

Review

Improved biochemical strategies for targeted delivery of taxoids

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Dr. David G. I. Kingston for his outstanding contributions in natural products and paclitaxel research.

Abstract—Paclitaxel (Taxol®) and docetaxel (Taxotere®) are very important anti-tumor drugs in clinical use for cancer. However, their clinical utility is limited due to systemic toxicity, low solubility and inactivity against drug resistant tumors. To improve chemotherapeutic levels of these drugs, it would be highly desirable to design strategies which bypass the above limitations. In this respect various prodrug and drug targeting strategies have been envisioned either to improve oral bioavailability or tumor specific delivery of taxoids. Abnormal properties of cancer cells with respect to normal cells have guided in designing of these protocols. This review article records the designed biochemical strategies and their biological efficacies as potential taxoid chemotherapeutics. © 2007 Elsevier Ltd. All rights reserved.

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1. Introduction

The diterpenoid natural product paclitaxel (PTX, Taxol®, **1**) and its semi-synthetic analog docetaxel (DTX,

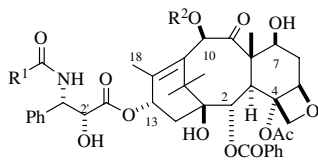
Taxotere®, **2**) are the leading anticancer drugs in clinical use today.^{1,2} Taxoids (PTX and DTX) bind to tubulin polymer in a stoichiometric ratio and promote tubulin polymerization. This phenomenon completely disrupts

Abbreviations: EEDQ, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; DCC, *N,N'*-dicyclohexylcarbodiimide; DMAP, 4-*N,N*-dimethylaminopyridine; DIC, DIPC, *N,N'*-diisopropylcarbodiimide; EDCI, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride; PEG, poly(ethylene glycol); DMF, *N,N*-dimethylformamide; DMSO, dimethylsulfoxide; TBAF, tetrabutyl ammonium fluoride; TBSCl, *tert*-butyldimethylsilyl chloride; TIPSCl, triisopropylsilyl chloride.

Keywords: Taxoid; Prodrug; Drug conjugate; Drug targeting; Receptor; Antibody; TPT; ADEPT; Colloidal gold nanoparticles; Cancer cells.

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tubulin polymerization dynamics, leading to cell cycle arrest at the G₂/M phase and thence to apoptosis.^{3,4} These two taxoid drugs were approved by the FDA for the treatment of several carcinomas including breast, advanced ovarian, non small cell lung (NSCLC), head and neck, colon, and AIDS-related Kaposi's sarcoma.⁵ The combined annual sales of these two taxoids were well over \$1 billion in the year 2001.⁶ While these taxoids perform well in the clinic for various types of cancer, several combination therapies of these drugs together with other anti-cancer agents that operate by different mechanisms of action to these taxoids (e.g., cisplatin and doxorubicin) have been explored to unravel synergistic effects that could lead to more effective therapy for tumor malignancies.⁷ These two taxoid drugs have also become the benchmark chemotherapeutic agents to evaluate the *in vitro* and *in vivo* efficacy of several newly discovered anticancer natural products such as the epothilones,⁸ discodermolide,⁹ eluetherobin,¹⁰ laulimalide,¹¹ peloruside,¹² and others that have a similar mechanism of action to that of PTX.



1: R¹ = Ph, R² = Ac
2: R¹ = Me₃CO, R² = H

Despite the successful performance exhibited by these taxoids for several cancers, their utility in the clinic is hampered by severe limitations such as: poor aqueous solubility, non-selective toxicity to tumor cells, and inactivity against drug resistant (MDR) cell lines.¹³ Due to the poor water solubility of PTX (**1**), it had to be formulated and administered with Cremophor EL[®] (polyethoxylated castor oil and ethanol, 50:50) as a surfactant. Unfortunately, this formulation caused severe adverse allergic reactions due to histamine release¹⁴ and hypersensitivity reactions.¹⁵ Although DTX (**2**) was more soluble in water than PTX,¹⁶ it still had to be formulated with 15% dry ethanolic solution containing 80% polysorbate for clinical use. In an effort to eliminate the solubility issues associated with these taxoids, significantly more water soluble taxoids have been developed, some of which have entered into the preclinical development.^{17,18}

The second limitation exhibited by these taxoids is their systemic toxicity, due to their non-selective cytotoxicity action on tumor cells over normal cells. This property resulted in severe adverse side effects, including bone marrow suppression, febrile neutropenia, neurotoxicities, mucositis, ulceration to mouth, throat, as well as a variety of other cardiac abnormalities.¹⁹ This undesirable toxicity has restricted the administration and dose levels, which often lead to incomplete tumor eradication.

The ideal way to eliminate the side effects caused by these taxoids would be to target them to the tumor site, in the right amount and also at the right time by means of a 'magic bullet'. The success of this strategy would have significant implications for future chemotherapy, such as the elimination of systemic toxicity and an increased bioavailability of drug to the tumor, thus leading to a lower dose of drug being required. To achieve this goal it is essential to recognize the intrinsic physiological and morphological differences between normal and tumor cells. Fortunately some broad differences in cancer cells have been observed, such as a higher metabolic rate when compared to normal cells. This then leads to a hypoxic environment in tumor cells, which in turn induces anaerobic metabolism that ultimately leads to a lower pH in the cancer cells. Cancer cells also consume more primary metabolites, due to rapid cell proliferation. For this to occur, cancer cells overexpress several receptors that attract several primary metabolites (such as peptides, polysaccharides, fatty acids, etc.). Cancer cells also possess leaky vasculature that allows small molecules to enter tumor cells much more rapidly than normal cells.²⁰ Tumors of breast and ovarian cancers also overexpress steroid hormone receptors which have specific interactions with the female hormone estrone.

Based on the above broad differences, several taxoid prodrug and targeted delivery protocols have been developed, and some of them have yielded better anti-cancer properties compared to the parent compound. Some of them have already entered into the preclinical development. In this article, we examine the prodrug strategies that have been explored to improve the oral bioavailability, and targeting methods which were developed for tumor specific delivery of the taxoids (PTX and DTX). For the purpose of this review, the protocols are divided into two classes namely, prodrug protocols and drug targeting protocols.

Prodrugs in general consist of a taxoid with a hydrolyzable and or a self immolative linker unit that would release the active drug at the tumor cells under the influence of the tumor environment such as pH or bio-reductive agents present in the cancer cells or a structurally modified inactive drug that rearranges to an active drug upon reaching the cancer cell (Fig. 1, path A). On the other hand, drug delivery protocol makes use of tumor markers' affinity to certain ligands. The drug delivery would be realized by a drug conjugate which is made up of a cytotoxic drug (e.g., taxoid) connected directly or through a linker to a tumor recognizing agent (Fig. 1, path B). The tumor recognizing agent could be a hormone, peptide, polysaccharide, enzyme, vitamin, gold nanoparticle, fatty acid, antibody, etc. that have a certain affinity to tumor cells. Ideally, the drug conjugate should have the following properties: selective binding affinity to cancer cells and essentially non-toxic to normal cells, stability throughout the blood stream, and upon reaching to cancer cell, it should be cleaved readily to release the active drug.

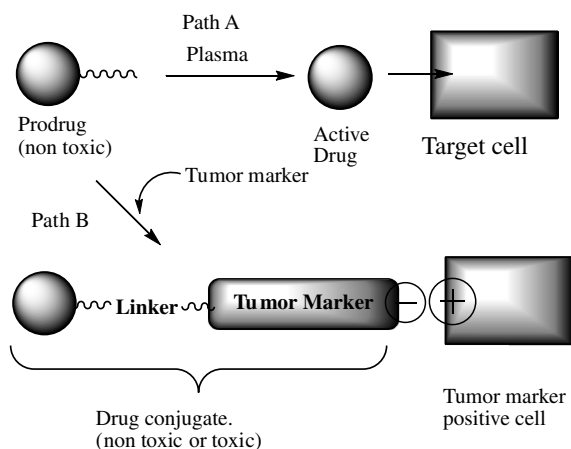


Figure 1. Schematic representation of prodrug and drug conjugate delivery to tumor cells.

2. Taxoid prodrug protocols

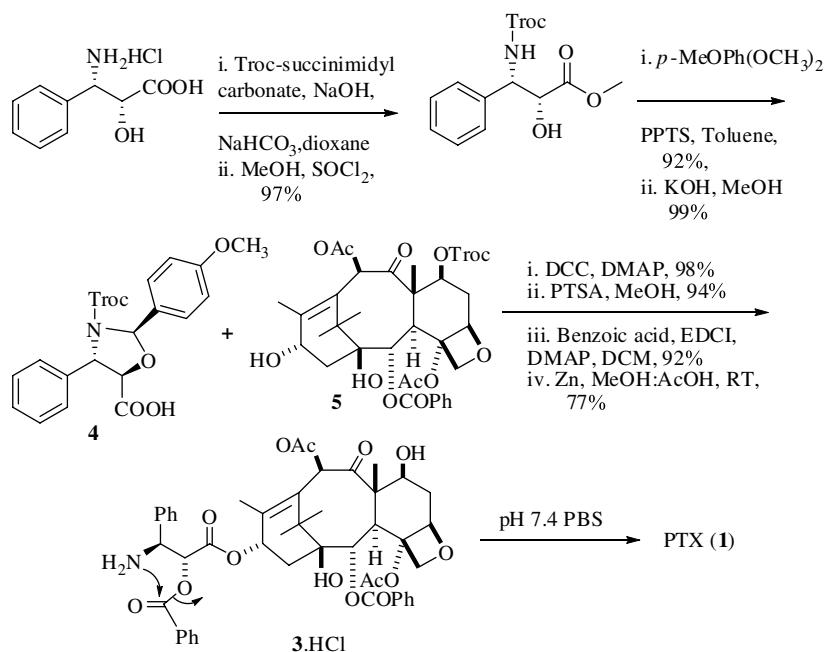
As outlined in the introduction, an ideal prodrug should have better aqueous solubility, prolong the circulation time, release the active drug only at the tumor site and also should not generate any byproducts which might show unpleasant properties. In this respect several prodrug protocols have been envisioned, based on the premise that when the prodrug is passing by the tumor site, the physiological conditions of the cancer cells would cause release of the active drug.

A noteworthy point is that a majority of the taxoid prodrugs or drug conjugates are generated by linking to the C2'-OH position of PTX or DTX.²¹ The SAR studies²² and pharmacophore modeling analysis²³ have determined that a free C2'-OH is essential for the bioactivity

of taxoids, and either esterification or derivatization of this C2'-OH could lead to inactive compounds. This is a requisite property of a prodrug. Additionally, C2'-OH derivatives (esters, with carbamates, etc.) are found to be much more accessible to various enzymes in the body and are able to undergo hydrolysis to release active drug. In few cases derivatives by alterations at C10-OAc (OH) and C7-OH of the taxoids have also been reported, however, these derivatives may or may not be able to release active taxoids, because they were found to be relatively more stable compared to C2'-esters of taxoids.²⁴

We begin with the recent report by Kiso et al.²⁵ who synthesized a water soluble PTX derivative called isotaxel (**3**) as shown in Scheme 1. The synthesis was accomplished in a convergent manner utilizing the troc-protected derivatives **4** and **5**, respectively, which were prepared from a phenylisoserine and baccatin III starting materials, using DCC as a coupling reagent in the crucial step. This prodrug **3** has no additional water soluble functionalities and would not generate any byproduct during its conversion to parent drug PTX, but would merely undergo a rearrangement to produce the active drug PTX. Remarkably the isotaxel (**3**) showed 1800-fold greater aqueous solubility, due to the ionized amino group. This prodrug was also found to be stable in the pH range 2–5. Very importantly this prodrug released PTX at physiological pH 7.4 with a half-life ($T_{1/2}$) of 15 min. This time was almost within the suggested systemic distribution period of the drug. However, no in vivo efficacy of this isotaxel has been reported.

Recently, Scheeren et al.²⁶ reported few attractive PTX prodrugs with malic acid attached at the C2' as well as the C7 positions. The synthesis of prodrug **6** as well as its sodium salt **7** and also **8** was achieved



Scheme 1.

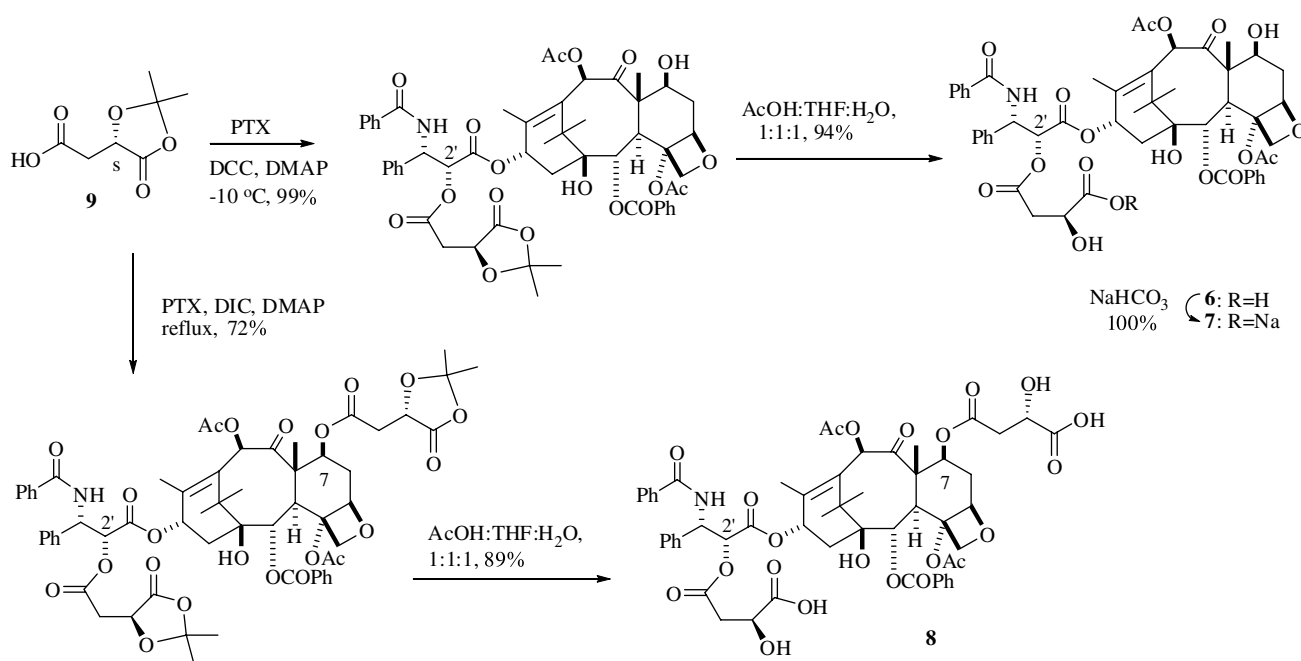
starting from a protected malic acid **9** and PTX (**1**), respectively, using the similar diimide mediated coupling, as shown in Scheme 2. Gratifyingly, all the prodrugs **6–8** exhibited 20- to 60-fold improved water solubility when compared to PTX and are stable during 48-h incubation in PBS buffer at pH 7.4 at 37 °C. No detectable amount of PTX was liberated from these prodrugs in the above time period. Interestingly, compound **6** and its sodium salt **7** generated PTX when they were incubated in human plasma, however the dimalic acid prodrug **8** did not produce any PTX under similar conditions. The latter result is consistent with the fact that C7-esters of PTX are much more stable than C2'-esters, because the C7 position of the PTX is in a hindered position and thus would not be exposed to external biological agents. Nevertheless the sodium salt **7** showed a half-life ($T_{1/2}$) of 4 h in human plasma, suggesting that it would increase the circulation time of PTX in the body.

The prodrugs **6–8** were investigated for in vitro cytotoxicity against the MCF-7 and EVSA-T breast cancer cell lines (Table 1). Prodrugs **6** and **7** showed similar activity to PTX, whereas the dimalic acid PTX prodrug **8** was much less active than PTX. The bioactivity results are consistent with the above stability results encountered with this C7 ester prodrug **8**, so its reduced activity can be attributed to its inability to release PTX in cell culture medium. The prodrug **7** was further evaluated for in vivo bioactivity against murine leukemia P388 tumor model, and it was found to cause dose-dependent increase of survival time. Optimum results were obtained with the compound injected at 25 mg/kg, and the results were significantly better than those with PTX. In this test no mouse survived after 30 days period with PTX, whereas there were 2/6 survivors after 45 days with compound **7**.

Table 1. Aqueous solubility, stability in plasma, and in vitro cytotoxicities of prodrugs **6–8**

Compound	Water Solubility mg/ml	$T_{1/2}$ (h)		IC ₅₀ (ng/mL)	
		pH 7	Plasma	MCF-7	EVSA-T
PTX	0.01			<3	<3
6	0.2	>24	20	<3	<3
7	0.6	No PTX	4	<3	<3
8	0.5	No PTX	No PTX	69	59

To maximize drug bioavailability, and minimize the systemic toxicity poised by the cytotoxic drug, numerous drug delivery systems (DDS), such as polymeric micelles,²⁷ colloidal nanoparticles,²⁸ dendrimers,²⁹ aerosol,³⁰ and liposomes,³¹ have been sought with an effort to enhance the circulation time of the drug in plasma.³² These agents would protect the drug from plasma-induced transformations and also transport an adequate amount of drug to the appropriate site.³¹ Particularly relevant to the present topic are liposomes. These colloidal particles are made of biodegradable natural material, found to be non-toxic to the body, and composed of a lipid bilayer surrounding the aqueous pocket making a capsule-like carrier. Liposomes could carry both hydrophobic as well as hydrophilic molecules to the appropriate site. However, these drug delivery systems are recognized by mononuclear phagocytic system (MPS) and are thus trapped by the reticuloendothelial system (RES). In this process various plasma proteins and enzymes were found to be involved and to enhance the clearance of liposomes from the site, while releasing the encapsulated molecule at the site. To circumvent this rapid clearance of liposomes by the RES, various modified liposomal alternatives, especially with polyethylene glycol (PEG), glucuronic acid derivatives, palmitoyl-D-glucuronide (PGLcUA), were developed. All



Scheme 2.

these liposomal delivery systems did indeed improve the circulation time and prevented RES-mediated trapping and clearance.³³

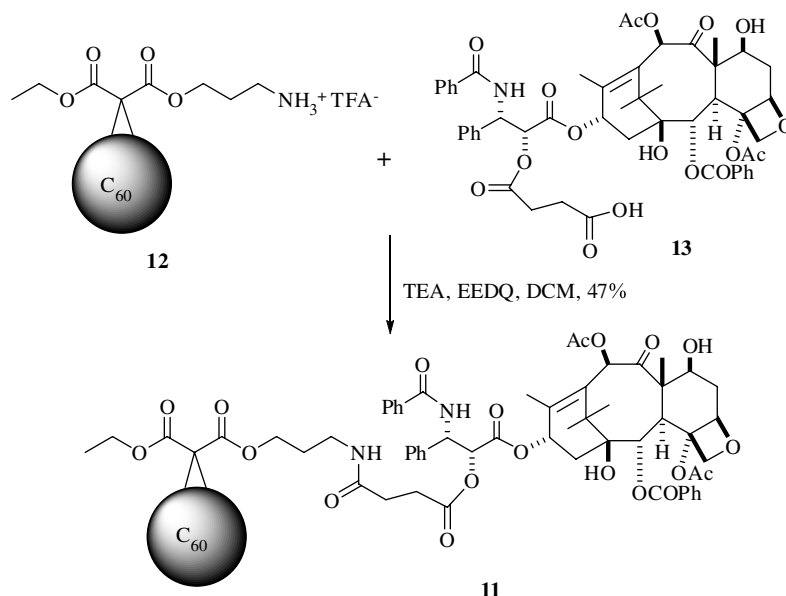
It is worth pointing out here that the size and charge of the liposomes could be controlled during their formulation, depending upon the requirements poised by the potential drug. Previously, various cytotoxic drugs were administered for lung cancer treatment by liposomes as a delivery vehicle.³⁴ This method has several advantages over other methods of drug administration such as increasing the drug concentration in the lungs, and has lower dosage requirements with overall reduced systemic toxicity.³⁵ However, by this method the active drug would be released at once at the tumor site, which often causes the drug clearance by efflux pumps. In this respect, a slow releasing prodrug would have greater impact than a single bolus delivery.

Recently, Wilson et al.³⁶ envisioned that a C₆₀ fullerene modified liposome could plausibly deliver the PTX in slow release fashion at the lungs, since fullerenes are found to be biologically stable and have the potential to form a slow drug releasing system. Thus the PTX prodrug **11**, with glutaroyl and amino ether linkages between a C₆₀ fullerene and PTX, was synthesized by an EEDQ-mediated coupling strategy between **12** and **13**, which were in turn prepared from a C₆₀ fullerene and PTX, respectively, as shown in Scheme 3. The prodrug **11** was investigated to determine its efficacies. First the biodistribution was measured comparing with parent drug PTX. These results indicated that **11** increases PTX aggregation in aqueous (10% DMSO) solution. The prodrug **11** was determined to be stable at physiological pH, however when it was incubated with bovine plasma at 37 °C, it released PTX with a half-life of 80 min. If **11** exhibits a similar half-life in the lungs, it could enhance PTX exposure to lungs at least 4-fold as compared with the previously measured half-life of

PTX in lungs (20 min) when the drug was delivered by aerosol.³⁵ Compound **11** was investigated for liposome formulation, and its antitumor activity against A549 (human epithelial lung carcinoma) cells was determined as a liposome suspension. The results show that it forms a stable liposome suspension with dilauroyl-phosphatidylcholine (DLPC), and this suspension did not let PTX precipitate. The cytotoxicity data of **11**-DLPC, PTX-DLPC, and intermediates **12**-, **13**-DLPC were measured, and the results indicated that the **11**-DLPC has almost equal potency to a PTX-DLPC formulation, with IC₅₀ values of 410 and 253 nM, respectively. In this study, the intermediates **12** with DLPC and DLPC alone were found to be inactive, clearly indicating the C₆₀ fullerene role in PTX delivery to the lung cancer cells. However, additional in vivo biological results are necessary to fully determine the efficacy of the prodrug **11**.

As stated previously, many solid tumors have enhanced metabolic rates that cause hypoxia (oxygen deficiency), a phenomenon that could activate several biological enzymes to metabolize bio-reductive prodrugs into toxic drugs. This metabolism occurs preferentially in absence of oxygen. Hypoxia also leads to anaerobic metabolism resulting in the formation of excess lactic acid and consequently lowering the intracellular pH.³⁷ This is a distinguishing property of cancer cells, and presents a tool for designing prodrugs that could conceivably be cleaved, by low pH environment to release the active drugs upon reaching the cancer cells. In this respect several attempts have been made to design taxoid prodrugs that could use the hypoxic environment of tumor cells to generate the bioactive taxoid drug.

For example, Scheeren et al.³⁸ have synthesized several PTX analogs, a typical structure is represented as **15**, which is a mixed carbonate derivative formed between C2'-OH of the PTX and *p*-nitrocinnamyl alcohol. The



Scheme 3.

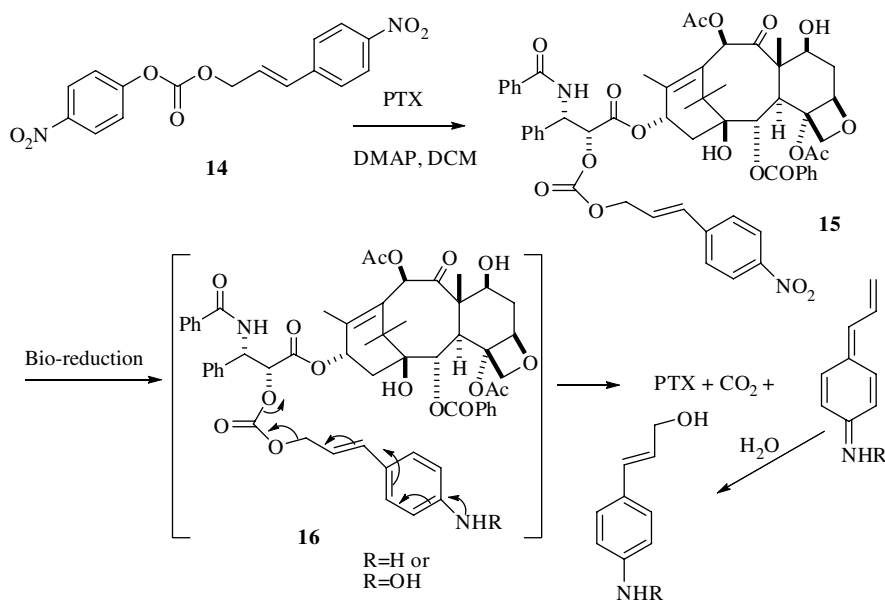
synthesis of **15** was achieved from the reactive mixed carbonate **14** and PTX. Conceptually, the nitro group is expected to be reduced to amino derivative **16** by bio-reductive agents present in the hypoxic conditions in the cell (Scheme 4). Once the amino group is formed, it would undergo self-immolation (electronic reorganization) to release the active PTX. In this study, several analogs similar to **15** were synthesized (varying aryl groups in place of *p*-nitrocinnamyl). Among them only **15** showed improved properties such as decreased cytotoxicity toward various cell lines with respect to PTX. All other derivatives (not shown) synthesized in this study showed almost equal cytotoxicity to PTX, because they were less stable and have released the PTX by esterases present in the assay cell culture. Only **15** showed less cytotoxicity against various cancer cell lines when compared to the parent drug PTX (Table 2), and it was stable for 24 h in the pH 7 buffered solution at 37 °C. Reduction of **15** by chemical methods such as Zn/AcOH/MeOH resulted in the release of PTX, suggesting it could be a potential prodrug, however, no detailed in vivo efficacies of this prodrug have been reported to date.

Along similar lines Vrudhula et al.³⁹ have synthesized several C2'-ester-disulfide PTX derivatives (**17a–c**) (Scheme 5), based on the similar notion that the disulfide bond could be reduced to free thiol in the hypoxic environment of the tumor site. Subsequently the free thiol intermediate would undergo a self-immolative

cyclization to release the bioactive PTX. The synthesis of disulfide prodrugs was accomplished as shown in Scheme 5, by reaction of excess of thioglucose, glutathione, and captopril separately with pyridyl disulfide-2'-O-PTX ester (**18**) under basic conditions to furnish **17a–c**, respectively.

After having synthesized the prodrugs **17a–c** and **18**, they examined whether these prodrugs could release PTX in a reductive environment. Thus, treatment of prodrugs **17a–c** and **18** with dithiothreitol (DTT) resulted in reduction followed by self-immolation to release PTX with half-life periods from 4 min to 1 h. The cytotoxicity of the prodrugs was determined against the L2987 lung cancer cell line, and results indicated that prodrugs alone were significantly (30- to 650-fold) less cytotoxic than PTX (Table 3). Interestingly, when tested in combination with reducing agent DTT, they all exhibited similar bioactivity to parent drug PTX except **17c**, which is about ten times less than PTX, indicating they could serve as prodrugs.

All these prodrugs were also tested for their in vivo biological profile in athymic nude mice that had subcutaneous L2987 human lung adenocarcinoma xenografts. Except for prodrug **17c**, containing a captopril unit on PTX, all other prodrugs proved to be not as effective as PTX at the dose range from 85 to 150 mg/kg. One possible reason could be that the C2'-ester hydrolysis of the prodrugs might have not occurred in mice at

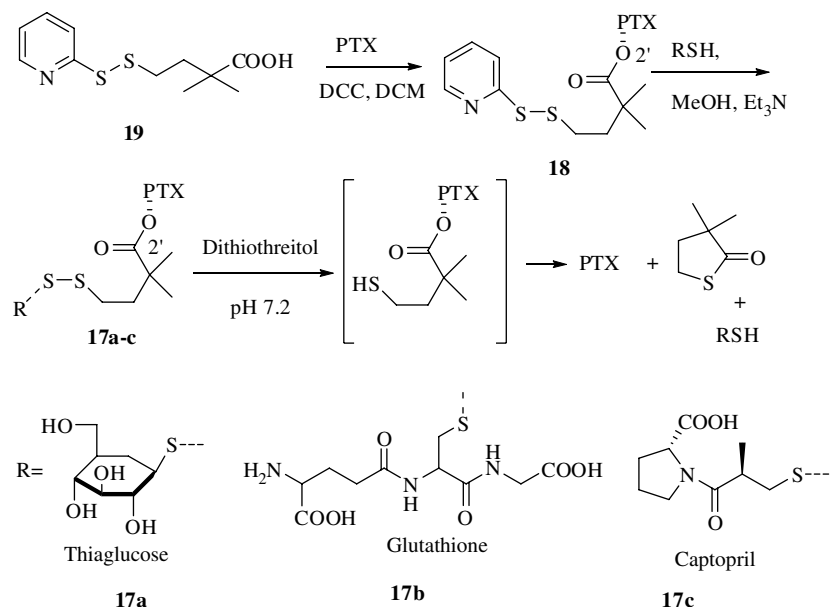


Scheme 4.

Table 2. In vitro cytotoxicity of PTX and prodrug **15**

Compound	IC ₅₀ ^a (ng/mL)						
	H226	IGROV	MCF-7	EVSA-T	WIDR	M19	A498
PTX	<3	10	<3	<3	<3	<3	<3
15	187	620	186	221	308	384	187

^a MCF-7, EVSA are breast carcinoma, WIDR are colon, IGROV are ovarian carcinoma, M19 are melanoma, A498 renal carcinoma, H226 non small cell lung carcinoma cell lines.



Scheme 5.

Table 3. Half-lives in DTT buffered solution (pH 7), in vitro cytotoxicity of the PTX and dithio-PTX prodrugs **17a–c** against L2987 lung carcinoma cells

Compound	Half-life of prodrug to release PTX in DTT buffer (min)	IC ₅₀ (μM) of the compound	IC ₅₀ (μM) of the compound + DTT
PTX		0.2	
17a	12	5.7	0.2
17b	60	10.1	0.4
17c	25	130.0	2.3
18	4	6.2	0.2

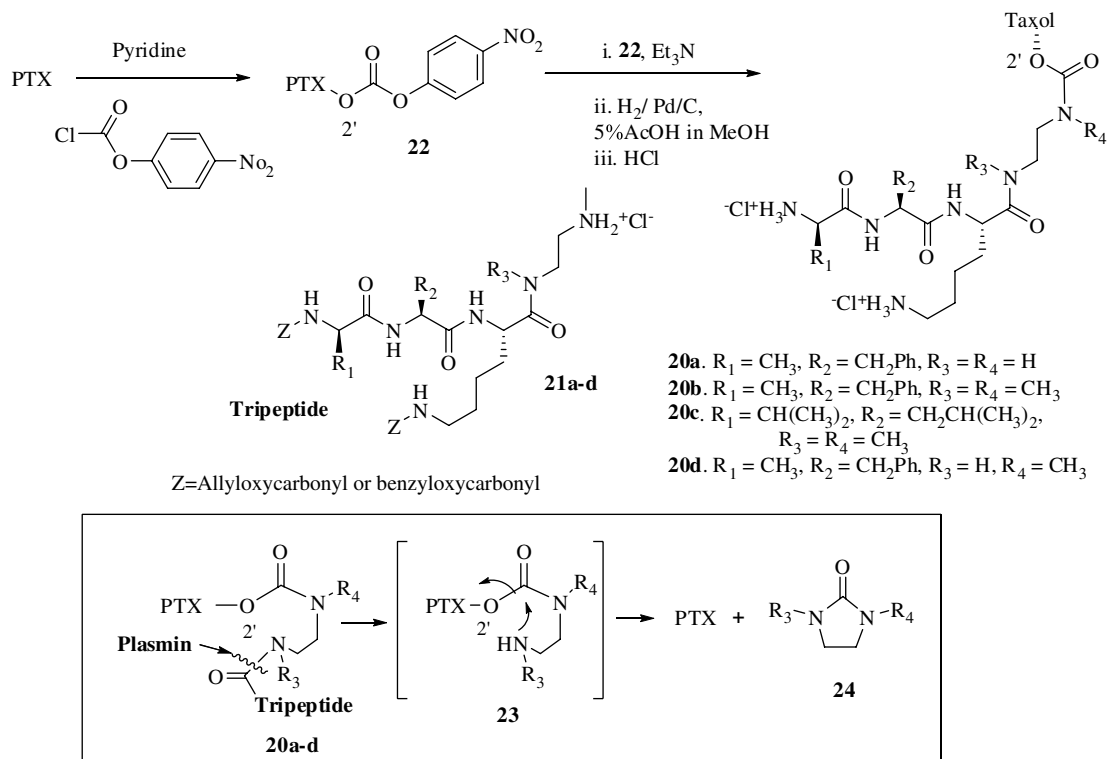
the tumor site. However, the prodrug **17c** showed superior in vivo bioactivity when compared to PTX. First **17c** was well tolerated at a 125 mg/kg dose with only 10–20% body weight loss, and this prodrug cured 60% of the tumors at the same dose. PTX had only a 30 mg/kg maximum tolerated dose, and this dose did not regress the tumor as was found with **17c**. The possible reason for the better activity of **17c** may be due to the captopril moiety, which was found to inhibit angiogenesis and slows down the tumor growth in rats.⁴⁰

The serine protease, plasmin, is overexpressed in tumor cells and plays a significant role in the various stages of cancer.⁴¹ Normal cells do possess the enzyme plasmin, but inhibitors such as the α_2 -antiplasmin of normal cells inhibit its activity. So the presence of high concentrations of plasmin in tumor cells could well serve as a guide to design prodrugs which could be selectively cleaved by plasmin at tumor cells. This hypothesis was previously applied very effectively for the design of prodrugs of several other cytotoxic agents.⁴² Based on this principle, recently Scheeren et al.⁴³ have synthesized the PTX-peptide prodrugs **20a–d**, starting from the carbonate **22**, which was derived from PTX and *p*-nitrophenyl chloroformate, and a tripeptide **21** (Scheme 6). The key concept here is that plasmin would attack and

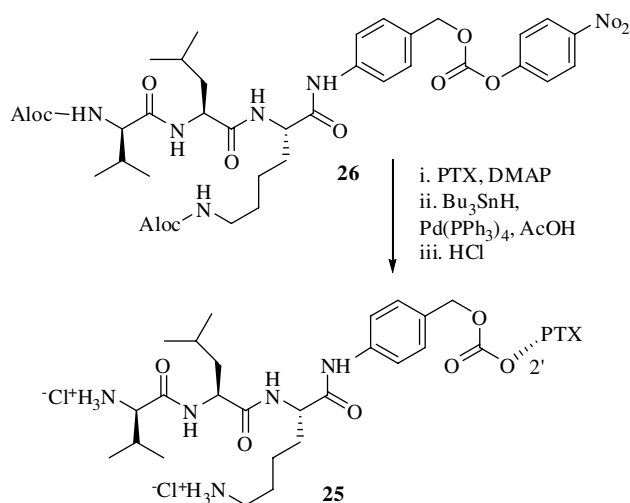
cleave the peptide bond of **20** to generate intermediate spacer **23**, which would immediately undergo 1,6-elimination reaction to release the active PTX as depicted in Scheme 6. The driving force here (and in the previous case Scheme 5) is the formation of entropically favored cyclic 5-membered ring **24**, with the expulsion of PTX. In a separate synthesis they also synthesized the prodrug **25** from the starting material **26** and PTX (Scheme 7).

The prodrugs **20a–d** were tested for their stability in Tris-buffer at pH 7.3 and were found to be stable for 3 days, except the prodrug **20a** which was degraded to liberate baccatin III. To test the plasmin effect on the prodrugs **20a–d**, they were incubated with human plasmin (in Tris-buffer at the same pH 7.3 for up to 3 days); only the prodrugs **20d** and **25** released PTX, but not **20a–c**, under the same conditions. The determined half-life in the above medium for carbamate prodrug **20d** was only 3.5 min. However, it only produced peptide cleaved spacer **23**, which has showed prolonged half-life period of 23 h to undergo complete 1,6-elimination to yield PTX. In contrast the carbonate prodrug **25** has shown 42 min half-life with no detectable spacer unit indicating as soon as the plasmin hydrolysis had occurred it underwent instantaneous 1,6-elimination reaction.

All the prodrugs were subjected to in vitro cytotoxicity evaluations against various cell lines (Table 4), and were found to be several hundred orders of magnitude less potent than PTX. It is worth reiterating the point here that many of the C2'-O-esters and carbonates of PTX were found to be as active as PTX, since they undergo hydrolysis under cell culture medium to generate PTX,⁴⁴ however, in this case the carbamate derivatives **20a–d** and **25** possessed significant stability against the cell culture medium. The stability of the prodrugs **20a–d** and **25** can be ascribed to blocking effects caused by the tri-peptide on the carbamate bond, which prevents the attack of several esterases present in the cell culture



Scheme 6.



Scheme 7.

medium. Overall compound **25** could be potential pro-drug candidate, if not on its own, by the ADEPT technology (discussed in Section 3.3) which is developed to localize enzymes at tumor site, since it possesses less cytotoxicity against a panel of cell lines and prolonged half-life period in the cell cultures.

3. Taxoid drug targeting strategies

Prodrugs described in the previous section could only improve the oral bioavailability of drug, however, there

is no guarantee that they may be selective for cancer cells. So, the aim of identifying a magic bullet, which is extremely stable during the circulation in the entire body and exclusively releases the active drug at tumor cell, is highly ambitious. Therefore it would be highly advantageous, if we could identify a tumor marker exclusively localized at the tumor (i.e., proteins/receptors/hormones etc.) and attach a drug molecule to it. On this premise, several drug targeting protocols have been envisioned and few attempts in this direction have already produced promising results. The following sections would describe some of the efforts and achievements.

3.1. Estradiol as a taxoid targeting agent

Nuclear proteins, such as Estrogen Receptors (ERs), are found to selectively attract and localize their ligands and/or ligand cognates in cells, where they are overexpressed. ERs are also found to play a pivotal role in modulating several biological effects of estrogens and anti-estrogens.^{45,46} They are overexpressed in cancer cells such as breast, ovarian, and gonads that are the primary target organs for estrogen action.⁴⁷ Thus ERs provide us with a plausible target for the selective delivery of cytotoxic agents, such as taxoids, through affinity of the ligands like estradiol (**26**). In fact this concept has been explored for the selective targeting of other cytotoxic drugs in the past⁴⁸ and the results indicate that the selective toxicity to ER positive MCF-7 breast cancer cells has occurred, when compared to ER negative MDA-MB-231 cells.⁴⁹

Compound	ID ₅₀ ^a (ng/mL)						
	H226	IGROV	MCF7	EVSA-T	WIDR	M19	A498
PTX	<0.003	0.010	<0.003	<0.003	<0.003	<0.003	<0.003
20a	41	19	16	11	13	45	40
20b	21	11	6.6	7.2	10	17	38
20c	25	26	6.9	5.2	11	24	45
20d	48	45	>63	>63	47	47	>63
25	16	27	9.2	9.3	11	20	>60

known that the derivatization at C2' and C7 could lead to less bioactive taxoids and modifications at the C10 position may or may not change the bioactivity of PTX.

There are various factors contribute to lower activities of the above drug conjugates. First, the conjugates should be able to be hydrolyzed to release the PTX in the cell culture, as it happened with many other cell lines in the case of C2' esters of PTX. Similarly, the fact that C2' ester conjugates **31** and **32** exhibited slightly higher activities when compared to **33** and **34** (which are C7 and C10 esters of PTX, respectively, found to be much



Table 5. In vitro cytotoxicity of PTX and drug conjugates **31–34**

Compound	IC ₅₀ (nM)		IC ₅₀ (nM)	
	A2780 ER-β(-)	PC-3 ER-β(+)	MDA-MB-231 ER-α(-)	MCF-7 ER-α(+)
PTX	25	77	4.5	4.9
31	180	73	22	39
32	680	40	51	62
33	8300	120	2200	1600
34	1900	68	304	103

more stable than C2'O-PTX esters) confirms the previous assertion that they might have undergone hydrolysis to release the PTX, which actually contributes to the cytotoxicity. The estradiol might have lost its binding affinity to ER positive receptors, since it was structurally modified and conjugated to PTX. Nevertheless, additional biological evaluations are necessary to fully understand the latter point.

The success of this strategy would depend on the conjugate's ability to bind to ER positive cells, internalize, thence to release the active drug. However, the conjugates (except **34**, which showed 3-fold selective toxicity to ER (α) positive cells) were unable to kill ER positive breast cancer cells indicating that they bind poorly to ER positive cells. If the latter point is true, their binding ability could be improved by coupling the taxoid drug at 17α position of estradiol rather than C11 and C16 positions, since other cytotoxic agents coupled from this position retained the binding abilities comparable to estradiol.⁵¹ Likewise, increasing the length of the linker between taxoid and estradiol would also help conjugates to bind well to the ER positive cells.

3.2. Antibody as a taxoid drug targeting agent (TPT and or PMT technologies)

Antigens are overexpressed at the surface of various tumor cells and have a high binding affinity to certain antibodies. Conceptually, this is an attractive feature aiding in the design of drug conjugates by attaching the cytotoxic drug to an antibody and selectively targeting the drug to tumor cells.⁵² In fact, this concept has led to the development of the various recombinant antibodies that would selectively localize tumor antigens. The field has been rapidly expanding and is now in advanced stages since the development of monoclonal antibodies (mAbs).⁵³ mAbs are highly specific antibodies, created by discrete colonies of hybridized cells, and show high binding specificity for tumor-specific antigens that could transport the cytotoxic drugs very well to the tumor site.⁵² When the mAb–drug conjugate (also called as immunoconjugate) reaches the tumor site it will be internalized by an antigen-mediated process called endocytosis.^{54,55} The latter process would trigger several biochemical processes in the cells to release the active drug. This strategy was previously coined as either tumor-activated prodrug therapy (TPT) or prodrug monotherapy (PMT).

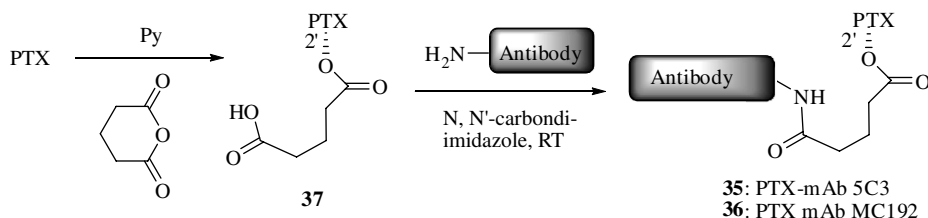
The success of the TPT strategy depends heavily on the internalization of the immunoconjugate, release of the active drug, the nature of cytotoxic agent, and finally

on tumor specific affinity of mAb. It was previously discovered that only a fraction of mAbs are localized to the tumor site from the total injection dose, and to achieve satisfactory therapeutic levels from immunoconjugate therapy, the drug conjugate must have a higher potency than the clinically used free drug (estimates indicate that the drug must have an IC₅₀ ≤ 1 × 10⁻¹⁰ M).⁵² In the past these mAbs were effectively used to target other cytotoxic drugs such as doxorubicin, calicheamicin, and maytansinoids to the tumor cells and laid the foundation to exploit various other new cytotoxic drugs such as taxoids for TPT delivery.^{56,57} Several efforts for taxoid drug targeting by the above concept have been reported thus far.

As one example, the tyrosine kinase receptors P140 TrKA and p75 are found to be overexpressed in many cancer cells such as neuroblastoma, non small cell lung, B-cell lymphoma, and melanoma.⁵⁸ They are also expressed in normal cells such as neurons, however in very low density. In this respect, separate investigations by Saragovi et al.⁵⁹ and Shooter et al.⁶⁰ developed anti-TrkA (mAb 5C3) and anti-p75 (mAb MC192) antibodies as tumor marking agents.

Recently, Guillemard and Saragovi⁶¹ synthesized two PTX-immunoconjugates, **35** and **36**, by a carbodiimide mediated coupling of C2'-glutaric acid PTX-ester **37** separately with two antibodies, mAb53 and mAbMC192. The precursor **37** was generated from a simple reaction between PTX and glutaric anhydride as shown in Scheme 9. In this report, they have systematically investigated the various properties of the immunoconjugate PTX-MC192 (**36**) as delineated below.

First, the binding affinity of PTX-MC192 immunoconjugate (**36**) against two cancer cell lines 4–3.6 and B104, that express p75 receptors, was investigated in FACS (fluorescent activated cells scan) assays. Conjugate **36** was found to retain the overall binding ability of the antibody itself. The in vitro cytotoxicity of **36** was determined and results indicated that it was better than free PTX against B104 cells at equimolar doses. Selective bioactivity of the **36** was evaluated against the cancer cells that do not express p75 receptors. Results indicated that **36** was inactive, whereas parent PTX showed dose dependent cytotoxicity against the same cancer cells. Together the data confirm the selectivity of **36** toward cells that express p75 receptors. Its specificity was established by competition experiments. Thus, 10nM concentrations of **36** (equivalent to free 10nM PTX) showed effective B104 cell death, whereas addition of the 40 nM antibody MC192 itself, which competes for p75, blocked the effect of **36**. And at the same time addition of a non-specific antibody, which does not compete for p75, did not show any effect on the conjugate bioactivity. Similar experiments on free PTX, with 40 nM of MC192, did not have any effect on the PTX cytotoxicity confirming the **36** specific binding ability to the cells that express p75 receptors. In this study, they investigated the parent antibody (MC192 alone) pharmacological role as adjuvant in enhancing the cytotoxicity of the immunoconjugate **36**, compared



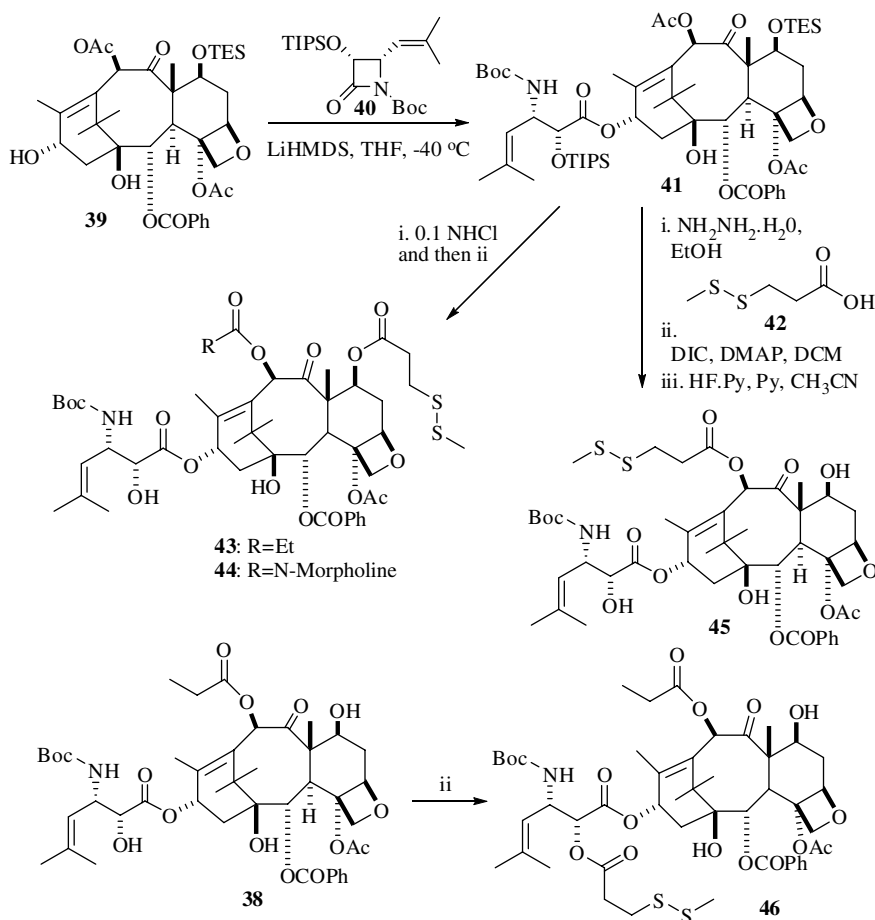
Scheme 9.

to free PTX. Results showed that mAb-MC192 alone neither enhances nor decreases the cytotoxicity of the free PTX at various concentrations and also the fixed concentration of PTX with varying concentration of the antibody. Lastly, **36** was subjected to *in vivo* evaluations against neuroblastoma xenograft in nude mice. Studies indicate that the conjugate was effective in reducing the tumor growth, compared with control, whereas free PTX alone or in combination with MC192 was not able to reduce the tumor growth. Additionally, the immunoconjugate **36** has prolonged the survival of the mice on average by 30% compared with free PTX. Likewise, *in vivo* experiments corroborate their *in vitro* findings of **36**, making it a potential candidate for the treatment of tumors expressing p75 receptors.

It is worth mentioning here that investigators prepared immunoconjugates **35** and **36** by very simple synthesis

and established all its biochemical properties, so that it could well serve as a guide for newly discovered antibody mediated drug delivery protocols to establish and identify an ideal candidate for TPT chemotherapy.

Along similar lines Ojima et al.⁶² also reported a successful protocol, through use of mAbs as taxoid drug delivery agents. For the execution of their strategy, they have used highly active taxoid **38**, a second generation DTX (**2**) analog, which exhibited remarkably 2–3 orders of magnitude higher potencies than DTX against various cell lines *in vitro*.⁶³ In order to be able to couple the taxoid **38** to antibodies, they had to be functionalized synthetically with a 3-methyldisulfide (MDS)-propanoyl linker unit. Initially, they resorted to developing a SAR study on **38** with a MDS linker unit by attaching it at the C2', C10, and C7 positions, respectively, to identify potential sites for antibody incorporation. The synthesis was accomplished as shown in



Scheme 10.

Scheme 10, by a straightforward synthesis starting from a baccatin III derivative **39** and a β -lactam derivative **40**, which has resulted in the taxoid derivatives **43–46**.

The in vitro cytotoxicity of the taxoid analogs **43–46** against epidermoid A431, and non small cell lung A549 cancer cell lines, indicated that the C10-MDS-propanoyl taxoid **45** showed higher bioactivity than other taxoids, so it was selected for coupling to an antibody. The disulfide bond of the taxoid **45** was readily cleaved by treating with dithiothreitol (DTT) to furnish taxoid **47** with a free thiol unit. And at the same time the antibodies were also functionalized with *N*-succinimidyl-4-(2-pyridylthio)-pentanoate to provide the intermediates, which were then treated with **47** in a buffered solution at pH 6.5 to yield immunoconjugates **47a–c** (**Scheme 11**).

The epidermoid growth factor receptor (EGFR) is found to be overexpressed in several cancers such as head and neck, lung, breast, and human squamous cancers.⁶⁴ Several immunoglobulin class G antibodies such as KS61 (IgG2a), KS77 (IgG1), and KS78 (IgG2a) were identified which could be localized at EGFRs on the tumor cell. In this regard, Ojima et al. selected the three antibodies and individually coupled them to a taxoid **47** to yield immunoconjugates **47-KS77**, **47-KS61**, **47-KS78**, respectively, as shown in **Scheme 11**. For the comparison purpose an immunoconjugate of **47** with antibody mN901, which does not bind to EGFR positive cancer cells, was also prepared.

The in vitro cytotoxicity evaluations of the immunoconjugate **47-KS78** were conducted against the EGFR expressing human epidermoid carcinoma cell line A431, and results indicated that the immunoconjugate **47-KS78** exhibited an IC_{50} of 1.5 nM (**Table 6**). For the same cell line the immunoconjugate **mN901-47** did

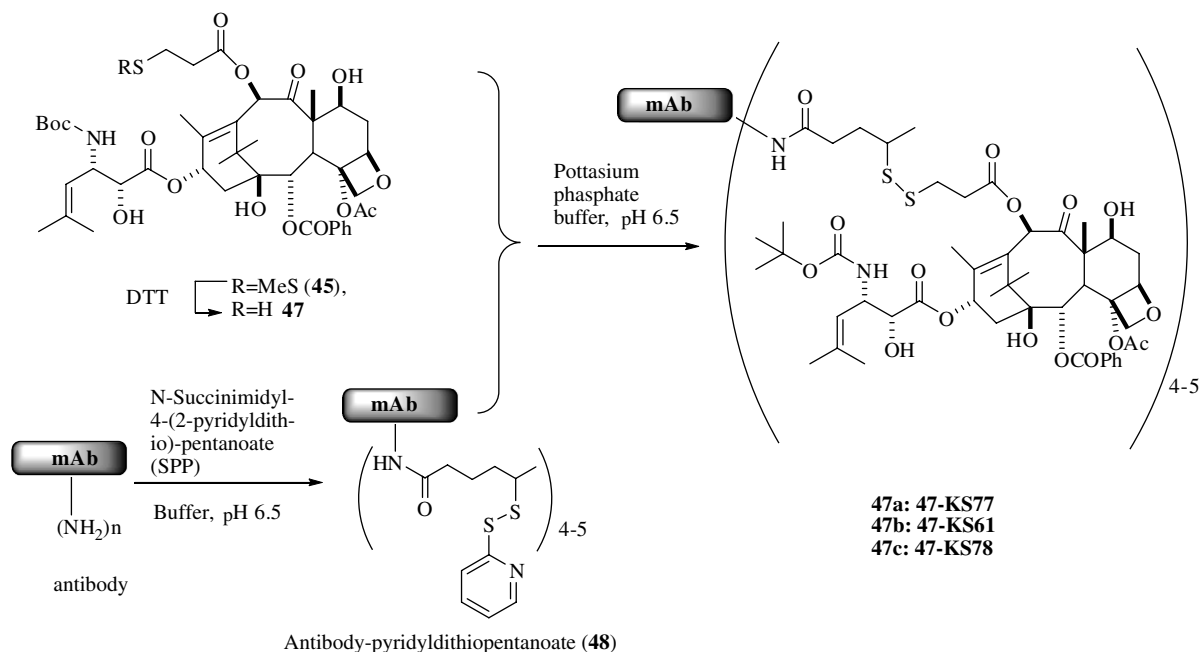
Table 6. In vitro cytotoxicities of taxoid derivatives **43–46** and immunoconjugate **47**

Taxoid	IC_{50} (nM)	
	A431	A549
38	0.09	0.1
43	2.0	ND
46	3.0	0.9
45	0.5	0.8
44	>3.0	ND
47-KS78	1.5	ND

not show any cytotoxicity. The addition of a large excess of anti-EGFR antibody (e.g., KS61) to the **47-KS78** eliminated the cytotoxicity caused by the immunoconjugate **47-KS78**, clearly demonstrating the antibody mediated binding had occurred.

The in vivo efficacies of the immunoconjugates **47-KS61** and **47-KS77** were evaluated against human xenografts in severe combined nude mice (SUID) inoculated with A431 cancer cells. The immunoconjugates inhibited tumor growth in all the treated animals for the duration of experiment. In contrast, the mAb free taxoid **45** showed no therapeutic effect. Additionally there was no systemic toxicity to the mouse was noticed at the administered dose (10 mg/kg) of immunoconjugate **47-KS77**.

One point to be noted here is that the conjugate described above is a C-10 derived taxoid (rather than usual C2'-derived taxoid) so additional experiments need to be fully investigated, about the exact nature of the conjugate internalization and release of the active taxoid drug after endocytosis. Nonetheless, the principal concept of antibody mediated drug targeting (TPT) seems very promising. As demonstrated by the above



Scheme 11.

two reports discussed in this section, the results raise the hope for better taxoid chemotherapies for various cancers.

3.3. Taxoid targeting by ADEPT technology

The challenge for highly effective targeted delivery protocols is to suppress the non tumor cytotoxicity and improve the cytotoxic drug concentrations at the tumor site (vide infra). An alternative way to address this issue in a broader sense is an antibody directed enzyme prodrug therapy (ADEPT) technology. Envisioned two decades ago,⁶⁵ this technology continues to gain momentum.⁶⁶ By ADEPT technology an enzyme would be targeted first to the tumor site by means of an enzyme–antibody conjugate. Once this conjugate is localized at tumor site and cleared out from the systemic, then a less cytotoxic prodrug would be administered. When the prodrug reaches the tumor site the enzyme thus concentrated mainly at the tumor site would act on the prodrug to release the active drug. The ADEPT technology offers several advantages over other prodrug and targeted delivery protocols (TPT) described above such as, it would allow us to design and develop more water soluble prodrugs with better pharmacokinetic properties, however, it also would suffer from a limitation such as the number of enzymes would be limited, since very few enzyme–antibody conjugates would be localized at the tumor cell. Unless the enzyme turnover number is very high, sufficient quantity of the active drug may not be made available by the enzyme present at the tumor site.

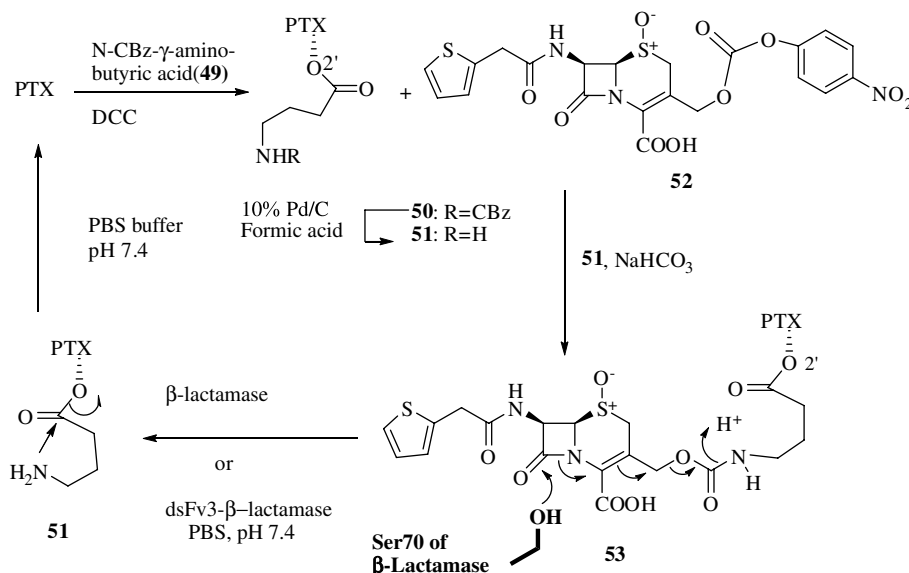
Conceptually, it is also possible to prepare a ‘super conjugate’ with all three (i.e., prodrug–antibody–enzyme) units as a cargo, and test the tumor specific delivery by the antibody interaction with the corresponding antigens present in the tumor cells. This idea is based on perspective that the enzyme would be in an inactive form in antibody–enzyme conjugate, which then can be coupled

to a prodrug with a spacer unit. The active enzyme would be released only after endocytosis at tumor cell. In this case the cargo would carry sufficient number of enzymes per molecule of prodrug, however, the synthesis of such a conjugate would be complicated one and thus this protocol has not been explored so far.

There are several enzymes such as β -lactamase, β -glucuronidase, carboxypeptidase-G2, and carboxypeptidase-A, cytosine deaminase, penicillin amidase, alkaline phosphatase, and cysteine proteases (cathepsin B, -L) that could be bound to antibodies. These enzymes have been used in the past to specifically target tumor cells and explored in ADEPT for other cytotoxic drugs.⁶⁷ Rodrigues et al. have prepared a fusion protein, dsFv3- β -lactamase, from the humanized antibody (hu-mAb4D5-8) and β -lactamase (REM-1) to target the other cytotoxic drugs in the past.⁶⁸ In that study they observed that β -lactamase shows high catalytic activity, large substrate specificity and would act on the β -lactam ring moiety of the prodrug to release the bioactive drug.⁶⁸ They have now explored the PTX for the tumor specific delivery through this technology.⁶⁹

The synthesis of PTX–prodrug conjugate **53** was achieved as shown in Scheme 12, by a coupling reaction between β -lactam derivative, cephalosporin **52**, and PTX-2'-O-aminobutyryl ester **51**, which in turn was prepared from PTX and N-CBz- γ -aminobutyric acid (**49**).

Once the PTX–prodrug conjugate **53** was synthesized they investigated the free β -lactamase and a tumor targeting fusion protein dsFv3- β -lactamase to mediate the hydrolysis of **53** to release the PTX linker **51**. The results show that **53** was rapidly hydrolyzed by both the above enzymes to release **51**, which showed longer half-life period of 16 h to release PTX as it was observed with the spacer unit **23** (cf. Scheme 6). It is interesting to see the same spacer unit **51** when treated with β -lactam



Scheme 12.

derivative **52** under mild basic conditions (with NaHCO_3) did not release any PTX. This can be attributed to the changes in the chemical reaction to a biological environment.

The conjugate **53** was subjected to *in vitro* cytotoxicity and tubulin polymerization activity in the presence and absence of β -lactamase. The results show that conjugate on its own had no effect on the tubulin polymerization, however, when it was activated overnight with the β -lactamase enzyme, it showed similar tubulin polymerization activities. A brief period of exposure of the **53** to β -lactamase did not show any tubulin activity, and this result is consistent with the slow expulsion mechanism of PTX from linker **51**.

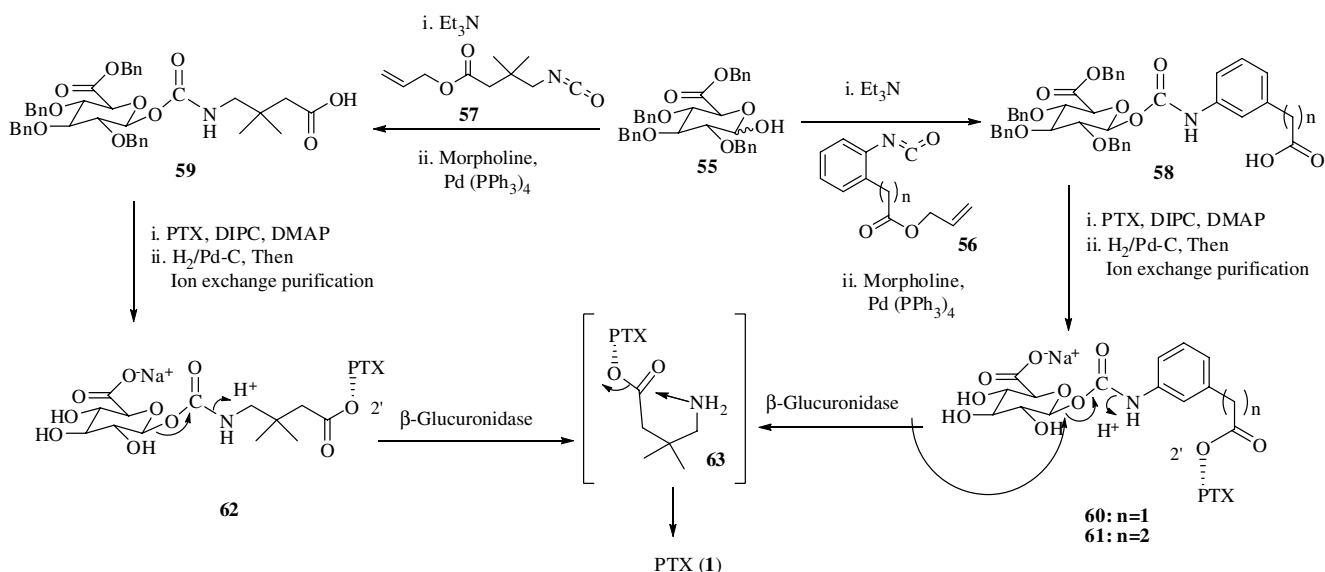
The cytotoxicity against breast cancer cell line, SK-BR-3, revealed that conjugate **53** is 10-fold less potent than PTX. In sharp contrast, similar activities to PTX were observed in the presence of targeting fusion protein dsFv-3- β -lactamase. Thus, proof of concept for the PTX drug targeting by ADEPT seems to be working, though the *in vivo* results are to be published on this conjugate **53**.

Recently Scheeren et al.⁷⁰ also investigated the PTX drug targeting by ADEPT technology using a human enzyme β -glucuronidase, which is a lysosomal enzyme and found to have no activity in the blood pH. In this study they synthesized the prodrug conjugates **60–62** as shown in Scheme 13, by DIPC mediated coupling of the PTX to the intermediate carbohydrate linked carboxylic acids **58** and **59**, which in turn were prepared from the protected sugar unit **55**. The prodrug conjugates **60–62** have 6–8 spacer atoms between PTX and the carbohydrate moiety. Like before, the β -glucuronidase enzyme is expected to cleave glycosidic bond as shown in Scheme 13, to liberate the PTX linker **63** at the tumor site.

The biological results revealed that the prodrugs **60–62** were several hundred times more soluble in water when compared with PTX, and were found to be stable at pH 6.8 at 37 °C, except the prodrug conjugate **60**, which was readily cleaved by the non specific hydrolysis. As a result only conjugates **61** and **62** were tested further in their study. The enzyme-catalyzed prodrug activation experiments were carried out with 100 μM prodrugs **61–62** versus 10 μM of human β -glucuronidase at 37 °C. The prodrugs, **61** and **62**, released the PTX with a half-life of 45 min and 2 h, respectively. It is worth mentioning here, that in this experiment no **63** was detected by HPLC indicating that the spacer unit in **63** had spontaneously undergone cyclization to release PTX. This result is in sharp contrast with results obtained by linker **53** in Scheme 12. This clearly emphasizes how important the linker design is in this tumor delivery protocol. The only difference in **63** is an extra *gem*-dimethyl group compared to **53**.

The *in vitro* cytotoxicities of the prodrugs **61** and **62** against OVCAR-3 cells were tested and the IC_{50} values of the prodrugs **61** and **62** indicate that they were about 2 orders of magnitude less cytotoxic than parent PTX, but prodrug **60** had the same activity as PTX. Importantly the prodrugs upon activation with enzyme β -glucuronidase exhibited nearly equal cytotoxicity to the parent (Table 7).

They further determined the activities of prodrug conjugates **61–62** against OVCAR-3 cells, which were pretreated with a conjugate derived from murine antipancarcinoma monoclonal antibody 323/A3 and human β -glucuronidase. The results revealed that antibody–enzyme conjugate specifically targeted to OVCAR-3 cells and showed enzyme activity on the prodrugs, which showed IC_{50} values only 2-fold higher than the prodrug. Nonetheless, it was found that the amount of enzyme bound to the above cells was too low to completely acti-



Scheme 13.

Table 7. In vitro cytotoxicity of PTX and prodrug conjugates **60–62**

Compound	OVCAR-3 cells IC ₅₀ (nM)	
	Without β -glucuronidase	With β -glucuronidase
PTX	0.2	NA
60	0.23	0.23
61	27	1.1
62	20	0.6

vate the prodrugs. This observation is quite consistent with speculated limitation by the ADEPT technology now witnessed by present investigation.

Monneret and Schmidt et al.⁷¹ have made extensive efforts in designing prodrugs of both PTX and DTX for tumor specific delivery by ADEPT technology. They have synthesized PTX conjugate **66** as shown in Scheme 14, starting from carbohydrate-*p*-nitro phenyl ether **63**.^{71a} They have also synthesized the PTX conjugate **74** as shown in Scheme 15, starting from **69** in a straightforward synthesis.^{71b} Recently they also synthesized DTX conjugates **75** and **76** by similar synthetic reactions depicted in Scheme 15.^{71c} It is worth clarifying a point here that conjugates **66** and **74–76** are two different classes, the former has only one linker unit, while the latter have two linker units between carbohydrate moiety and taxoid. They have also envisioned testing these prodrugs by β -glucuronidase mediated activation.

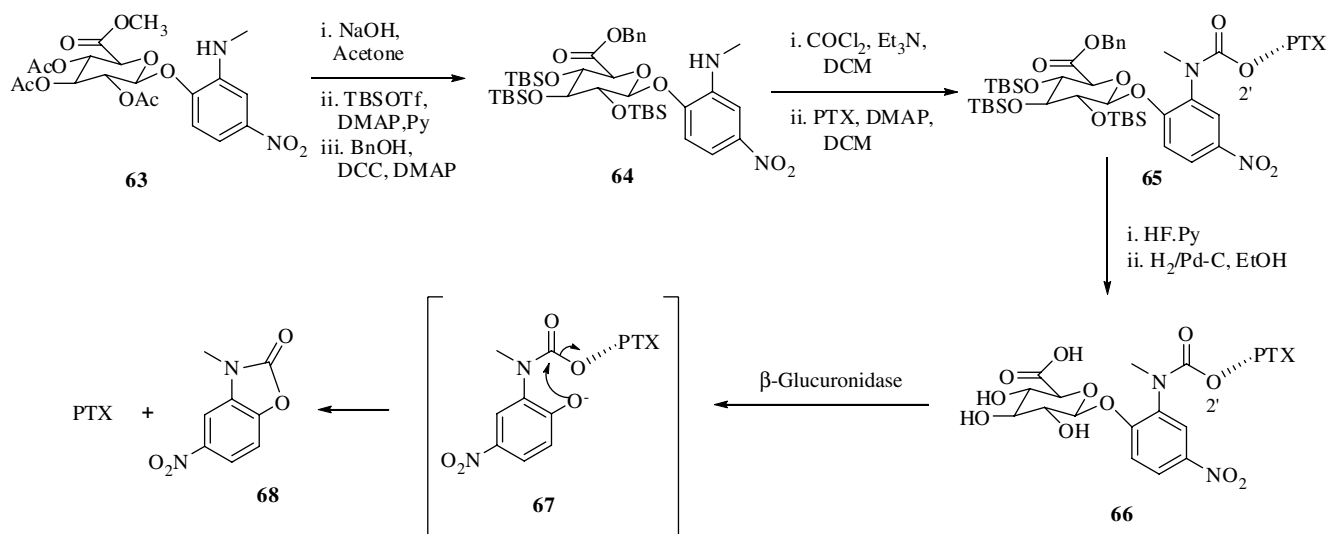
In vitro cytotoxicity assay results of the prodrug conjugates **66**, **74–76**, against LoVO (human colon cancer) cell line, indicated that they all have significantly lower cytotoxicities, when compared to their respective parents (PTX or DTX), and more importantly, upon activation with β -glucuronidase enzyme, the prodrugs showed equal cytotoxicity to PTX (Table 8). The liberated byproduct **68**, however, has very low cytotoxicity compared to prodrug IC₅₀ values suggesting that it does not have much impact on the values obtained by the prodrugs after β -glucuronidase mediated activation. In addition, the conjugates **66** and **74–76** showed inherent

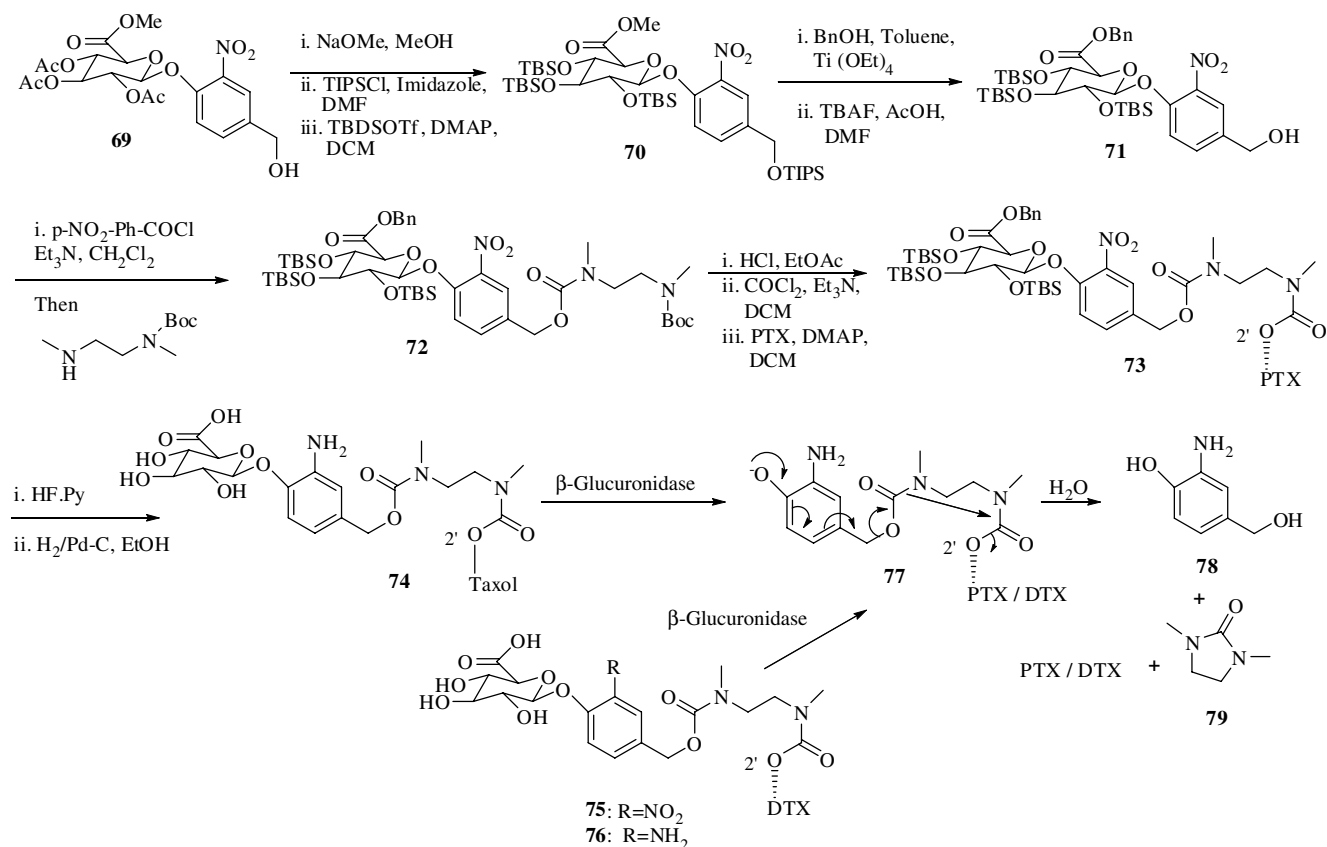
prodrug properties, for instance, they were found to be stable in a phosphate buffer at pH 7.2 at 37 °C for 24 h, and also found to be more aqueous soluble than PTX. Special mention should be made for the prodrug conjugate **66** showing 2000-fold more aqueous solubility than PTX, so it was selected to test further for its efficacy in ADEPT technology.

To determine the kinetic properties of the prodrug **66** in vitro, they used *Escherichia coli* β -glucuronidase enzyme that was found to be analogous to human β -glucuronidase.⁷² At the concentration of 100 μ g/mL of β -glucuronidase per 250 μ g/mL of prodrug **66**, the enzyme catalyzed reaction had occurred and prodrug **66** released the PTX with half-life near 2 h. In this experiment, no trace of intermediate (PTX-linker **67**) was found, clearly representing that as soon as the enzyme activation had happened (cleavage of sugar unit), the resulting intermediate **67** underwent rapid self immolative cyclization to release PTX and a byproduct **68**.

In the case of **74–76**, the enzyme activation experiments were conducted at low concentrations due to their low solubility. At 30 μ g/mL β -glucuronidase per 30 μ g/mL of prodrug, all of them showed only 10 min of half-lives even at such low enzyme concentration. The difference in the half-life periods of **74–76**, compared to **66**, can be explained by steric factors. In the case of **74–76**, the carbohydrate unit and PTX were attached to *para*-position of aromatic ring which makes the glucuronate unit more accessible to enzyme compared to **66**, in which both units were attached at *ortho* position, which would make unfavorable interactions with approaching enzyme. Also in the case of **74**, the PTX-ethylene diamine linker unit **77** was detected (by HPLC). At enzyme concentrations 50 μ g/mL and up **77** showed half-life of 13 min, in sharp contrast to **67** which was not detected.

It is worth noting a point here that the prodrugs **74–76** contain a double spacer unit which upon initial cleavage by enzyme would undergo 1,6-elimination to generate a transient *N,N*-dimethyl ethylene diamine spacer unit,

**Scheme 14.**



Scheme 15.

Table 8. In vitro cytotoxicity of taxoid prodrug conjugates **66**, **74–76**

Taxoid	IC ₅₀ compound/IC ₅₀ of the parent	IC ₅₀ of compound + β -glucuronidase
PTX		90 nM
66	722	100 nM
74	153	NA
75	337 (DTX)	NA
76	187	NA
68	3000	NA

which was found to undergo a cyclization–elimination process much faster than a single N-substituted spacer unit present in **23d** (cf. Scheme 6).⁷³ Though the preliminary results of these conjugates are encouraging, a detailed in vivo bioactivity data would help to understand the efficacy of these prodrugs.

3.4. Folic acid as taxoid drug targeting agent

Various cancer cells, except prostate, pancreatic, bladder, and lymphoid cancer cells, overexpress folic acid receptors (FRs).⁷⁴ FRs, also known as folate binding proteins (FBPs), have high binding affinity to folic acid (FA, a vitamin B) and play pivotal role in cellular uptake of folic acid.⁷⁵ Folic acid was discovered to play prominent roles in the formation of new cells and tissues, especially at the prenatal and postnatal stages as well as during childhood. That is why gynecologists highly recommend FA to pregnant women. A hydrofolate form of folic acid is found to be abundant in the

blood, and does not cause any side effects and immunogenicity to the human body.

The FRs were found to exist in various forms such as α -FBP, β -FBP, γ -FBP, and δ -FBP. Among these α -FBP and β -FBP are membrane-associated folate receptors.⁷⁶ The high binding affinity interactions between FRs and FA⁷⁷ could conceptually enable us to design a cytotoxic drug–folic acid–complex and selectively target it to the FR expressing tumor cells. Excellent reviews covering the topic of folates (FA) and their role in the targeted therapy have recently appeared in a series of reports.⁷⁸ Once the folic acid–drug conjugate reaches the FR positive tumor cells, it will enter into the cells via a receptor mediated process called endocytosis.⁷⁹ The explicit mechanism of how endocytosis of the drug–FA conjugate occurs in the cell to release the bioactive drug has been represented by Leamon and Reddy.^{78a} In brief, once the folate–drug conjugate binds to the cell the plasma membrane surrounds the ‘folate drug conjugate–FR–complex’ forming an early stage endosome, then the pH of the endosome drops suddenly to ~ 5 by the action of proton pumps, that are localized in the endosome membrane. Subsequently, the protonation of the carbonyl groups of FR by changes in pH would enhance the conformational change of the receptor that would trigger the release of folate molecule.

In fact, dropping of cell pH upon FR mediated FA–drug conjugate internalization is a highly useful

property for designing prodrugs as well as linkers that could be selectively cleaved at lower pH 5. In the past the FA mediated drug targeting attempts have been explored for other cytotoxic agents.⁸⁰ Few reports on taxoid drug targeting by FA have appeared but Fuchs et al.⁸¹ have designed and synthesized several PTX–FA conjugates from the C2' and C7 positions of PTX.

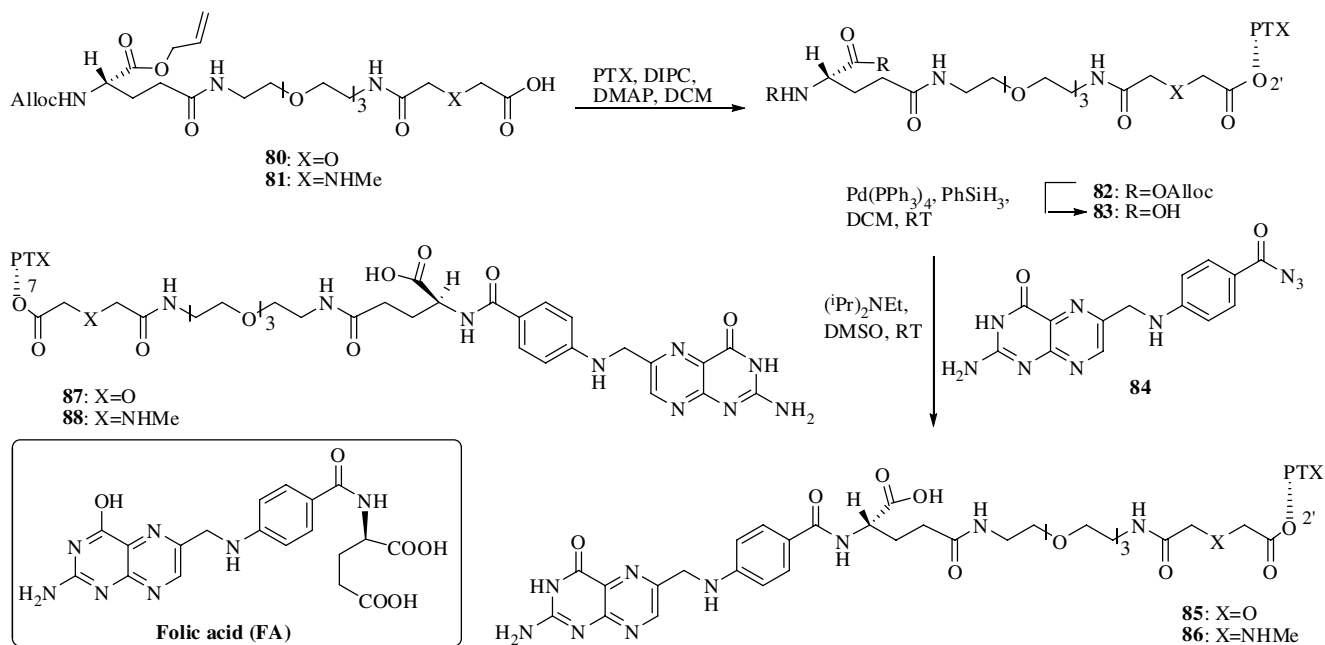
The synthesis of PTX–FA conjugates **85–88** was accomplished as shown in Scheme 16. Thus, the linker starting materials **80** and **81** were separately coupled to PTX by DIPC, to furnish the PTX linker intermediates **83** (after deprotection of **82**), which then were coupled to a FA derivative **84** to yield FA–PTX conjugates **85** and **86**. Likewise the conjugates **87** and **88** were also prepared by similar syntheses.

The PTX–C2'O–FA conjugates **85** and **86** showed shorter half-lives at pH 7 and pH 5 when compared to the corresponding conjugates **87** and **88** that were connected from the C7 position of the PTX (Table 9). These observations are consistent with the previous point that the C2'O-esters of PTX are more labile than the C7 esters. Cytotoxicity results of the PTX–FA conjugates indicated that they were significantly more potent than PTX against 3 different cell lines in vitro (Table 9). The binding affinity of **88** was investigated against labeled ³H-folic acid, in comparison with free folate.

Conjugate **88** retained most of the binding affinity of free folic acid against receptor positive murine M109 and human KB tumor cell lines. Efforts to discover any receptor-mediated specific cytotoxicity (in vitro) against receptor positive KB tumor cells (by comparing with parent PTX) revealed that the conjugate **88** was about 50-fold less cytotoxic than PTX. At the same time concurrent addition of a 500-fold excess of free folic acid did not change the bioactivity of the conjugate, meaning that the folate moiety attached in the conjugate **88** is not responsible for cell internalization.

At the same time **88** and PTX were treated with KB and M109 cells, and FA negative A549 cancer cells at constant drug concentrations. In this experiment conjugate **88** neither showed more potency than PTX, nor any folate receptor mediated selectivity in the above cell lines (its activity was found to be nearly equal against 3 different cell lines). Likewise, in vivo experiments on mice implanted with folate receptor positive M109 tumors did not show any useful level of increase in the life span of the mice, when compared to PTX.

The only interesting feature observed by the conjugate **88** is its lack of toxicity in tumor-free mice. PTX treated mice showed 20% body weight loss, whereas mice treated with **88** showed no body weight loss. Overall, **88** is the best of the four PTX–FA conjugates synthesized in



Scheme 16.

Table 9. In vitro cytotoxicity of PTX–FA conjugates **85–88**

	(ED ₅₀ µg/mL)			T _{1/2} (h) at 37 °C	
	A549 lung	MCF-7 breast	HT-29 colon	pH 7	pH 5
PTX	2 × 10 ⁻²	5 × 10 ⁻²	3 × 10 ⁻²		
85	1.6 × 10 ⁻³	3 × 10 ⁻³	1.2 × 10 ⁻³	1	17
86	1.2 × 10 ⁻³	2.4 × 10 ⁻³	1 × 10 ⁻³	9	38
87	1.7 × 10 ⁻³	3.3 × 10 ⁻³	2.9 × 10 ⁻³	40	>300
88	1.6 × 10 ⁻³	2.6 × 10 ⁻³	1.1 × 10 ⁻³	109	197

this study, but it failed to selectively kill the receptor expressing cells in vitro and did not show any superior activity in vivo against the FR positive tumor cells when compared to PTX. Thus, the concept of taxoid drug targeting by FA mediated drug delivery has not yielded conclusive evidence with the above conjugates. The results led to investigation of other types of linkers for making the PTX–FA conjugates, that might be cleaved at lower pH and also increase the folate mediated selective internalization, rather than non-selective entry into the cells. From Table 9, it is clear that the conjugates **85**–**88** were cleaved much faster at pH 7 rather than pH 5, and this result is in contrast to anticipated results knowing that tumor cell pH drops to 5 upon FA-mediated internalization.

Very recently, Majoros et al.⁸² reported a FA-mediated successful PTX drug delivery protocol to FR positive cell lines. In this study they have utilized a dendrimer PAMAM (polyamidoamine) as a linker unit between the PTX and FA as well as a fluorescein isothiocyanate moiety (FITC) used as a fluorescent probe for cancer cell imaging.

The conjugate **89** was synthesized as shown in Scheme 17 starting from the advanced intermediates of PTX **90** and folic acid–dendrimer–complex **91**. The average number of PTX molecules attached to conjugate **89** was determined to be 3. The PTX–FA–PAMAM–FITC conjugate **89** was subjected to the cytotoxicity assay

against FA receptor positive KB cells, at various concentrations (100, 50, 25 nM). A FA conjugate without PTX was also prepared in this study and used as a control in a parallel experiment. The results indicated that the conjugate **89** showed cytotoxicity against FR positive KB cells (human epidermoid carcinoma) at the highest concentration (100 nM). Flow cytometry analysis of cells revealed that the PTX–drug conjugate and drug-free conjugate were taken up into the FR positive cells, however, only the PTX–conjugate **89** killed the cancer cells, but not the drug-free conjugate. Importantly, when subjected the same drug and drug-free FA conjugates to the FR negative cells, they neither internalized into the cells nor showed any cytotoxicity. The properties clearly emphasize that the FA-mediated targeted delivery has occurred.

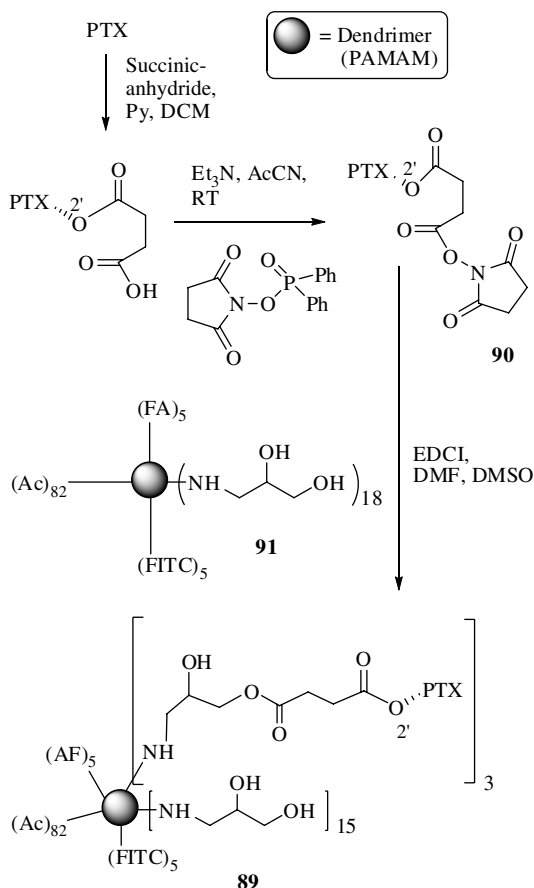
Furthermore XTT proliferation assay was performed to verify the efficacy of the drug conjugate **89** and drug-free control. At the concentration of 200 nM, the PTX–FA conjugate showed cytotoxic effects against both FR positive and FR negative cells, however, at lower concentrations (50 nM) the conjugate showed selective cytotoxicity against positive cells, leaving the FR negative KB cells unaffected. The control (drug-free conjugate), however, had no cytotoxic effects at concentration of 200 nM.

Several alluring properties of the dendrimer (multi-functional polymeric units) used in this study are noteworthy. First, they not only are capable of transporting more than one drug molecule per molecule of the conjugate to the tumor site. Second, they have many additional synthetically available sites for attaching the fluorescent or radio-labeled probes to monitor the properties of a drug conjugate. Third, when they were subjected to FR positive and FR negative cells without FA molecule (in the above study), they had virtually no association between the cells, thus explaining that they do not interfere with FA mediated drug conjugate binding efficiency. Overall, the taxoid drug targeting concept by folic acid seems promising, however, detailed in vivo efficacies are still to be reported.

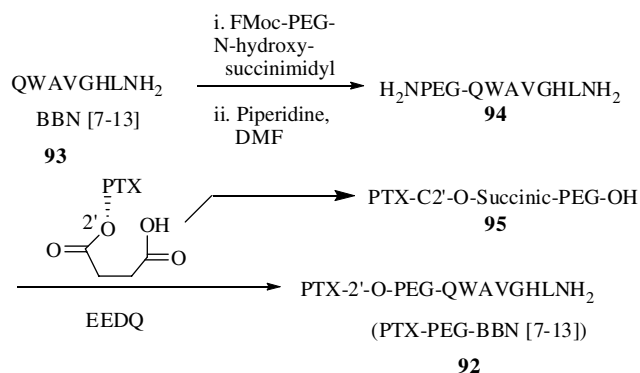
3.5. Peptide as taxoid drug targeting agent

Numerous proteins and peptides (such as $\alpha v \beta 3$, $\alpha v \beta 5$, APN, VEGFR, MMPs) have been identified which are selectively localized in the tumor endothelial cells.⁸³ These proteins are found to play a very important role in tumor angiogenesis and have specific affinity to various peptides.⁸⁴ In particular, the GRP (gastrin releasing peptide), which belongs to bombesin (BBN) peptide family, is overexpressed in small cell lung cancer cells (SCLC) when compared to normal cells.⁸⁵ In this regard, efforts have been directed to achieve tumor specific delivery of cytotoxic agents by means of a peptide conjugate.

Thus, Safavy et al.⁸⁶ synthesized a PTX–PEG–GRP conjugate **92** as shown in Scheme 18 starting from peptide **93** and PTX–C2'–O-succinate. This conjugate **92**



Scheme 17.



Scheme 18.

was expected to be localized by a specific receptor peptide binding at the tumor cell surface, then release the active drug upon hydrolytic cleavage of the peptide at tumor site. For the comparison study they also synthesized a peptide-free PTX-PEG derivative **95** by a similar method used in the synthesis of **92**.

The conjugate **92** showed an interesting solubility profile. For example, consistent with previous reports, that resulted in significantly more soluble PTX derivatives when coupled PEGs, the conjugate **92** also showed better solubility than PTX, and it was readily soluble in aqueous media at 25 mg/mL concentrations. Additionally it was soluble in organic solvents such as methylene chloride, chloroform, and methanol whereas the unconjugated peptide **93** (without PEG) was only soluble in dimethyl formamide and methanol, clearly showing the PEG's role in solubilizing the PTX as well as peptide units.

To determine the tumor specific cell binding efficacy of **93**, it was tested against two different cell lines. BNR-11 cell line, which was previously used to evaluate the binding affinity of the [^{125}I]-Try³-BBN, and NCI-H1299 cell line, which has relatively lower number of BBN receptors.⁸⁷ The results indicated that the conjugate **92** had the same impact on the inhibitory effect of [^{125}I]-Try³-BBN to the above cell lines when compared to PTX-free peptide **93** (Table 10), indicating neither PTX nor the PEG units had any effect on the peptide binding. Then conjugate **92** was tested for its stability at physiological pH and in human plasma. In both of the above conditions, the conjugate released the PTX with half-lives of 154 and 133 min, respectively. The in vitro cytotoxicity of **92** along with parent PTX and

as well as peptide-free drug **95** was investigated against NCI-H1299 cells and results indicated that the conjugate **92** exhibited 50% better cytotoxicity at both 15 and 30 nM concentrations, respectively, when compared to peptide-free PTX **95**.

The IC₅₀ values were determined for **92** at various time points against the same cell line and are shown in Table 10. The results clearly indicates that the cytotoxicity values obtained for **92** are due to receptor mediated drug localization, since the peptide-free taxoid **95** and parent PTX had weaker IC₅₀ values in the same experiment. Overall the results clearly demonstrate proof of concept targeting a taxoid drug by a peptide. The in vivo efficacy of this drug conjugate has yet to be reported.

3.6. Gold nanoparticles as taxoid delivery agent

Nanoparticles and nanomaterials continue to gain great deal of attention because of their significant biomedical applications.⁸⁸ Particularly, gold nanoparticles initially discovered in nearly a century and half ago by Faraday⁸⁹ are now rediscovered for interesting physicochemical properties. In this regard numerous syntheses for the gold nanoparticles, with controlled size and shape in aqueous and non aqueous environments have been developed.⁹⁰ At the same time the chemistry related to their surface modification has been investigated. Ligands containing either a thiol or amine groups strongly bind to the surface of the gold nanoparticles, presumably through a dative covalent bond formation between either thiol or amine functionality and surface of colloidal gold nanoparticles (Fig. 2).

The biocompatibility of the gold nanoparticles was also investigated and studies report that they do not cause any cytotoxicity to the body, instead they exhibit useful properties such as: dose dependent reduction in cellular RNS (Reactive Nitrite Species) and ROS (Reactive Oxygen Species) levels when administered at 10 μM and

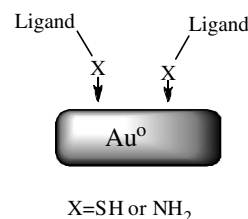


Figure 2. Colloidal gold complex with thiol (amino) ligands.

Table 10. In vitro cytotoxicity and binding efficacy of **92** and peptide-free PTX-PEG-OH (**95**)

Compound	% binding of [^{125}I]-Try ³ -BBN to		IC ₅₀ (nM)	
	BNR 11 cell line	NCI-H1299 cell line	NCI-H1299 cell line	
			12 h	96 h
PTX	—	—	35	15
No inhibitor	70	19	ND	ND
BBN ⁷⁻¹³	14	3.6		
PTX-PEG-OH (95)	ND	ND	72	44
PTX-PEG-BBN (92)	5.4	4.5	14	6

above in RAW264.7 macrophage cells. They additionally cease the stress induced secretion of proinflammatory cytokine such as $\text{TNF-}\alpha$, $\text{IL-1}\beta$ inside the macrophage cells, suggesting that they could be potentially implicated in various future therapies.⁹¹ In addition, gold nanoparticles possess attractive characteristics such as generally small size (as low as 32 nm diameter), large surface area to accommodate vast number of organic molecules, and more importantly, they have a propensity to evade phagocytic clearance by the reticuloendothelial system (RES) especially when coordinated with polymeric units such as thiolated polyethyleneglycol (PEG-SH).⁹⁰ It is also now known that the gold nanoparticles enter the tumor interstitium via its leaky vasculature (passive cellular uptake) and tend to reside at the interstitial space in tumor tissue, which is called as EPR (enhanced permeability and retention) effect. It was also reported that the gold nanoparticles could enter the tumor cells by endocytosis (active cellular uptake) especially when they are combined to another tumor marker such as $\text{TNF-}\alpha$.⁹²

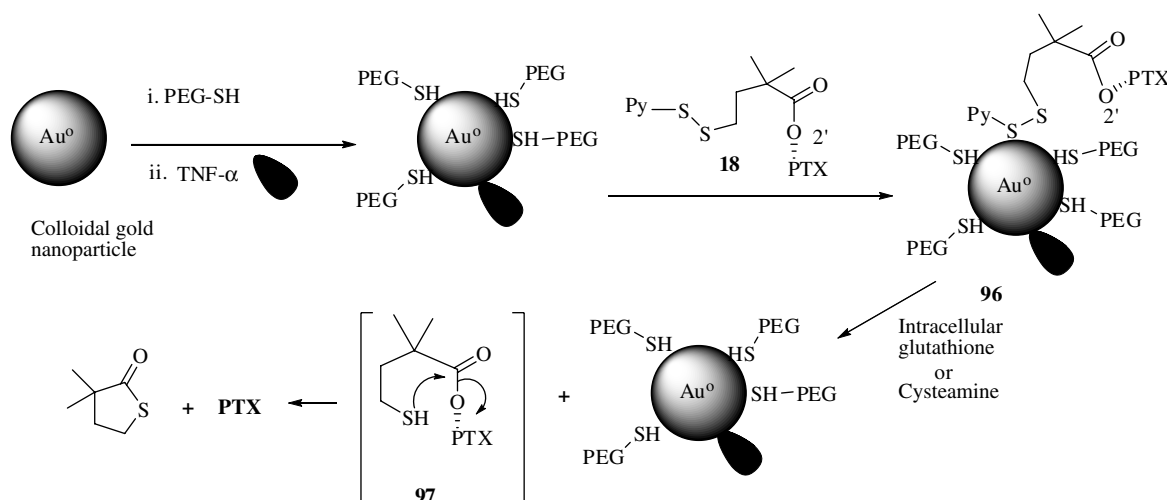
In this respect Paciotti et al.⁹² have envisioned gold nanoparticles as a plausible alternative platform for the tumor targeted delivery of cytotoxic agents. $\text{TNF-}\alpha$ (tumor necrosis factor- α), a potent cytokine anticancer agent which causes severe systemic toxicity, was subjected to tumor specific delivery by means of gold nanoparticles (cAu) and results revealed that the cAu- $\text{TNF}\alpha$ vector was effective in reducing the tumor burden; however, it was rapidly cleared out by RES.⁹² Subsequent efforts from the same investigators, a vector of TNF and thiolated polyethylene glycol (PT) embedded onto gold nanoparticles, resulted in the synthesis of a PT-cAu- TNF vehicle. They demonstrated that this vehicle escapes the RES-mediated rapid clearance and specifically increases the concentrations of $\text{TNF-}\alpha$ within the solid tumor.⁹² An attractive factor of gold nanoparticle mediated delivery is the relative ease of tracking the vehicle once it is injected into the body. In each physical state, the gold nanoparticles will have a typical color. For example in completely stabilized monodispersed

state the particles will appear as a red-burgundy color, while in a precipitated state they appear black.⁹²

Encouraged by the above results, very recently Paciotti et al.^{93,94} prepared a PTX-PT-cAu- TNF vehicle (**96**), which was composed of both $\text{TNF-}\alpha$, thiolated PTX prodrug **18**, and peglated gold nanoparticles (PT-cAu). The synthesis of the vector **96** is achieved as shown in Scheme 19. When the PTX-PT-cAu- TNF vehicle (**96**) localized into the solid tumor, it is expected to release the PTX by the intracellular glutathione mediated cleavage via the transition state **97** in a similar fashion discussed in Scheme 5.

The PTX-PT-cAu- TNF vehicle (**96**) was extensively investigated for its biological efficacy. First, the dithiothreitol (DTT) induced liberation of PTX from **96** and subsequently the bioactivity of liberated PTX was determined in vitro, against B16F10 melanoma cells. Second, for determining the extent of tumor localization of **96**, 500 μL of it was injected to B16/F10 tumor burdened C57/BL6 mice. Tumor and blood samples were collected after animals were sacrificed and estimated. The results indicate that **96** had delivered at least 7 times more of $\text{TNF-}\alpha$ and 10 times more of PTX to tumors, when compared $\text{TNF-}\alpha$ and thiolated PTX prodrug **18** alone. Third, to find out the role played by $\text{TNF-}\alpha$ in the same tumors they prepared a PT-cAu-PTX vector (without TNF), and administered with equal doses of vectors (one with TNF and without TNF). These results indicated that the $\text{TNF-}\alpha$ does play a role in the tumor uptake of the vector (by active targeting).

Given the fact that, DTT is not available inside the tumor and also in the event that the intracellular glutathione may not be available to release the PTX from **96** inside the tumor cell; they have envisioned to conduct the prodrug activation experiment with an external disulfide bond activator by an intra-tumor injection on in vivo tumor model. At this juncture, 'cysteamine' (2-Aminoethane thiol) is an approved therapeutic, was found to activate disulfide bonds, posed it could be used



Scheme 19.

as an activator for the prodrug **96** in vivo.⁹⁵ The experimental results indicate that **96** indeed was activated by cysteamine and has released the active drug, in agreement with in vitro DTT-mediated observations. Finally, the PTX-PT-cAu-TNF **96** caused tumor regression in a TNF- α sensitive tumor model.

Overall the results of this study indicate two following points: First nanoparticle mediated drug delivery takes advantage of EPR effect due to gold nanoparticles as well as active cellular uptake (targeting) due to TNF- α . Also lay the foundation to investigate multiple cytotoxic drug delivery by colloidal gold nanoparticles for a combination therapy, since gold nanoparticles possess intrinsically vast surface area and accommodate several organic molecules.

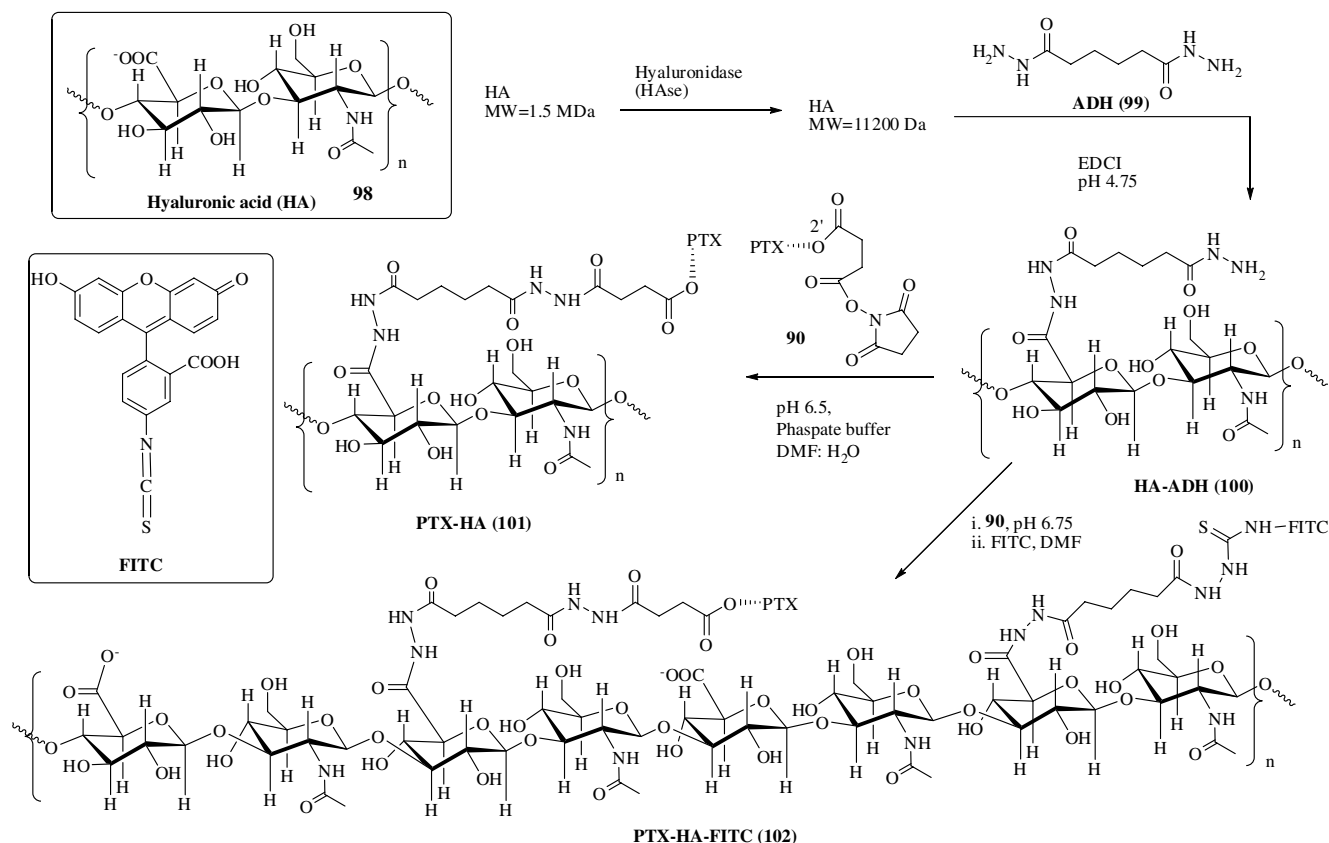
3.7. Hyaluronic acid as taxoid delivery agent

Extracellular and cell-surface-associated polysaccharide 'hyaluronic acid' (HA) was once believed as synovial fluid of joints, cartilages, and connective tissue, now has emerged as a very important target because HA mediates a vast array of biological functions such as cell proliferation, migration, inflammation, wound healing, and tumor development.⁹⁶ HA (**98**) is a polymer composed of repeating disaccharide units of glucuronic acid and *N*-acetyl glucosamine [β -(1,4)-GlcUA- β (1,3)-GlcNAc]_n, is ubiquitously found in extracellular as well as intracellular matrices of many types of tumor cells. Often, high HA levels in cells are useful in a prognosis

for malignant progression.⁹⁷ Additionally, cancer cells overexpress surface receptors such as CD44, HAS2, Hyal-2, and RHAMM, all of which are implicated in tumor invasiveness, cell migration, and proliferation.⁹⁸ The functions of the above receptors are found to be mediated through their specific interactions with HA (**98**). All the properties suggest that HA has potential as a targeting agent delivering cytotoxic agents to the tumor site. Since HA is produced by the body, no immunogenic responses are anticipated in using it as drug delivery agent. In this regard, several studies have used HA to directly target the cytotoxic drugs including taxoids and results seem encouraging.^{99,100}

Prestwich et al. have synthesized a PTX-HA conjugate with an adipic acid dihydrazide (ADH) linker as shown in Scheme 20, starting from a high molecular weight HA-polymer and PTX-2'-hemisuccinate-NHS ester.¹⁰⁰ In the synthesis, a low molecular weight HA was first generated from degradation of a high molecular weight HA-polymer by using the enzyme HASE. Then it was covalently linked to ADH (**99**) using EDCI as coupling reagent to provide HA-ADH (**100**). Finally, HA-ADH was coupled to PTX-hemisuccinate in buffer solution to yield the drug conjugate PTX-HA (**101**).

The conjugate PTX-HA (**101**) has been investigated for its in vitro cytotoxicities against four very different, SK-OV-3 (ovarian), HBL-100 (breast), HCT-116 (colon), and NIH-3-T-3 (untransformed fibroblast) cell lines, of which the first 3 cell lines overexpress HA receptors



Scheme 20.

CD44.¹⁰¹ The conjugate **101** showed activity toward only first 3 cell lines, but not toward NIH-3-T-3 cell line. This suggests HA–PTX conjugate has an affinity to HA-receptors. They have tested the activity of conjugate **101** in detail, relative to free PTX as well as premixed PTX and HA-ADH sample, against HCT-116 colon cancer cell line. The results show that conjugate **101** had better activity compared to latter. In a control experiment, HA-ADH did not show any activity at the concentrations 10 times more than conjugate **101** concentration. To further confirm the activity of conjugate **101** was due to HA-receptor mediated cellular uptake, they recently synthesized a fluorescently labeled PTX–HA–FITC drug conjugate (Scheme 20) by stepwise addition of PTX-NHS ester (**90**) and FITC to the HA-ADH adduct (**100**), which enabled them to measure the cellular uptake by using flow cytometry.¹⁰² In these experiments, the labeled drug conjugate **102** was found to be strongly absorbed by the tumor cells, SK-OV-3, HBL-100, HCT-116, that express the HA receptors, as indicated by a dose dependent shift in the fluorescent peak in FACS. No fluorescence binding or shift was noticed when **102** was treated with NIH-3-T-3 cells, clearly indicating that PTX conjugated to HA was selectively binding to and taken by HA receptor positive cells. These results further confirm the selective cytotoxicity results obtained by non-fluorescently labeled PTX–HA conjugate.

Additionally, in a competition experiment, the drug conjugate **101** did not show activity against cancer cells which were preincubated (2 h) with 100-fold excess high molecular weight HA, whereas the same drug conjugate together with 100-fold HA, or PTX alone with 100-fold excess HA showed cytotoxicity. These experiments conclude the receptor mediated binding of PTX–HA conjugate to the tumor cells and leave the notion that high molecular weight HA has slow binding properties compared to low molecular weight HA, because simultaneous addition of HA with free PTX and or PTX-HA did not protect the cells. Also in other experiment a synthesized low molecular weight HA has been rapidly absorbed by the tumor cells.¹⁰⁰

In this study, they have also carried out how an active drug (PTX) could be released after HA-PTX has been taken up by the cells. PTX-HA was incubated in 3 different cell culture medias (cell culture media with HASE enzyme, esterases, human plasma). All these media released PTX from the PTX–HA conjugate, among which esterase's media released the PTX at a faster rate compared to others, suggesting that when HA-mediated cellular uptake has been achieved the active drug could be released hydrolytically from the conjugate inside the cells by enzymatic hydrolysis. The results from this study are encouraging and raise the hope for better chemotherapeutics.

3.8. Fatty acid as taxoid drug targeting agent

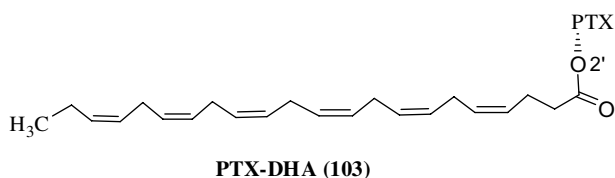
As pointed out in the introduction, rapidly proliferating cancer cells require more energy than normal cells and consequently consume increased amounts of primary metabolites including natural fatty acids, as biochemical precursors.¹⁰³ Most human diets contain a variety of fatty acids and these are categorized as saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids (PUFAs). PUFAs are considered as essential fatty acids because human body cannot synthesize them and they are not found adequately in most diets, so they must be provided as supplements. These PUFAs were subdivided into several classes based on location of the first double bond from ω -methyl group located at opposite end to the carboxylic acid (Table 11).¹⁰⁴ Essential PUFAs such as linolenic acid (LNA), docosahexaenoic acids (DHA) were previously associated with increased risk of several types of cancers such as breast, colon, and prostate. Also reported was that ω -6 PUFAs enhance the tumorigenesis and metastasis in animals by several mechanisms of action, whereas ω -3 PUFAs inhibit the growth of initiated cancer cells.¹⁰⁵ All these properties suggest that PUFAs could serve as tumor marking agents and could selectively deliver cytotoxic drugs to the tumor environment. In this regard attempts have already been directed toward targeting cytotoxic drugs such as 4'-dimethyldeoxydophyllotoxin while

Table 11. Representative examples of fatty acids

Class	Name	Structure of fatty acid	No. of carbons (double bonds)	Subclass
Saturated fatty acids	Lauric acid	$\text{H}_3\text{C}-\text{CCCCCCCCCCCCCCCCOOH}$	12	
	Palmitic acid	$\text{H}_3\text{C}-\text{CCCCCCCCCCCCCCCCCCCCOOH}$	16	
	Stearic acid	$\text{H}_3\text{C}-\text{CCCCCCCCCCCCCCCCCCCCCCCCOOH}$	18	
Mono Unsaturated fatty acids	Oleic acid	$\text{H}_3\text{C}-\text{CCCCCCCC=CCCCCCCCCOOH}$	18 (1)	ω -9
Poly unsaturated fatty acids	Linoleic acid (LA)	$\text{H}_3\text{C}-\text{CCCC=CCCC=CCCCCCCCCOOH}$	18 (2)	ω -6
	Linolenic acid (LNA)	$\text{H}_3\text{C}-\text{C=C=C=CCCC=CCCCCCCCCOOH}$	18 (3)	ω -3
	Eicosapentanoic acid	$\text{H}_3\text{C}-\text{C=C=C=C=C=CCCC=CCCCCCCCCOOH}$	20 (5)	ω -3
	Docosahexaenoic acid (DHA)	$\text{H}_3\text{C}-\text{C=C=C=C=C=C=C=CCCC=CCCCCCCCCOOH}$	22 (6)	ω -3

other side taxoids drugs are being explored and the results seem very encouraging.^{106–108}

Bradley et al. have synthesized a PTX–DHA conjugate (**103**) in a single step reaction between PTX and docosa-hexaenoic acid using DCC as a catalyst and formulated in 10% Cremophor EL-P/10% ethanol/80% normal saline.¹⁰⁷



Two very important points are worth mentioning here. The PTX is a hydrophobic molecule and found to be a very good substrate for P-glycoprotein (Pgp) efflux pumps, which is why it was inactive against several resistant (MDR) cancer cells. Conjugation of a fatty acid to PTX could make the drug a much better substrate for efflux pumps hence, the therapeutic effects could be limited. PTX is already found to be insoluble in aqueous solvent and conjugation with a fatty acid could make it much less soluble in aqueous media therefore the conjugate must be formulated in a vehicle which may develop undesirable side effects.

PTX–DHA conjugate **103** was tested against NCI's 56 human tumor cell lines which are derived from human leukemias, melanomas, lung, colon, ovarian, renal, and breast tumors. Results indicated that the conjugate **103** showed at least 10 orders of magnitude lower cytotoxicity than PTX. PTX–DHA conjugate (**103**) showed very interesting biochemical properties including no microtubule assembly activity (in vitro) at 10 μ M concentration, however, microtubule bundle formation when SKOV3 cells were treated with 5 μ M concentration of **103**. Very interestingly, it was found to be a relatively (4 times) weaker substrate for Pgp-efflux pumps, compared to PTX. The PTX–DHA conjugate has also increased the maximum tolerated dose to 4.5, 3.6, 2.9 times compared to PTX for mice, rats, and dogs, respectively.

In in vivo experiments on female mice inoculated with M109 lung tumor, the PTX at optimum dose (OD) (20 mg/kg \times 5 days) has shown some tumor growth inhibition but tumors continued to grow as in control experiment after 10 days and also PTX developed severe hind-limb paralysis. However, the PTX–DHA (**103**) at OD (120 mg/kg) eliminated all the tumors for 60 days on 10/10 mice and did not show any hind-limb paralysis. One point to be noted here is the activities of PTX–DHA mentioned here are only at higher doses, since at equimolar concentration to PTX (i.e., 27.4 mg/kg) the conjugate **103** did not eliminate any tumors. In the same study it was investigated that free DHA was also lethal to CD2F1 mice

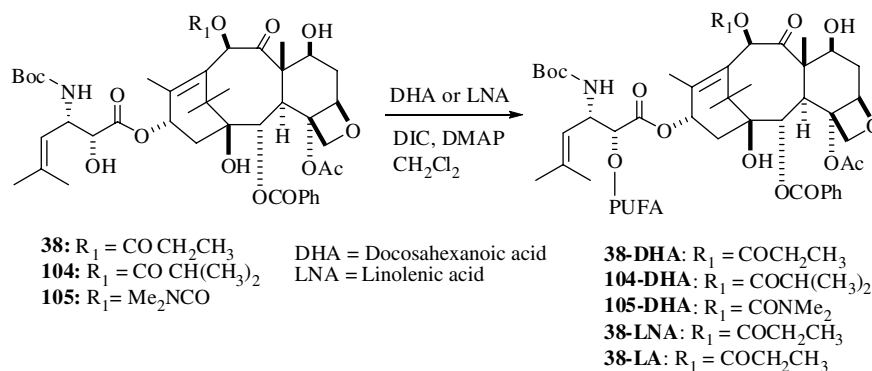
at >60 mg/kg (iv for 5 days) and a ethyl ester of DHA was non toxic.

Further in vivo results of PTX–DHA (**103**) are very promising. When it was tested on H-29 human colon carcinoma implanted into BALB/c-nu/nu mice, it showed superior activity with 40% complete responses and 60% partial responses, whereas PTX showed no response. To verify the toxicity of PTX–DHA was due to PUFA mediated targeting, they have conducted several pharmacokinetic studies of tumors, plasma, and muscle of M109 tumor bearing mice. They concluded that PUFA actually targeted the drug to tumors. The PTX–DHA (**103**) has shown several improved biochemical properties and entered into phase II clinical study, with trade name 'Taxoprexin[®]' for several types of cancer.¹⁰⁹

Recently Ojima et al. have also reported the synthesis of several PUFA–taxoid conjugates.¹⁰⁸ Their main purpose was to target the taxoid drugs to resistant (MDR) cancer cells. Though **103** proved to be a weaker substrate for Pgp-efflux pumps, there is a chance that, once PTX is released slowly from the conjugate, it will be captured and eliminated by efflux pumps. For this reason they have selected to use highly active taxoid (**38**) showing activity against resistant tumor cells (which express Pgp-efflux pumps). They have also investigated other PUFAs including DHA which already produced efficacious results.¹⁰⁷ The synthesis of fatty acid conjugates was carried out by coupling between taxoid and a PUFA using DIC and DMAP as catalysts. Scheme 21 shows only conjugates with improved performance. In this study they have also resynthesized PTX–DHA (**103**) for comparison studies.

The taxoid–fatty acid conjugates were tested against drug sensitive human ovarian tumor xenograft A121 (Pgp negative) cell line and drug resistant human colon tumor xenograft DLD-1 (Pgp positive) in SUID mice. The **38**–DHA conjugate showed complete tumor regression of the DLD-1 tumor in 5/5 mice at 80 mg/kg dose administered at 3 day interval starting from day five after tumor implant. It also showed 187 days of growth delay time whereas PTX–DHA conjugate (**103**), as expected, was ineffective against the same tumor xenograft. Likewise **38**–DHA showed delayed tumor growth for more than 186 days and complete tumor regression in 5/5 mice of drug sensitive A121 xenograft at 30 mg/kg \times 3 doses. Similar results were obtained with **105**–DHA, whereas PTX–DHA in this case cured only 2/5 mice and tumors appeared after 150 days in 3/5 mice. The relatively low efficacies of PTX–DHA compared to previous study can be attributed to relatively low dose used in the latter study as well as low cytotoxicity of PTX compared to taxoid **38**.

Prompted by the results of taxoid–DHA conjugates, they have also investigated the other PUFA conjugates for the activity. Notable is the **38**–LNA conjugate tested against drug resistant human colon xenograft DLD-1 and results showed that it caused complete tumor regression in 2/5 mice tested with tumor growth delayed



Scheme 21.

up to more than 109 days. Authors indicated that **38-LNA** exhibited overall better activity compared to **38-DHA** conjugate, despite some toxicity noted with **38-LNA**. Moreover **38-LA** conjugate synthesized in this study did not show fruitful results. Clearly, the results obtained from the above two studies, that have utilized fatty acids to transport taxoid drugs to tumor cells, are very promising and could lead to better and alternative chemotherapeutics compared to parent taxoid drugs.

Owing to time and space constraints, the literature cited in this review article is not exhaustive, so the reader is referred to a review article published with a broader scope, during the preparation of this article,¹¹⁰ and also a perspective article appeared during the revised stage of this manuscript.¹¹¹

4. Conclusions

Significant efforts have already appeared to improve the aqueous solubility and stability of the taxoid drugs in plasma and also circumvent toxic side effects posed by them. Such efforts have resulted in two different sets of protocols as categorized in this article; they are prodrug and drug targeting methods. In many studies both of these methods led to taxoid derivatives with significantly enhanced biochemical properties. As outlined at the end of every section several drug conjugates demonstrated potential success in normal taxoid chemotherapy. Noteworthy are the antibody and fatty acid mediated drug targeting protocols already entered into the clinical trials not only for the taxoid drug, but also for several other cytotoxic drugs such as doxorubicin.

The designed linkers that couple the drug and tumor marking agent to form a drug conjugate so far belong to a restricted subclass. Very importantly, the self-immolating linker strategy, which was originally designed by Carl et al.¹¹² in the early '80s, has now been carefully applied in several cases, however the approaches still warrant some new classes of linkers that would increase the stability of the drug in the plasma and liberate the active taxoid drug readily upon reaching of the conjugate to the tumor site and or by the tumor microenvironment such as low pH. This was often the

limitation encountered by some of the drug conjugates described in this article.

We strongly believe that any anticancer drug or potential anticancer agent that ultimately operates by cellular death mechanism is not fully devoid of side effects due to non-selective toxicity. Therefore prodrug and drug targeting protocols reviewed in this article will play a pivotal role in future generations of anticancer agents that either belong to taxoid class (tubulin binders such as epothilones, discodermolide, eluetherobins, peloruside, etc.) or other classes. There is little doubt that the successful protocols described in this article, eventually, could be applied to newly discovered drugs.

Acknowledgments

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