble prodrugs of peptidomimetics and paclitaxel.

Yoshiaki Kiso received his Ph.D. in 1974 from Kyoto University. In 1975, he joined Professor K. Hofmann's group at The University of Pittsburgh School of Medicine as Research Associate. In 1977, he was appointed Associate Professor at the Faculty of Pharmaceutical Sciences, University of Tokushima. In 1983, he moved to Kyoto Pharmaceutical University as Full Professor. He received The Society Award of Japanese Peptide Society and Cathay Award and served as Dean of Graduate School of Pharmaceutical Sciences. He is currently Director of Center for Frontier Research in Medicinal Science, President of Japanese Peptide Society, and Program Leader of Kyoto Pharmaceutical University 21st Century COE (Center of Excellence) "Development of Drug Discovery Frontier Integrated from Tradition to Proteome".

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