Recent Advances in the New Generation Taxane Anticancer Agents

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Abstract: Recent advances in the design and preclinical evaluations of promising new generation taxane anticancer agents are reviewed in this article. Paclitaxel and docetaxel are two of the most important anticancer drugs today. However, recent reports have shown that treatment with these drugs often encounters undesirable side effects as well as drug resistance. Therefore, it is important to develop new taxane anticancer agents with fewer side effects, superior pharmacological properties, and improved activity against drug-resistant human cancers.

Structure-activity relationship (SAR) studies led to the discovery of a series of highly active second-generation taxanes. One of them, "Ortataxel" (SB-T-101131, IDN5109, BAY59-8862), exhibits excellent activity against a variety of drug-sensitive and drug-resistant cancer cell lines, as well as human tumor xenografts in mice. It is orally active and is currently in phase II clinical trials.

Photoaffinity labeling of microtubules and P-glycoprotein using photoreactive radiolabeled taxoids has disclosed the drug-binding domain of tubulin as well as Pgp. Together with information on microtubule-bound fluorine-labeled taxoids obtained by solid-state NMR studies, the bioactive conformation of paclitaxel and taxoids appears to emerge.

Novel taxane-monoclonal antibody (mAb) immunoconjugates, have shown highly promising results for the tumor-specific delivery and release of an extremely cytotoxic, second-generation taxane. Also, another novel series of second generation taxanes conjugated with n-3 polyunsaturated fatty acids, e.g. decosahexaenoic acid (DHA), has exhibited impressive antitumor activity with minimum general toxicity against the highly drug-resistant DLD-1 human colon cancer xenografts in SCID mice.

Key Words: Taxane, Multidrug resistance, P-glycoprotein, MDR-reversal agents, Photoaffinity label, Tumor-activated prodrug, Monoclonal antibody, Fatty acids.

INTRODUCTION

Taxol® (paclitaxel), a naturally occurring diterpenoid isolated from the bark of the Pacific yew tree (*Taxus brevifolia*), is currently considered one of the most important drugs in cancer chemotherapy. Paclitaxel has been approved by the FDA, for the treatment of various human tumors such as metastatic breast cancer, advanced ovarian cancer, and Kaposi's sarcoma [1,2]. A semisynthetic analog of paclitaxel, Taxotère® (docetaxel), has also been approved by the FDA, for the treatment of advanced breast cancer. These two taxane anticancer drugs are currently undergoing clinical trials worldwide for the treatment of other cancers (e.g. lung, head and neck, prostate, and cervical cancers) as well as in combination with other anticancer agents. Paclitaxel and docetaxel are the first members of a new class of microtubule-stabilizing anticancer agents. These drugs bind to the β-tubulin subunit of the tubulin heterodimer, accelerate the polymerization of tubulin, and stabilize the resultant microtubules to inhibit their depolymerization. This inhibition results in the arrest of the cell division cycle mainly at the G2/M stage, which triggers the cell-signaling cascade, leading to apoptosis of the cancer cells [3-6].

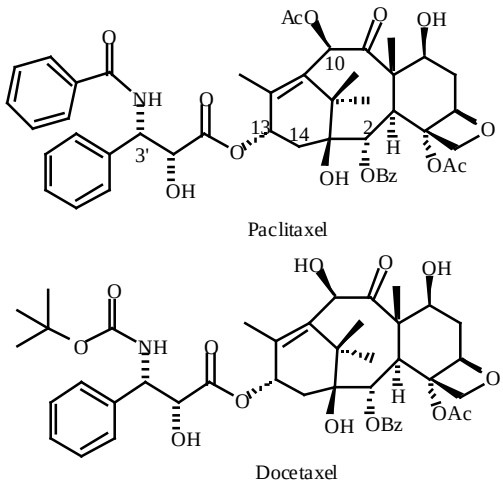

Fig. (1). Structures of paclitaxel and docetaxel.

Despite their potent antitumor activity, the extensive clinical use of these taxane anticancer drugs, has been experiencing severe side effects as well as drug resistance.

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Accordingly, it is essential to develop new anticancer agents with fewer side effects, improved pharmacological properties, and activity against cancers, not effectively treated by existing anticancer drugs.

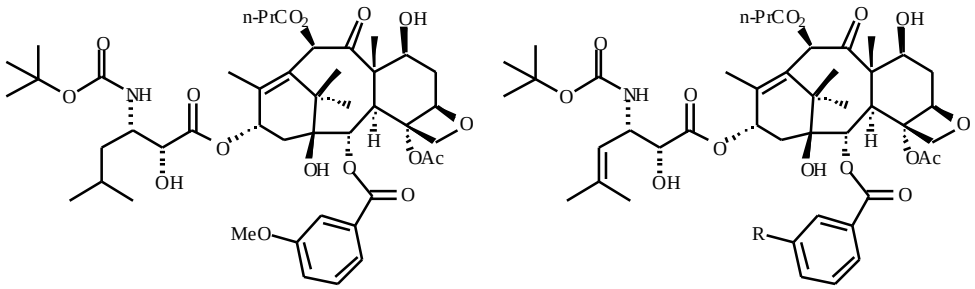
In this review, we describe our recent approaches to overcoming multidrug resistance (MDR) and circumventing undesirable side effects associated with taxane chemotherapy.

DEVELOPMENT OF SECOND-GENERATION TAXANE ANTICANCER AGENTS

Our early structure-activity relationship (SAR) study has shown, that the phenyl moieties of paclitaxel at the C-2, C-3', and C-3'N positions are not essential for its potent cytotoxicity and tubulin-binding ability [7]. It was found that the incorporation of a simpler alkyl or alkenyl substituent at C-3', considerably increased activity against drug-sensitive as well as drug-resistant cancer cell lines. More importantly, appropriate modifications at the C-10 and C-3' positions have led us to the development of the "second-generation" taxane anticancer agents. The most significant results with this series of taxanes was their substantially increased potency against drug-sensitive human cancer cell lines, as well as remarkable activity against drug-resistant cell lines, expressing MDR phenotypes (e.g., IC_{50} = 2.1-9.1 nM; paclitaxel IC_{50} = 300 nM against MCF7-MDR) [8]. Taking advantage of the reported finding that modifications at *meta* position of the C-2 benzoate considerably increased paclitaxel's cytotoxicity against the P-388 cell line [9-11], we designed and synthesized a series of second-generation taxanes with modifications at the C-2, C-3', and C-10 positions, and assayed their activities [12]. Some of these second-generation taxanes were found to possess 2–3 orders of magnitude higher potency against drug-resistant cancer cell lines LCC6-MDR and MCF7-MDR, as compared to paclitaxel and docetaxel. As Table **1** summarizes, three of these taxanes (SB-T-11033, 121303, and 121304, Fig. (**2**)) show essentially no difference in activity against drug-resistant and drug-sensitive cell lines, which are categorized as "advanced second-generation taxane anticancer agents" [12].

The low water solubility of paclitaxel and docetaxel is a significant drawback to their clinical use. 14β-Hydroxy-10-deacetylbaccatin III (14β-OH-DAB) (Fig. (**3**)), isolated from the needles of the Himalayan yew tree (*Taxus wallichiana Zucc*.), has substantially better water solubility than 10-deacetylbaccatin III (DAB) due to the presence of an extra hydroxyl group at the C-14 position. We expected that taxanes derived from 14β-OH-DAB would have improved water solubility, bioavailability, and reduced hydrophobicity-related drug resistance. Thus, various taxanes were synthesized from 14-OH-DAB and their cytotoxicity as well as antitumor activity *in vivo* against human cancer xenografts was evaluated [13].

Among the second-generation taxanes derived from 14-OH-DAB, SB-T-101131 was selected as the best compound for preclinical studies (with the code name of IDN5109) by Indena, S.p.A. and several medical institutions in the U.S. and Europe. This selection was made mainly because SB-T-101131 (IDN5109) did not show any appreciable



SB-T-11033

SB-T-121303 (R = OMe)
SB-T-121304 (R = N_3)

Fig. (2). Advanced Second-Generation Taxane Anticancer Agents.

Table 1. Cytotoxicity (IC_{50}, nM)[a] of Advanced Second-generation Taxane Anticancer Agents.

Taxane	LCC6-WT	LCC6-MDR	MCF7	MCF7-MDR
Paclitaxel	3.1	346	1.7	300
Docetaxel	1.0	120	1.0	235
SB-T-11033	0.9	0.8	0.36	0.33
SB-T-121303	1.0	0.9	0.36	0.43
SB-T-121304	0.9	1.2	0.9	1.1

a. The concentration of compound which inhibits 50 % of the growth of cancer cell line after 72 drug exposure.

neurotoxicity or cardiotoxicity and exhibited much less general toxicity while maintaining excellent cytotoxicity, which provides a wider therapeutic window. IDN5109 has shown much higher potency than paclitaxel, across the board against the NCI's panel of drug-resistant cancer cell lines. IDN5109 exhibited 50-80 times higher potency than paclitaxel in inducing apoptosis against human cancer cell lines MCF-7 (breast) and CEM VBL-R (leukemia) [13,14]. IDN5109 has been found to be highly active orally with excellent bioavailability (50.4% in mice), which provides the first highly effective orally active taxane anticancer agent [15-17] (Note: paclitaxel is not orally active). Fig. (**4**) illustrates the comparison of antitumor activities of IDN5109 and paclitaxel *via* i.v. and p.o. routes.

IDN5109 also exhibits remarkable antitumor activity against drug-resistant tumors expressing Pgp [14] such as human colon carcinoma SW-620 xenograft in mice in sharp contrast with paclitaxel, which is not effective even by i.v. at its maximum tolerated dose (MTD) [17]. Moreover, it has been observed that the best antitumor efficacy of IDN5109 is often reached with doses lower than MTD, which indicates an excellent therapeutic index of this anticancer agent [18]. It is noteworthy that IDN5109 has ability to inhibit the function of the Pgp efflux pump, i.e., IDN5109 has a built-in ability to modulate Pgp efflux pump, which brings about superior growth inhibition activity against Pgp-expressing drug-resistant tumors [17]. IDN5109 (SB-T-101131) was advanced to human clinical studies and is currently in multi-centered phase II trials sponsored by Bayer Corporation under the name BAY59-8862 or "ortataxel".

MDR CAUSED BY OVEREXPRESSION OF P-GLYCOPROTEIN (PGP)

The major mechanism of resistance to hydrophobic drugs involves, overexpression of ATP–dependent transporter proteins that act as efflux "pumps" and thus lower the

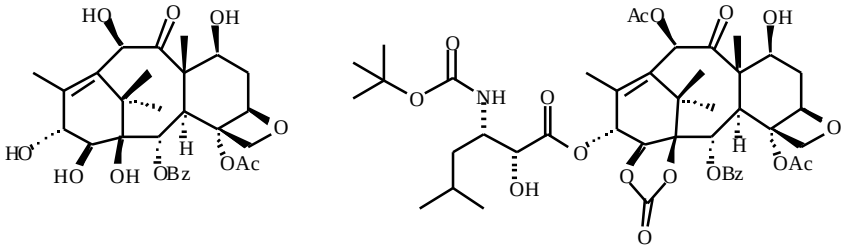


Fig. (3). Structures of 14β-OH-DAB and Ortataxel (SB-T-101131, IDN5109).

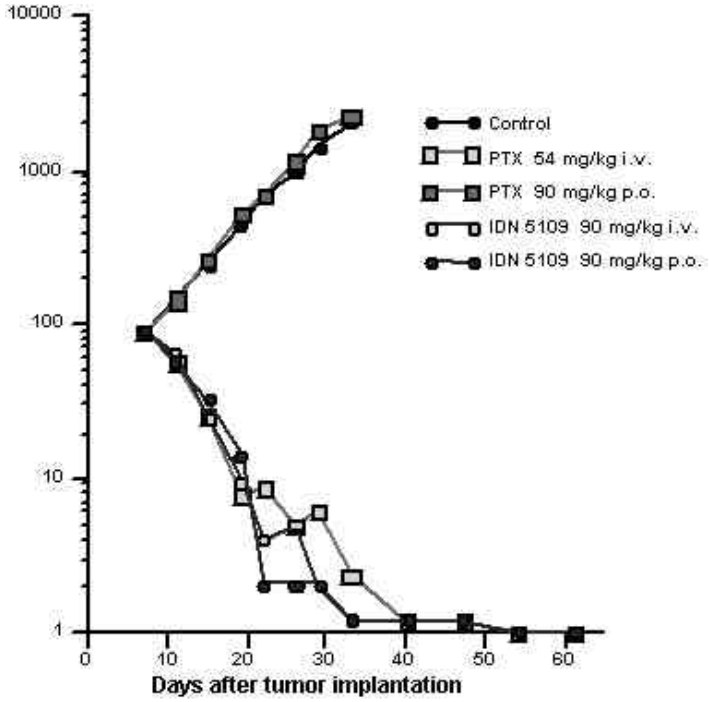


Fig. (4). Growth curves (mg) of human MX-1 mammary carcinoma xenografts in mice after treatment with IDN5109 (PTX = paclitaxel; i.v. = intravenous administration; p.o. = oral administration).

intracellular drug concentration to non-toxic levels. ATP-binding cassette (ABC) transporters known to be involved in the MDR phenomenon include Pgp (MDR1 or ABCB1 gene), MRP1 (ABCC1) and ABCG2. Other ABC transporters (ABCA2, ABCB11, ABCC2-5) have also been shown to transport hydrophobic drugs, but further studies are required to link them with MDR [19]. These proteins are widely expressed in the peripheral tissues and at the blood-brain barrier (BBB), where they play a key role in regulating the permeability to the central nervous system.

P-Glycoprotein (Pgp), a 170 kDa transmembrane protein, is by far the most studied of these transporter proteins. Pgp is composed of two homologous halves, each containing six transmembrane domains and an ATP binding/utilization domain, separated by a flexible linker polypeptide (Fig. (**5**)). Various drugs are known to have the ability to modulate its action, acting as "MDR-reversal agents", e.g., verapamil, dexverapamil, cyclosporin A, the phenothiazines, quinine, quinacrine, quinidine, amiodarone, some neuroleptics, tamoxifen, progesterone, dexniguldipine, GF-902128, PSC-833, VX-710, etc. [19]. Most MDR-reversal agents, block drug transport by acting as competitive or noncompetitive inhibitor [20] and by binding to drug binding sites or other modulator binding sites [21], leading to allosteric changes. However, none of the modulators has been shown to disrupt ATP binding [22].

Benzophenone photophores are known to bind to α-carbons of polypeptide chains with very high selectivity, when activated by ultraviolet light (360 nm). In the labeling process, the oxygen radical of the benzophenone moiety abstracts a hydrogen atom to the protein, while the carbon radical binds covalently to an α-carbon of the protein; the adduct is a tertiary alcohol [23].

Critical new insight into the binding pocket(s) of hydrophobic anticancer drugs in Pgp has been obtained by our photoaffinity labeling study of Pgp using two tritium-labeled photoreactive paclitaxel analogs bearing a 4-benzoyldihydrocinnamate (BzDC) moiety. BzDC-paclitaxel analogs were found to bind to the C-terminal part of murine Pgp overexpressed in paclitaxel- and vinblastine-resistant cell lines, which was confirmed by Western blot analysis [24,25]. While the labeled regions of the protein spanned over 80 residues in each experiment, both probes labeled the same bundle of helices, suggesting the existence of a defined binding site for paclitaxel. [^{3}H]-7-BzDC-paclitaxel labeled a piece comprising TM7, the TM7-TM8 loop, and the first half of TM8 while [^{3}H]-3'N-BzDC-paclitaxel labeled a region comprising the last 15 residues of TM12 and the subsequent intracellular loop (Fig. (**5**)).

Recently, the structure of the *E. coli* lipid flippase MsbA has been determined at 4.5 Å resolution [26]. MsbA is the bacterial transporter of the MDR-ABC group most closely related to Pgp by sequence homology, in spite of the fact that Pgp is monomeric, while the functional form of MsbA is a homodimer. The sequence of MsbA is 36% and 32% homologous to the N- and C-terminal halves, respectively, of the human Pgp. Each monomer comprises a transmembrane (TM) domain (about 52Å in length) composed of six α-helices, linked to a nucleotide-binding domain (NBD) by a short bridging domain. When associated, two monomeric units form a cone-shaped cavity, 45 Å wide at its base, in the cellular membrane. The NBD's are situated about 50 Å apart in the MsbA dimer [26].

Based on the substantial sequence homology between murine Pgp and MsbA, we have extrapolated the hypothetical binding site of taxanes in the Pgp based on the MsbA structure. The most interesting and significant issue addressed by this study is to determine whether the two photoreactive paclitaxel probes, [^{3}H]-7-BzDC-paclitaxel and [^{3}H]-3'N-BzDC-paclitaxel, bind to two totally different areas or these probes bind to a specific region of the Pgp, indicating the existence of unique binding site for the paclitaxel core structure.

A hypothetical paclitaxel analog, "bis-BzDC-paclitaxel", bearing two BzDC groups at the C-7 and C-3'N positions was modeled. Both benzophenone groups were replaced by

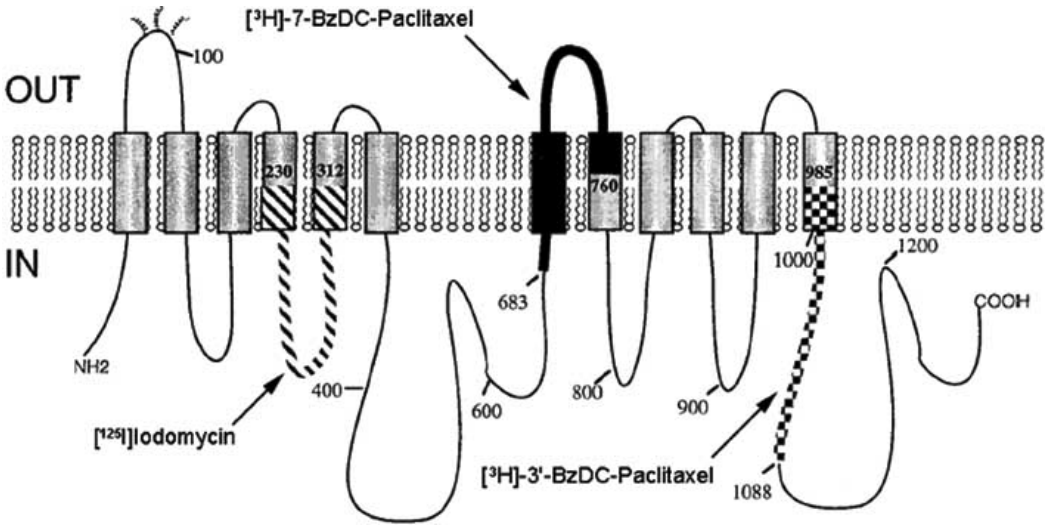


Fig. (5). Schematic illustration of the Pgp, showing the regions labeled by [^{3}H]-3'-BzDC-paclitaxel (checked), [^{3}H]-7-BzDC-paclitaxel (solid), [^{125}I]iodomycin (striped) [24].

1,1-diphenylethanol moieties as found in the post-labeling tertiary alcohol adduct. Molecular dynamics simulations (CVFF force field [27] in InsightII, Accelrys) were performed on this "bis-BzDC-paclitaxel" to probe a possible range of the intramolecular distance between the two methyl carbons mimicking the α-carbons of the labeled Pgp amino acid residues. Segments of MsbA corresponding to the labeled sections of the Pgp were identified from the Eco-MsbA/Human MDR1/Mouse MDR1 sequence alignment and localized onto the 3D structure of MsbA. As illustrated in Fig. (**6**), the major part of the two labeled segments is actually within the intramolecular distance between the two BzDC groups of "bis-BzDC-paclitaxel". This result strongly suggests the existence of a unique binding region for paclitaxel in the C-terminal half of the Pgp. Possible existence of drug-specific binding sites in Pgp is also indicated in the case of iodomycin, a daunomycin photoreactive analog, which labeled a totally different area of the Pgp, located in the N-terminal half between TM4 and TM5 [28] (Fig. (**5**)).

Besides Pgp, selected TRA's were found to modulate efflux pumps mediated by the MDR-associated ABC-transporters MRP-1 and BCRP [33,34]. Six TRA's have been identified as triple-modulators to date, which can effectively modulate mitoxantrone efflux from drug-resistant cancer cell lines overexpressing Pgp, MDR-1 and BCRP. Among these, SB-RA-310124 (Fig. (**9**)) exhibited the best activity as broad-spectrum modulator, and has been identified as the lead drug candidate for further pharmacological evaluations directed toward clinical applications [33,34].

EXPLORING THE TUBULIN-BOUND PACLITAXEL CONFORMATION, INSIGHTS FOR STRUCTURE-BASED DRUG DESIGN

Photoreactive paclitaxel analogs have also been used to map out the contact region of paclitaxel with its primary cellular target, β-tubulin [35-37]. Most notably, [^{3}H]-7-BzDC-paclitaxel has been shown to label exclusively a single amino acid residue (Arg^{282}) in the M loop of β-tubulin of microtubule. Initially combined with the electron

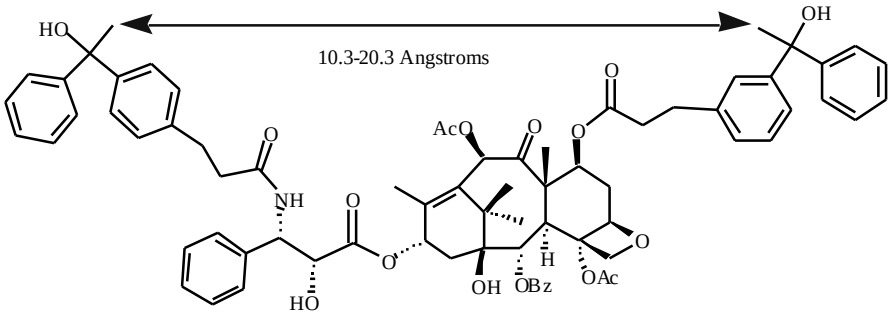


Fig. (6). Possible binding sites (in white and gray) of paclitaxel on Pgp and the structure of the modeled virtual double photoaffinity probe.

Taking into account the strong affinity of BzDC-paclitaxel analogs to Pgp and reports indicating that some naturally occurring taxanes from *Taxus cuspidata*, increased the accumulation of vincristine in MDR tumor cells [29,30], we developed a series of novel "taxane MDR-reversal agents (TRAs)" based on the baccatin III skeleton, which is a crucial component of paclitaxel and other taxane anticancer agents. These TRA's have substitutions at the C-7 and C-10 positions by Bz-DC, Bz-Cinnamoyl and other planar hydrophobic groups [31]. It should be noted that baccatin III itself is not a substrate for Pgp and does not show any appreciable MDR-reversal activity [24]. Several of these TRA's exhibited excellent MDR-reversal activity when administered in combination with paclitaxel against both LCC6 and MCF-7 MDR expressing cell lines (Fig. (**7**)). Another salient feature of these TRA's is their lack of appreciable cytotoxicity up to their solubility limit, which makes them ideal combination drugs with paclitaxel and other taxane anticancer agents in the treatment of MDR-expressing tumors [32]. The mechanism of action of these TRA's has been confirmed by comparing the accumulation of paclitaxel in Pgp-expressing cancer cells in the absence and presence of a TRA. Representative results are shown in Fig. (**8**) wherein the TRA is SB-RA-31012.

crystallographic structure of the αβ-tubulin dimer [38], these labeling results have provided the visualization of the orientation and insight about the conformation of paclitaxel in its tubulin binding site [37].

More recently, a computational docking protocol guided by the BzDc tether has been developed and combined with conformational information inferred from rotational-echo double-resonance solid state NMR (REDOR-NMR) experiments on microtubule-bound 2-(*p*-fluorobenzoyl)paclitaxel [39] to delineate a unique tubulin-binding structure of paclitaxel, coined "REDOR-taxol"[*]. While "REDOR-taxol" agrees with the "T-taxol" model proposed by Snyder *et al.* [40] on the location of the C-2, C-3' and C-3'N pharmacophoric groups of paclitaxel in the binding site, the C13 side chain adopts a markedly different conformation, favoring hydrogen bonding of the C-2' hydroxy group to His 227, rather than to Arg 359 (Fig. (**10**)). A conformationally constrained macrocyclic taxoid bearing a linker between the C-14 and C-3'N positions has been designed and synthesized to enforce this "REDOR-taxol" conformation. This novel

[*] Sun, L.; Geney, R.; Geng, X.; Ojima, I. Abstracts of Papers of the American Chemical Society 228: 97-MEDI, Aug. 22 2004

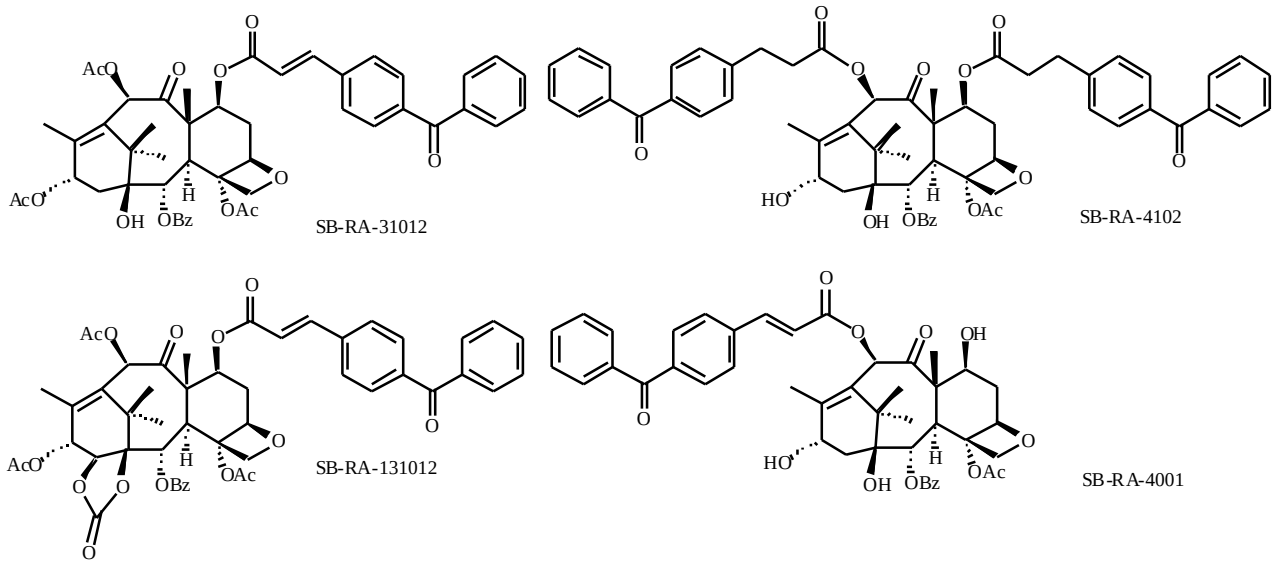


Fig. (7). Taxane MDR-reversal agents and their reversal activity modulation on two human breast cancer cell lines. %Recovery = [1-[IC_{50}(reversal agent+paclitaxel)/IC_{50}(paclitaxel)]]x100.

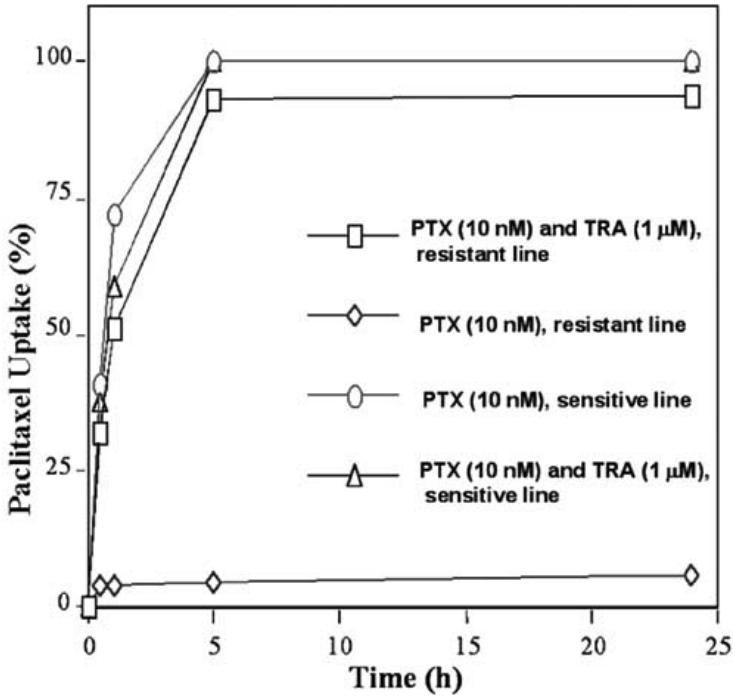


Fig. (8). Paclitaxel (PTX) accumulation with a TRA (SB-RA-31012) in MDA-435/LCC6 Cells.

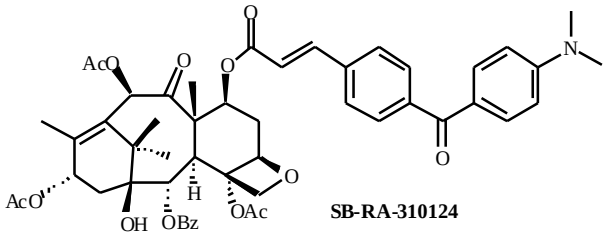


Fig. (9). Structure of broad-spectrum taxane reversal agent SB-RA-310124.

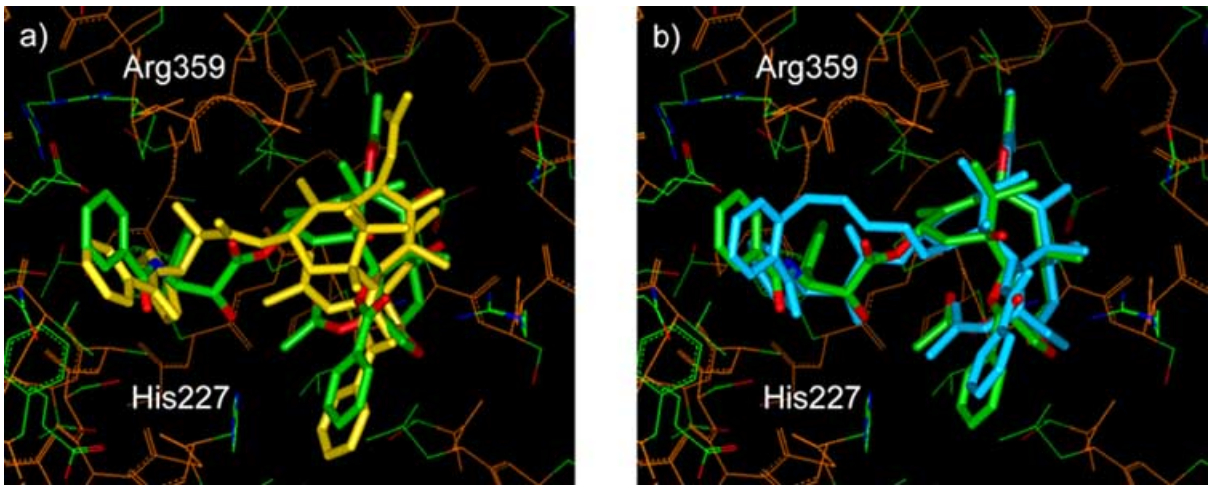


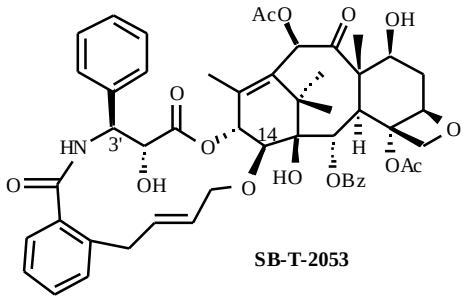
Fig. (10). Proposed tubulin-binding models of paclitaxel. (a) Overlay of the docked "REDOR-taxol" (green) and "T-taxol" (yellow) structures. (b) Overlay of "REDOR-taxol" (green) with SB-T-2053 (blue).

taxoid, SB-T-2053 (Fig. (**11**)), exhibits cytotoxicity on the same order of magnitude as paclitaxel against MCF-7 and LCC-6 human breast cancer cells (wild type and drug-resistant). More importantly, SB-T-2053 induces *in vitro* tubulin polymerization at least as well as paclitaxel, which more directly validates our drug design process. These recent results, together with the disclosure by Kingston *et al.* of highly active constrained analogs of paclitaxel [41], open a new avenue for designing next generation taxoids and other microtubule-stabilizing agents based on the refined structural information of tubulin-drug complexes, in accordance with typical enzyme-inhibitor medicinal chemistry approaches.

TUMOR SPECIFIC NOVEL MAB-SECOND GENERATION TAXOID CONJUGATES

Current cancer chemotherapy is based on the premise that rapidly proliferating tumor cells are more likely to be killed by cytotoxic drugs. However, in reality, the difference in activity of current drugs against tumor tissues in comparison to healthy tissues is relatively small. In fact, it is well known that representative cytotoxic chemotherapeutic agents like paclitaxel, cisplatin, doxorubin, and other widely used anticancer drugs cannot distinguish cancer cells from normal cells. This unfortunate feature constitutes a major basis for a variety of undesirable side effects associated with these drugs in cancer chemotherapy, i.e., the amount of drug required to achieve clinically effective level of cell kill often causes severe damage toward actively propagating non-malignant cells such as cells of the gastrointestinal tract and bone marrow, resulting in a variety of undesirable side effects [42]. Therefore, a continuing challenge in cancer treatment is to develop new cytotoxic agents with greater selectivity for the tumor.

Tumor-targeted delivery approach is one important approach to achieve this goal. In this approach, the inherent differences between normal and cancer cells have to be



SB-T-2053

Taxoid	LCC6-WT[b]	LCC6-MDR[c]	MCF7-S[d]	MCF7-R[e]
Paclitaxel	4.5±0.8	323±23	3.0±0.3	518±71
SB-T-2053	15±1.6	455±38	42±2.3	1066±59

a. The concentration of compound which inhibits 50% of the growth of human tumor cell line after 72 h drug exposure; b. LCC6-WT: human breast carcinoma; c. LCC6-MDR: MDR1 transduced line; d. MCF7-S: human breast carcinoma; e. MCF7-R: MDR phenotype human breast carcinoma.

Fig. (11). Structure and *in vitro* cytotoxicity (IC_{50}, nM[a]) of conformationally constrained macrocyclic taxoid SB-T-2053.

identified first. Then, new chemotherapeutic agents with improved tumor specificity are developed by taking advantage of these differences. The basic premise of this method is that, the conjugation of a drug to a tumor-specific molecule renders the drug inactive until it reaches the target site. Once at the tumor site, the conjugated drug binds to the surface of tumor cells and is internalized *via* endocytosis or related mechanism, and release the original drug from the carrier to restore its potency. This type of drug-conjugates can be categorized as "tumor-activated prodrugs" (TAP's) [42].

The discovery of antigens that are particularly overexpressed on the surface of cancer cells suggests that by using certain antibodies to selectively bind to the tumor cells, malignant tissues could be distinguished from normal tissues [43]. Monoclonal antibodies (mAbs), which have shown high binding specificity for tumor-specific antigens, could be used as targeting agents [42,43].

The high potential of mAb-based TAP strategy was recognized in 1980s and early attempts were made. Methotrexate [44], daunorubicin [45], vinca alkaloids [46], mitomycin C [47], idarubin [48] and *N*-acetylmephalan [49] were linked *via* non-readily-cleavable amide bond to murine monoclonal antibodies. These mAb-drug conjugates displayed impaired affinity *in vitro*, and unfavorable pharmacokinetics in animals. Cleavable linkers, such as tetrapeptide spacer [50,51], *cis*-aconitic [52-54], hydrazide [55], thiocarbamoyl [56], have also been used to conjugate cytotoxic drug to antibodies. Although these "first generation mAb-drug conjugates" showed some impressive activity in animal models, they all failed in the human clinical trials. The summary of the in-depth analysis by Chari and his collaborators [42] on the failure is as follows: (a) The first generation conjugates were often not sufficiently "humanized"; (b) The cytotoxic drug component of those immunoconjugates was not sufficiently potent. Thus, these agents could not achieve therapeutic levels of conjugate in the serum of cancer patients. (c) The linkers used for conjugating the antibody and drug were not stable enough *in vivo* to provide the degree of antigen-specificity that antibody-targeting was expected to provide. Thus, the premature release of drug results in lower overall targeting-selective uptake of the drug.

Learning from the failures in the past, new generation immunoconjugates have recently been investigated with more advanced insight. For example, an mAb-calicheamicin conjugate, Mylotarg, has been approved by FDA for treatment of acute myeloid leukemia [57]. MAb-immunoconjugates with maytansinoids and CC-1065 analogs, which are extremely cytotoxic, were also developed to overcome the problem of insufficient potency [42,43,58,59]. The new generation immunoconjugates use humanized antibodies that are expected to be non-immunogenic, for use in the repeated cycles of therapy [60]. Following the success of "Mylotarg" that has a rather limited scope, some of the newer generation immunoconjugates are currently in the human clinical trials.

Calicheamicin, maytansinoids and CC-1065 are extremely toxic, having very narrow therapeutic windows, and thus have never become therapeutic antitumor drugs for clinical use by themselves. Accordingly, no clinical profiles for these agents including drug resistance are available. Thus, their immunoconjugates may have very limited applications. Also, it has been shown that each cancer is different and no single anticancer drug can be effective against all types of cancers. Therefore, it is very important to have clinically effective immunoconjugates, bearing cytotoxic "warheads" with different molecular targets in the chemotherapeutic arsenal.

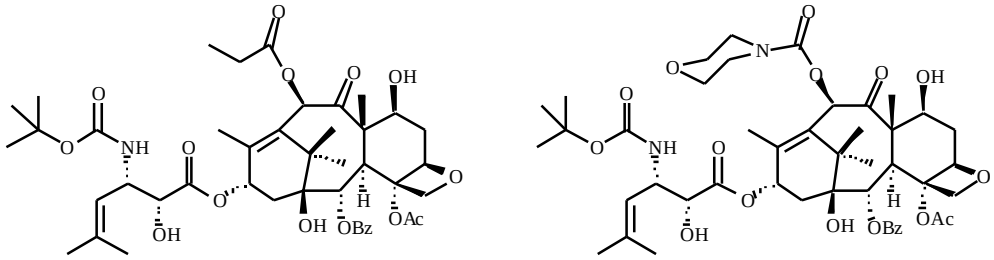
Design of mAb-Taxoid Conjugates

The efficacy of these immunoconjugates as chemotherapeutic drugs relies heavily both on the nature of the cytotoxic agents and the tumor specificity of mAbs. It has been shown that the number of tumor-associated antigens on the cancer cell surface is limited (estimated to be 10^5 molecules/cell). Thus, the cytotoxic agents that can be effectively used in these conjugates must have an IC_{50} value of 10^{-10} to 10^{-11} M against target cancer cells [42]. However, the IC_{50} of paclitaxel is 10^{-9} M, and thus not applicable to this target delivery prodrug approach. In addition, paclitaxel-immunoconjugates are anticipated to be inactive against tumors expressing MDR because of the inherent weakness of paclitaxel against drug-resistant cancer cells expressing MDR phenotypes. In a sharp contrast, most of second-generation taxoids developed in our laboratory exhibited one order of magnitude higher potency than that of paclitaxel against drug-sensitive cancer cell lines, and some of them showed 2-3 orders of magnitude higher potency than that of paclitaxel against drug-resistant cell lines, which makes them highly promising clinical candidates for cancer chemotherapy [61-65]. Accordingly, novel chemotherapeutic agents with high potency and exceptional tumor specificity by linking these second-generation taxoids with mAbs were developed [66-68].

Use of an appropriate linker between a taxoid and a mAb is crucial for the efficacy of the resulting immunoconjugate. It is required that the linker is stable for an extended period of time upon storage and also in circulation *in vivo*, while it is readily cleavable inside of cancer cells. Among possible linker units reported, a disulfide linker was chosen to be employed because of its favorable characteristics [42].

In order to synthesize an mAb-taxoid conjugate, both a taxoid and an mAb need to be modified to form a disulfide linkage by disulfide-thiol exchange reaction. As a logical precursor for a sulfhydrylalkanoyl group in a methyldisulfanyl(MDS)-alkanoyl group, we decided to synthesize MDS-alkanoyltaxoids. Two of the second generation taxoids possess cytotoxicity in the required range, i.e., SB-T-1213 and SB-T-12162 [61], as shown in Fig. (**12**). Thus, these two taxoids were chosen for modification with an MDS-alkanoyl group.

Since incorporation of an MDS-alkanoyl group into these taxoids may affect the cytotoxicity of the resulting taxoids, a structure activity relationship study was necessary to determine the optimal position for the introduction of a MDS-alkanoyl group. Thus, we synthesized new taxoids bearing an MDS-alkanoyl group at the C-2, C-10, C-7, C-2' positions and their cytotoxicity was assayed.



SB-T-1213 **SB-T-12162**

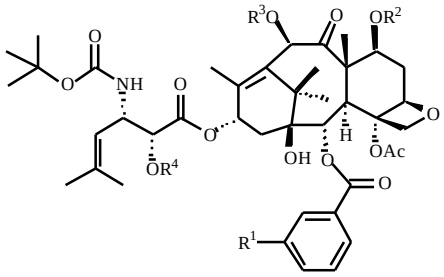
Fig. (12). SB-T-1213 and SB-T-12162.

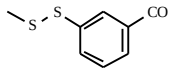
Syntheses of MDS-alkanoyltaxoids

The syntheses of taxoids employed the β-*Lactam Synthon Method* [69-72], featuring the highly efficient β-lactam ring-opening coupling reaction with a properly modified baccatin. However, we found that introducing 3-(MDS)propanoyl group at the C-10 position of the taxoids is not a trivial matter. This group is rather sensitive to basic conditions, which are used in the coupling reaction of β-lactam and baccatin. Therefore, this moiety was introduced at later stage of the synthesis. Accordingly, three C-10-modified taxoids were synthesized. Those are SB-T-12136 and SB-T-12137, possessing two different length MDS-alkanoyl groups at the C-10 position, and SB-T-1219001 bearing a 3-MDS-benzoyl group (Table **2**).

Synthesis of 7-MDS-alkanoyltaxoids was less demanding to afford SB-T-1213C701 and SB-T-1216C701 in excellent yields. Introduction of an MDS-propanoyl group at the C-2' position was straightforward to give SB-T-1213C2'01. In a

Table 2. ***In Vitro*** **Cytotoxicity (IC_{50}, nM)[a] of Taxoids.**



Taxoid	R^1	R^2	R^3	R^4	A431[b]	A549[c]	LCC6-WT[d]
SB-T-1213	H	H	CH_3CH_2CO	H	0.09	0.1	-
SB-T-12162	H	H		H	0.2	0.6	-
SB-T-12136	H	H	$CH_3SSCH_2CH_2CO$	H	0.5	0.8	-
SB-T-12137	H	H	CH_3SSCH_2CO	H	0.7	0.8	-
SB-T-1213C701	H	$CH_3SSCH_2CH_2CO$	CH_3CH_2CO	H	2.0	-	6.9
SB-T-1216C701	H	$CH_3SSCH_2CH_2CO$		H	>3.0	-	4.6
SB-T-1213C2'01	H	H	CH_3CH_2CO	$CH_3SSCH_2CH_2CO$	>3.0	0.9	-
SB-T-1219001	H	H		H	>3.0	>3.0	-
SB-T-1212003	CH_3SS	H	CH_3CO	H	>3.0	>3.0	-

a. The concentration of compound which inhibits 50% of the growth of cancer cell line after 72 h drug exposure. b. Human epidermoid carcinoma. c. Non-small cell lung carcinoma. d. Human breast carcinoma.

similar manner, we synthesized SB-T-1212003 bearing a 3-MDS-benzoyl group at the C-2 position, replacing the benzoyl group (Table **2**).

Biological Evaluation

Cytotoxicity of new taxoids thus synthesized was assayed against A431 (epidermoid), A549 (non-small cell lung) and LCC6 (breast) cancer cell lines. Their parent taxoids were also assayed for comparison. The IC_{50} values of these taxoids are summarized in Table **2**. As Table **2** shows, the 10-MDS-alkanoyl analogs of SB-T-1213 (SB-T-12136 and SB-T-12137) retain sub-nanomolar IC_{50} values, making each of them very promising as a cytotoxic component for taxoid-mAb conjugates.

Previous SAR studies of taxoids have shown that the C-7 position is well tolerated for modifications [73]. To our surprise, taxoids with MDS-propanoyl group attached to the C-7 position, SB-T-1213C701 and SB-T-1216C701, exhibit compromised cytotoxicity. Interestingly, when the MDS-propanoyl group is attached to 2'-OH, the resulting taxoid (SB-T-1213C2'01) is much less active against A431 cell line while maintaining its potency against A549 cell line. Taxoids bearing a 3-MDS-benzoyl group at C-2 (SB-T-1212003) or C-10 (SB-T-1219001) show substantial loss of activity.

Thus, the SAR study has clearly demonstrated that cytotoxicity can be retained when an MDS-propanoyl group is attached to the C-10 position of a taxoid. This is an important finding, which is totally unexpected.

Based on the *in vitro* study described above, SB-T-12136 was selected for linking to mAbs to form immunoconjugates. Murine monoclonal antibodies directed against the human EGFR were used as the tumor-targeting moieties in immunoconjugates. Three such IgG monoclonal antibodies, KS-61 (IgG2a), KS-77 (IgG1) and KS-78 (IgG2a), were linked to SB-T-12136 via disulfide bonds with a 4-thiopentanoyl linker, as shown in Fig. (**13**). A conjugate of SB-T-12136 with monoclonal antibody mN901 that does not bind to EGFR, was also prepared for comparison.

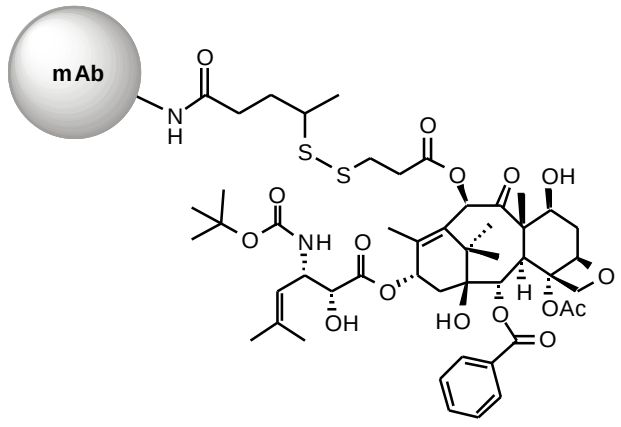


Fig. 13. MAb-SB-T-12136 Conjugate.

In vitro cytotoxicity was determined in a clonogenic assay [74] after a continuous exposure of the cells to the conjugates. It is expected that antigen-expressing cancer cells could only be "targeted" by an immunoconjugate bearing an mAb specific to the antigen. In fact, as Fig. (**14**) shows, mN901-SB-T-12136 exhibits no cytotoxicity against the A431 cell line, expressing EGFR. In sharp contrast, KS-78-SB-T-12136 shows high potency (IC_{50} = 1.5 nM) against the same A431 cell line (Fig. (**14**)). These results demonstrate that the binding of anti-EGFR mAb-taxoid conjugate to EGFR is highly specific. Moreover, it is strongly recommended that the immunoconjugate KS-78-SB-T-12136 generates highly cytotoxic agent (10-HS-propanoyl-SB-T-12136) upon binding to EGFR, followed by internalization and the subsequent cleavage of the disulfide linkage.

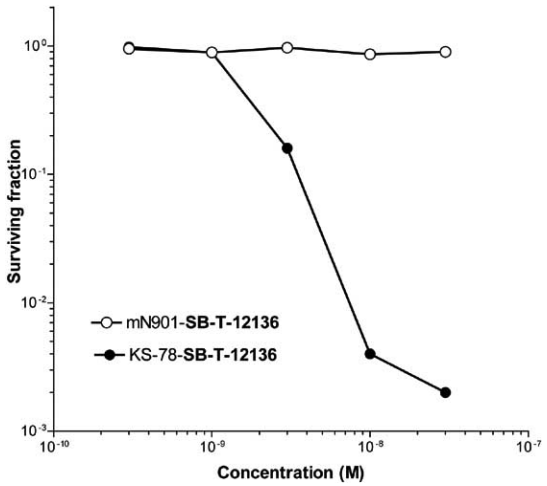


Fig. (14). Cytotoxicity of taxoid-mAb conjugates on A-431 cells.

The anti-tumor activities of two anti-EGFR-mAb-taxoid conjugates, KS-61-SB-T-12136 and KS-77-SB-T-12136, were evaluated against human tumor xenografts in severe combined immune deficiency (SCID) mice. Each mouse was inoculated with 1.5 x 10^6 A431 human squamous cancer cells, and the tumors were allowed to grow for 11 days to an average size of 100 mm^3 (range of 54-145 mm^3). The mice were then randomly divided into four groups. The first group received KS-61-SB-T-12136 conjugate (10 mg/kg, qd x 5, administered i.v). The second group received KS-77-SB-T-12136 conjugate in the same manner. The third group received free taxoid (i.e., SB-T-12136) (0.24 mg/kg, qd x 5, i.v.) at the same dose as that is present in the conjugate. A control group of mice received PBS using the same treatment schedule as in the groups 1-3.

The tumors in the control group of mice grew to a size of nearly 1000 mm^3 in 31 days. Treatment with free taxoid SB-12136 showed no therapeutic effect, and the tumors in this group grew at essentially the same rate as in the untreated control group of mice. In contrast, both anti-EGFR-mAb-taxoid conjugates showed remarkable antitumor activity, resulting in complete inhibition of tumor growth in all the treated animals for the duration of the experiment. Necropsy on day 75, followed by histopathological examination showed residual calcified material at the tumor site, but no evidence of tumor cells. The data also unambiguously indicates that targeted delivery of the taxoid using a tumor-

specific mAb is essential for the activity, since an equivalent dose of unconjugated taxoid shows no antitumor activity (Fig. (**15**)). Notably, the doses of antibody-taxoid conjugates used are non-toxic to the mice as demonstrated by the absence of any weight loss.

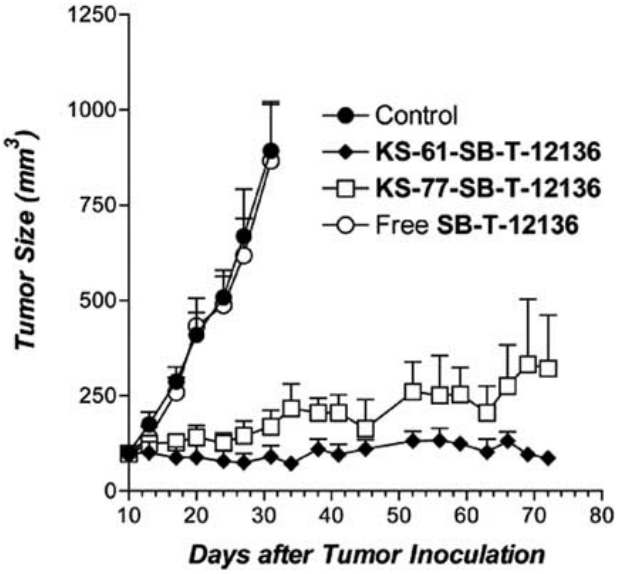


Fig. (15). Anti-tumor activity of anti-EGFR-mAb-taxoid conjugates in an A-431 Xenograft Model in SCID Mice.

NOVEL FATTY ACID-SECOND GENERATION TAXOID CONJUGATES AS PROMISING ANTICANCER AGENTS

As discussed above, a meaningful approach to more successful chemotherapy is to find a unique property of tumor biochemistry or physiology, that can be exploited to target drugs to tumors. The preceding section dealt with the use of tumor specific mAbs, targeting tumor surface antigens, as the vehicle for highly cytotoxic taxoids in the form of mAb-taxoid immunoconjugates. The uptake of fatty acids and other metabolic precursors has been studied by using tissue-isolated hepatomas with a single arterial inflow and a single venous outflow. In these systems, it has been found that some particular natural fatty acids are taken up greedily by tumors from the arterial blood, presumably for use as biochemical precursors and energy sources [75,76]. This observation makes fatty acids attractive for use in tumor targeting conjugates.

Essential fatty acids (EFA's) are unsaturated fatty acids that can only be obtained from the diet source. There are two different classes of EFA's, n-3 and n-6, where the number indicates the position of the first double bond from the methyl. The most abundant n-6 fatty acid is linoleic acid (LA), originated from vegetable oils, such as corn oil, sanflower, soybean oils, and meat [77,78]. The n-3 fatty acids may be found in vegetable oils, especially canola, soybean oils, and leafy vegetables as α-linolenic acid (LNA) and in cold-water fish as eicosapentenoic acid (EPA) or docosahexenoic acid (DHA) [77,78].

DHA-paclitaxel conjugate (Taxoprexin®) has been synthesized by researchers in Protarga and tested against different human cell lines and tumor xenografts [79]. Taxoprexin® exhibits increased antitumor activity compared with paclitaxel (optimum dose for paclitaxel is 20 mg/kg with complete or partial regression of tumor, while Taxoprexin® 's optimum dose is 120 mg/kg with complete regression) [79]. Taxoprexin® is stable in plasma and high concentrations are maintained in animal plasma for long time, slowly releasing active compound. In this way, conjugate can be more effective and kill slow cycling or residual tumor cells and also reduce side effects [79].

The Importance of Developing Fatty Acid-second-generation Taxoid Conjugates

As discussed earlier, paclitaxel and docetaxel are effective against breast, ovary, and lung cancer, but do not show efficacy against colon, pancreatic, melanoma, and renal cancers. Human colon carcinoma is inherently multi-drug resistant due to the overexpression of P-glycoprotein (Pgp), which is an effective ATP-binding cassette (ABC) transporter, effluxing out hydrophobic anticancer agents including paclitaxel and docetaxel. Paclitaxel does not show any appreciable efficacy against colon cancer in humans [17]. In sharp contrast, some second generation taxoids showed 2-3 orders of magnitude higher activity against drug-resistant cancer cells and tumor xenografts in mice, expressing MDR phenotypes [17,80]. DHA does not seem to be a good substrate of Pgp, since DHA-paclitaxel (Taxoprexin®) was found to be a weak substrate of Pgp as compared to paclitaxel. However, if the cancer cells are overexpressing Pgp, paclitaxel molecule, even when released slowly, will be caught by the Pgp effluxing pump and eliminated from cancer cells. Therefore, it will be beneficial to conjugate DHA to the second-generation taxoids that possess built-in Pgp modulating ability.

Synthesis of Fatty Acid-second-generation Taxoid Conjugates

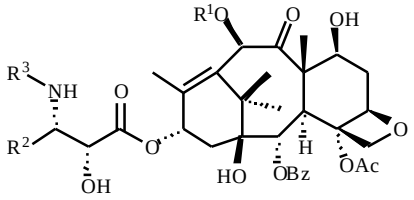
Among the second-generation taxoids, SB-T-1213, SB-T-1214, SB-T-1216, and SB-T-1217 exhibited exceptional cytotoxicity (Table **3**), with IC_{50} value two orders of magnitude higher than paclitaxel and docetaxel against resistant cancer cells overexpressing Pgp efflux pump. Those, together with SB-T-1103, SB-T-1104 and ortataxel (IDN5109) were chosen to be conjugated to fatty acids.

Coupling of taxoids with fatty acids has been carried out under the standard conditions (DIC, DMAP) to give the corresponding conjugates in good yields (Table **4**). The reaction takes place at the C-2' OH group that is more reactive than the C-7 OH group.

Biological Evaluation

Successfully synthesized fatty acid-second-generation taxoid conjugates were then evaluated for their antitumor activity against the drug-sensitive human ovarian tumor xenograft (Pgp-) A121, and the drug-resistant human colon tumor xenograft (Pgp+) DLD-1 in SCID mice.

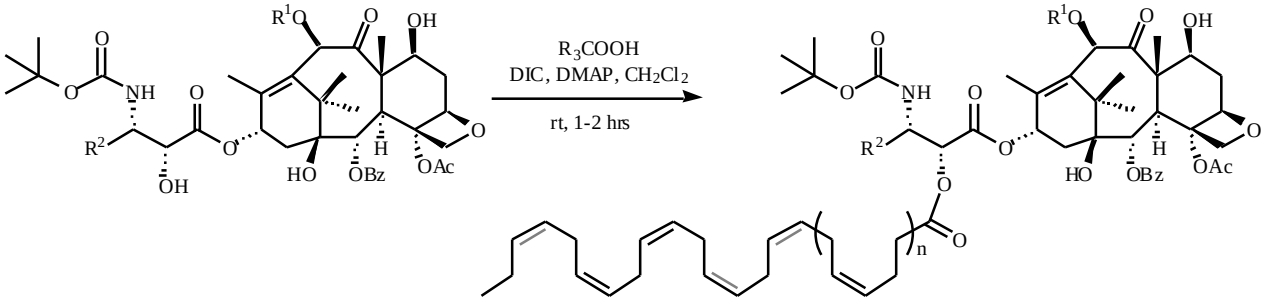
Table 3. Cytotoxicity of Selected Second-generation Taxoids Against Human Breast Carcinoma Cell Lines[a].



Taxoid	R^1	R^2	R^3	IC_{50}(nM)[b]			
				MCF7-S	MCF7-R	LCC6-WT	LCC6-MDR
Paclitaxel	Ac	C_6H_5	C_6H_5CO	1.7	550	4.0	360
Docetaxel	H	C_6H_5	*t*-Boc	1.0	723	1.3	125
SB-T-1103	EtCO	isobutyl	*t*-Boc	0.35	5.1	-	-
SB-T-1104	c-PrCO	isobutyl	*t*-Boc	0.51	7.9	-	-
SB-T-1213	EtCO	isobutenyl	*t*-Boc	0.18	4.0	-	-
SB-T-1214	c-PrCO	isobutenyl	*t*-Boc	0.20	3.9	-	-
SB-T-1216	Me_2NCO	isobutenyl	*t*-Boc	0.13	7.4	-	-
SB-T-1217	MeOCO	isobutenyl	*t*-Boc	0.14	9.7	-	-

a. Human mammary tumor cell lines MCF7-S (Pgp-), MCF7-R (Pgp+), MDA-435-LCC6-WT (Pgp-), MDA-435-LCC6-MDR(mdrl transferred line). b. Concentration of drug which inhibits cell growth by 50% (72 h continuous exposure).

Table 4. Synthesis of Fatty Acid-second Generation Taxoid Conjugates.



Taxoid	R^1	R^2	R^3
DHA-docetaxel	H	C_6H_5	$C_{21}H_{31}$
DHA-SB-T-1213	EtCO	isobutenyl	$C_{21}H_{31}$
DHA-SB-T-1103	EtCO	isobutyl	$C_{21}H_{31}$
DHA-SB-T-1214	c-PrCO	isobutenyl	$C_{21}H_{31}$
DHA-SB-T-1104	c-PrCO	isobutyl	$C_{21}H_{31}$
DHA-SB-T-1216	Me_2NCO	isobutenyl	$C_{21}H_{31}$
DHA-SB-T-1217	MeOCO	isobutenyl	$C_{21}H_{31}$
LA-SB-T-1213	EtCO	isobutenyl	$C_{17}H_{31}$
LNA-SB-T-1213	EtCO	isobutenyl	$C_{17}H_{29}$
LA-SB-T-1214	c-PrCO	isobutenyl	$C_{17}H_{31}$
LNA-SB-T-1103	EtCO	isobutyl	$C_{17}H_{29}$

In the case of the sensitive tumor A121, the efficacy of DHA-paclitaxel reported by Bradley *et al.* [79] was confirmed by our results. DHA-paclitaxel (80 mg/Kg q3dx3, total dose 240 mg/Kg) exhibited greater than a two-fold increase in tumor growth delay as compared with paclitaxel. Nevertheless, one of the new DHA-taxoids exhibited even better activity, i.e., DHA-SB-T-1213 (30 mg/Kg q3dx3) delayed the tumor growth for more than 186 days and caused complete regression of tumors in all surviving mice (4 of 5). The best compound in this experiment, DHA-SB-T-1213 together with DHA-SB-T-1216 match and surpasses DHA-paclitaxel in anticancer activity. Both conjugates delayed the growth of the transplanted tumor for 200 days, while DHA-paclitaxel was effective for 160 days.

More exciting results were observed in the human colon tumor xenografts, (Pgp+) DLD-1. Not surprisingly, paclitaxel and DHA-paclitaxel (Taxoprexin®) were totally ineffective in this case. In sharp contrast, DHA-SB-T-1214 caused complete regression of the DLD-1 tumor in 5 of 5 mice at 80 mg/Kg dose administered on days 5, 8 and 11 (total dose 240 mg/Kg; tumor growth delay>187 days). This is a very promising result which promotes this compound as a lead candidate for further investigations. DHA-SB-T-1213 caused 54-day delay in tumor growth with total dose 75 mg/kg without serious toxicity to the animals (Fig. (**16**)).

Effect of different fatty acids on antitumor activity was also investigated. Conjugates of SB-T-1213 with DHA, LNA and LA were synthesized and tested against drug-resistant human colon tumor, xenografts (Pgp+) DLD-1. LA-SB-T-1213 and LNA-SB-T-1213 exhibited better antitumor profile than DHA-paclitaxel. LNA-SB-T-1213 exhibited the complete regression in 2 of 5 mice tested against drug-resistant human colon tumor xenografts (Pgp+) DLD-1 (tumor growth delay > 109 days). Although the toxicity of LNA-SB-T-1213 to the animals was higher than DHA-SB-T-1213, the former exhibited overall better activity than the latter. On the basis of these results, we can infer that DHA is not absolutely required in the conjugate, and that LNA, an n-3 fatty acid clearly showed advantages over the corresponding n-6 fatty acid, LA.

CONCLUSION

Extensive SAR studies and rational drug design have led to the discovery of potent second-generation taxane anticancer agents that can substantially reduce MDR or virtually overcome MDR. Also, it has been found that these new generation taxane anticancer agents possess dual functions, i.e., cytotoxicity and MDR reversal activity, built in one molecule. Thus, these agents can modulate Pgp efflux pump in cancer cells as well as intestine. The latter leads to the high oral bioavailability observed for these new type anticancer agents. Some of the second-generation taxanes are in extensive preclinical evaluations and ortataxel (SB-T-101131; IDN5109; Bay59-8862) has advanced to the phase II human clinical trials. Ortataxel exhibits remarkable antitumor activity by i.v. and p.o. routes with excellent bioavailability. Photoaffinity labeling of Pgp with photoreactive BzDC-paclitaxel analogs provided critical information for a possible specific binding site of paclitaxel in Pgp. Taxane-based, highly efficient MDR reversal agents, TRA's, have been developed. TRA's are non-cytotoxic and exhibit impressive MDR reversal activity when coadministered with paclitaxel. It has been demonstrated that novel taxane-mAb immunoconjugates can deliver and release extremely potent taxane anticancer agents, in a highly tumor-specific manner against human squamous carcinoma xenograft in SCID mice, which provides an early promise for circumventing undesirable side effects associated with taxane cancer chemotherapy. In addition, the exceptional efficacy of DHA-second-generation taxoids against drug-sensitive and drug-resistant human tumor xenografts provides bright prospects for the application of the n-3 fatty acid-second-generation taxoid conjugates in cancer chemotherapy.

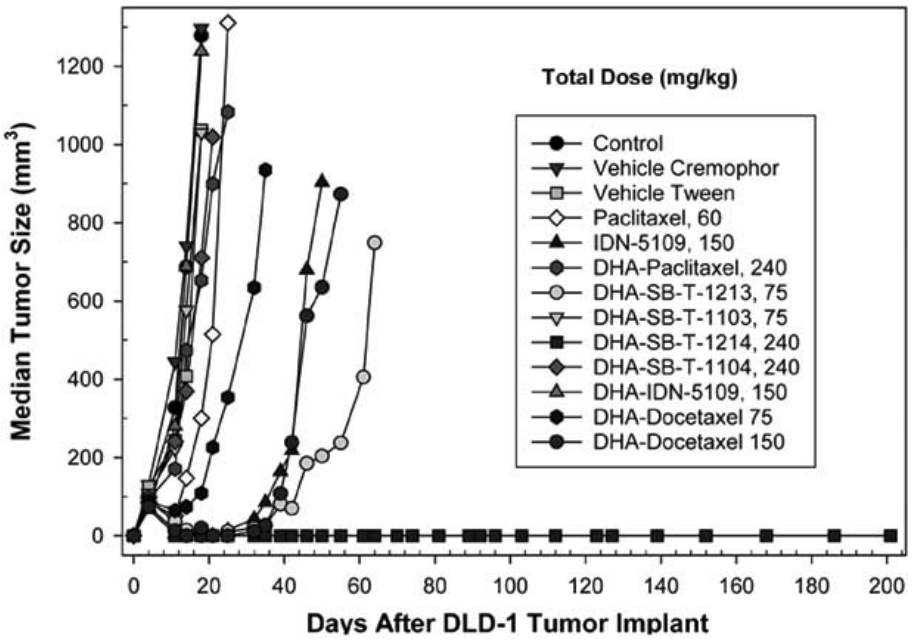


Fig. (16). Effect of DHA-taxoid conjugates on human colon tumor xenograft (Pgp+) DLD-1.

ABBREVIATIONS

14β-OH-DAB	=	14-hydroxy-10-deacetylbaccatin III
ABC	=	ATP-binding cassette
BBB	=	Blood-brain barrier
BzDC	=	Benzoyldihydrocinnamoyl
DAB	=	10-deacetylbaccatin III
DHA	=	Docosahexenoic acid
DIC	=	Diisopropylcarbodiimide
DMAP	=	4-(dimethylamino)pyridine
EFAs	=	Essential fatty acids
EGFR	=	Epidermal growth factor receptor
EPA	=	Eicosapentenoic acid
LA	=	Linoleic acid
LNA	=	Linolenic acid
mAb	=	Monoclonal antibody
MDR	=	Multidrug resistance
MDS	=	Methyldisulfanyl
MTD	=	Maximum tolerated dose
NBD	=	Nucleotide-binding domain
Pgp	=	P-glycoprotein
PTX	=	Paclitaxel
REDOR	=	Rotational-echo double-resonance
SAR	=	Structure-activity relationship
SCID	=	Severe combined immune deficiency
TAP	=	Tumor-activated prodrug
TM	=	Transmembrane
TRA	=	Taxane reversal agent

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