Recent Progress in Structure Activity Relationship and Mechanistic Studies of Taxol Analogues

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Abstract: Structure activity relationship (SAR) and mechanism of paclitaxel and its analogues in recent years are discussed in the following areas: SAR of paclitaxel analogues toward "normal" and multi-drug resistance tumors; paclitaxel prodrugs with improved water solubitily and specificity; mechanism of paclitaxel related to tubulin binding and quest for its pharmacophore.

Keywords: Anticancer drugs, paclitaxel, structure-activity relationship (SAR), multi-drug resistance (MDR), prodrugs, microtubule, pharmacophore.

Paclitaxel (**1a**) is a well-known anticancer drug currently used in clinics for the treatment of several kinds of metastatic tumors. Despite its success in chemotherapy, there are demands to improve its efficacy and lower its toxicity. Different approaches which can be applied include preparation of more active analogues, synthesis of conjugates or prodrugs with better bioavailability and specificity, preparation of new formulations with improved physical properties, combined use with other drugs, etc. The approaches regarding structure modifications will be addressed in Sections 1 to 3.

Mechanistic studies of paclitaxel not only reveal the molecular basis of its action, but also provide clues to the rational design of new analogues of paclitaxel and even the molecules with different structures. Progress in this area will be discussed in Section 4.

1. PACLITAXEL ANALOGUES ACTIVE AGAINST NORMAL TUMOR CELLS

SAR studies of paclitaxel analogues are still active in recent years. Many reviews on this topic appeared in periodicals, and some of which were written by leading researchers in this area [1a-c]. Instead of a systematic retrospect, this review will only concentrate on recent progress.

Our discussion will follow a left to right route in the molecular framework, i.e. C-13 side chain, A, B, C and D rings. Some results difficult to be categorized are located at the end of this section.

1.1. C-13 Side Chain

The importance of C-13 substituted phenylisoserine side chain to bioactivity of paclitaxel has been acknowledged for a long time. Replacement of 3'-Ph with other alkyl or alkenyl substitutions, especially 3'-isobutenyl and 3'-isobutyl groups, usually improves the activity of paclitaxel analogues. In combination with changes at C-10 acyl substitutions [4], as well as C-2 meta-substituted benzoyl groups [5], some promising taxoids were prepared.

Recently, Ojima *et al.* [2, 3] described the synthesis of taxoids with C-13 fluorine-containing side chains. Introduction of a fluorine atom to the *para* position of 3'-phenyl decreased activity in most cases, except in **2**, which are comparably cytotoxic to docetaxel in all tested cell lines. For 3'-CF_3 docetaxel analogues, in combination with the change of 10-Ac to other acyl groups, enhancement of activity several times were observed [2]. For 3'-difluoromethyl docetaxels, most derivatives with changes at 10-OH to 10-esters and 10-N,N-dimethylcarbamate were comparable to or modestly more active than docetaxel, similar to their 3'-CF_3 counterparts, whereas their 14β-OH counterparts were less active [3].

Another interesting example on C-3' modification is the synthesis of taxoids bearing 3'-cyclopropane and 3'-epoxide moieties [6]. Two 3'-cyclopropane (**3a-b**) and 3'-(R)-epoxide taxoid (**4a**), with IC_{50} less than 1 nM, is the most potent among them. Although 3'-(S)-epoxide (**4b**) is 10 to 30 times less active than **4a**, its cytotoxicity is still comparable to paclitaxel.

A Bristol-Myers Squibb research group found that 3'-*t*-butylaminocarbonyloxy paclitaxel analogue **5a** and **5b**, were several times less active than paclitaxel *in vitro*, while **5b** was equipotent to paclitaxel *in vivo* [7]. They also prepared 3'-N-thiocarbamate and 3'-N-thiourea bearing taxol analogues. While 3'-N-thiocarbamate were found to be more potent than paclitaxel and docetaxel in both tubulin polymerization and cytotoxicity assays, thioureas are usually less active [8].

Georg's group reported [9] the synthesis of a series of 3'-*t*-butyl paclitaxel analogues (Butitaxels) with 3'-N amides and carbamates, among which N-debenzoyl-N-(2-thienoyl) analogue is the most potent. Although equipotent to docetaxel (**1b**) and about 25 times more water soluble than paclitaxel, this taxoid is not superior to a Butitaxel analogue reported earlier [10].

Barboni *et al.* [11] prepared a conformationally strained paclitaxel analogue (**6a**), in which C-2' and ortho-position of

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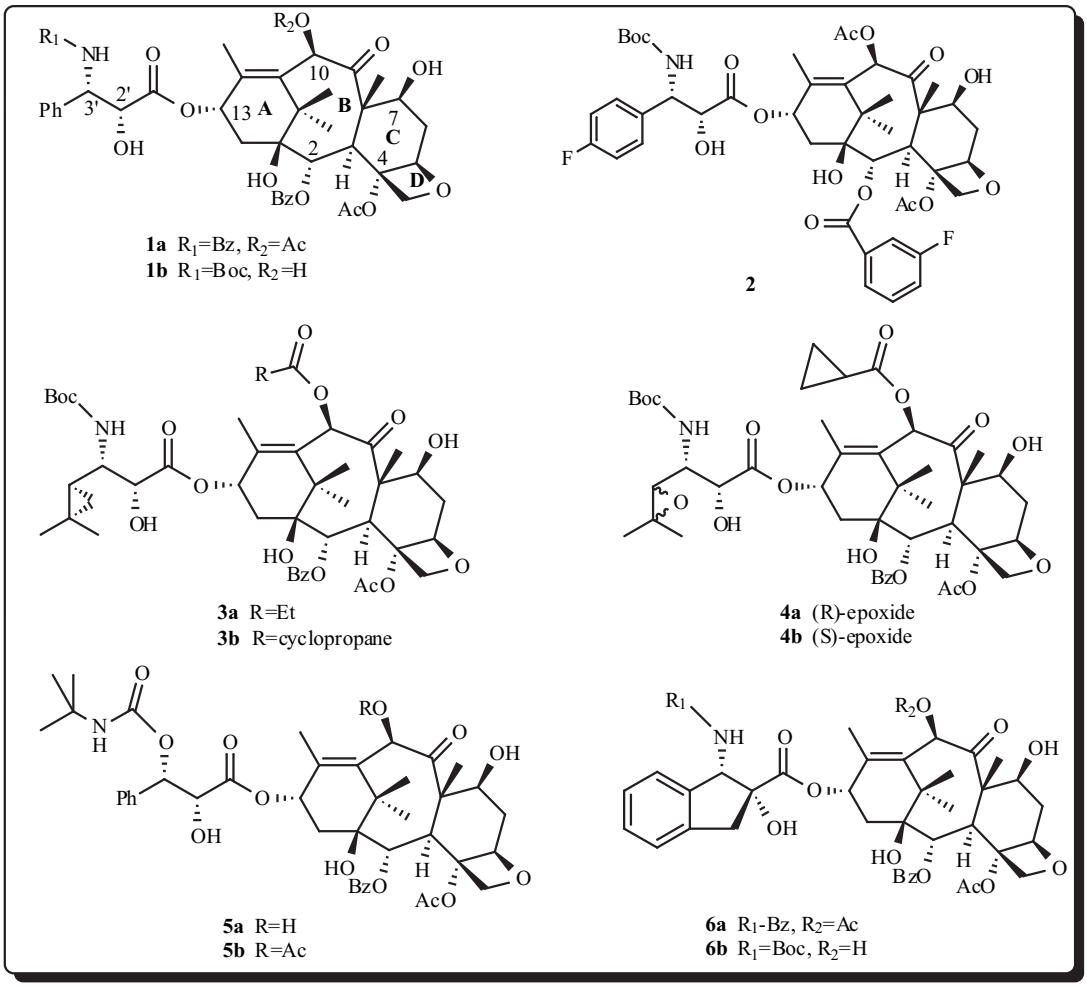


3'-phenyl is tethered with a methylene group. It exhibited comparable cytotoxicity to that of paclitaxel. After synthesizing several tethered analogues including docetaxel analogue **6b**, they observed ethylene linkage between C2'-C3' led to a drastic decrease in either tubulin or cytotoxicity tests. The analogue with reversed C2' and C3' configurations is totally inactive [12].

Cephalomannine is a congener of paclitaxel in several *Taxus sp*. plants, which showed comparable cytotoxicity to paclitaxel. Several bromine and chlorine adducts to the double bond of 3'-N-tigloyl in cephalomannine were prepared. The dichlorocephalomannine derivatives were one order of magnitude less active, while dibromocephalomannines better than paclitaxel against several colon, ovarian and breast cancer cell lines [13]. Epoxidation products of the double bond on 3'-N-tigloyl exhibited comparable activity [14].

1.2. A Ring and its Substitutions

Most A-nor analogues prepared by Kingston's group were far more less active than palitaxel in both cytotoxicity and tubulin polymerization tests [15]. Preliminary *in vivo* tests for this series of compounds are also disappointing.

From 14β-OH-10-DAB, a taxoid isolated from the needles of *T. wallichiana* Zucc., a series of A-nor-seco analogues were prepared [16]. Unfortunately, they were less active than paclitaxel by 1 to 2 orders of magnitude. The analogues with amide side chain at the position comparable to C-13 in paclitaxel are less active than their ester counterparts.

A series of 14β-hydroxy and 14β-acyloxy taxoids without C-13 oxygenations and oxetane D ring have been isolated from cell culture of *T. yunnanensis*. Attachment of N-benzoylphenylisoserine side chain to C-14, and further incorporation of 4(20), 5-oxetane ring and change of 2-acetate to benzoate didn't improve their activities. All derivatives were far less active than paclitaxel [17].

The role of 1-OH in SAR of paclitaxel is not clear until Kingston's group finished the synthesis of 1-deoxypaclitaxel from a naturally occurring taxoid 1-deoxybaccatin VI [18]. After comparison of 1-deoxy-9-dihydrodocetaxel analogue **7** and other 1-deoxy analogues with the structurally closed paclitaxel analogue, they concluded that 1-deoxygenation caused slightly reduced activity.

1.3. B Ring and its Substitutions

Cheng *et al.* [19] coupled different purine and pyrimidine riboside to the 9α-OH in 7-deoxy-9(R)-dihydropaclitaxel. These derviatives were less active toward all 5 human normal tumor cell lines in the assays. Enhancement of cytotoxicity was also observed for two of these analogues.

Walker *et al.* [20] prepared 10α-spiro epoxide (**8a**) and its 7-MOM ether(**8b**), which exhibited comparable cytotoxicity and tubulin assembly activity to paclitaxel. Compound **8b** is more active against a MDR tumor cell line HCT-VM46 than paclitaxel and **8a** almost by one order of magnitude.

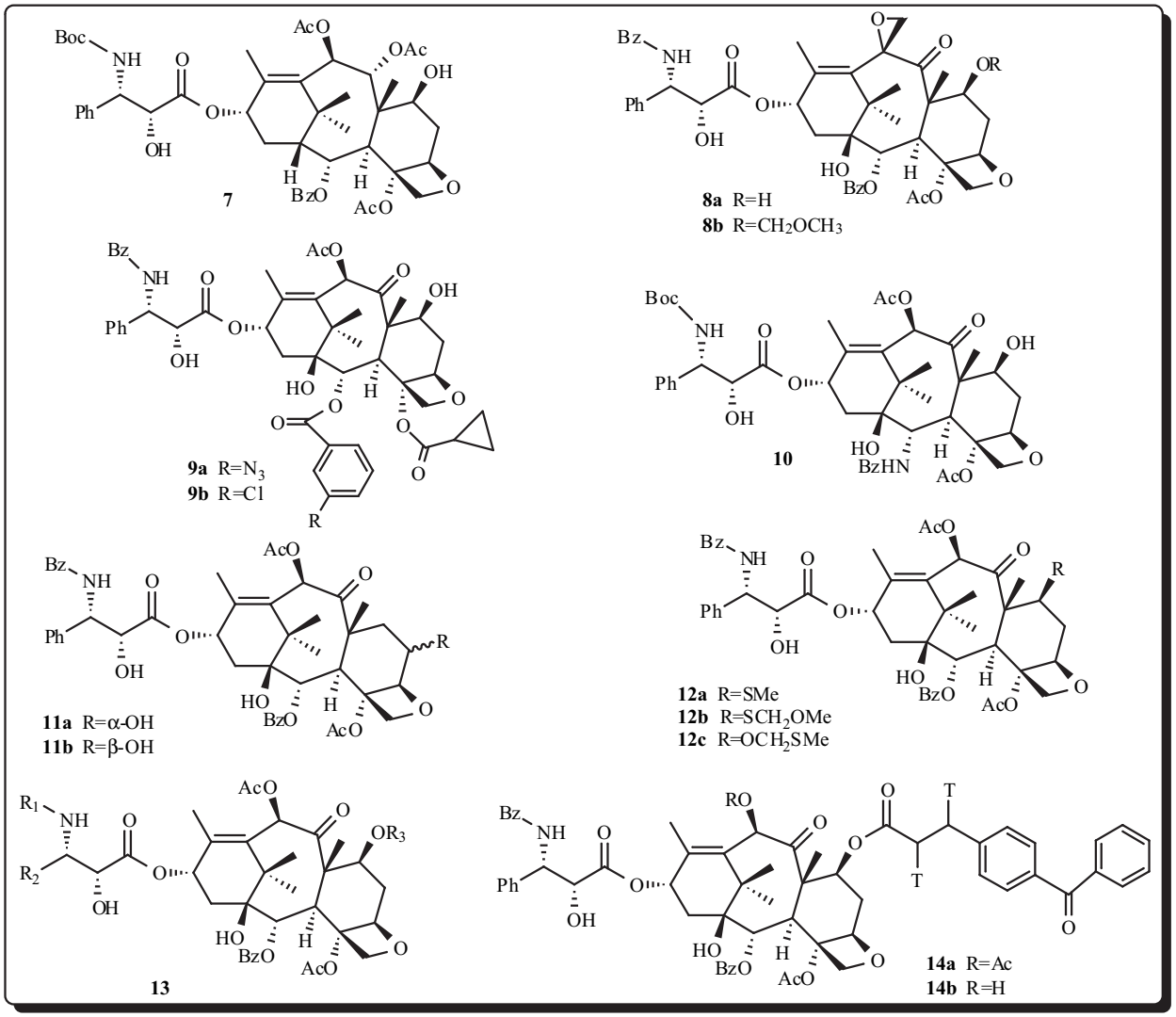
Kingston's group systematically explored [21, 22] the acyl substitutions at C-2, and found reasonable agreement in the correlation of cytotoxicity and tubulin polymerization activity for those active analogues. In general, *meta*-substituted compounds are more cytotoxic than paclitaxel. The *para*- and *ortho*-substituents usually have negative impact on activity, except for some specific compounds, e.g. 2-(*o*-azido)benzoyl analogue. Di-substituted benzoyl analogues were generally less active than their mono-substituted counterparts. For other heteroaromatic analogues tested, only thiophene analogues showed improved activity. The 2, 4-diacyl paclitaxel analogues were also prepared. Taxoids **9a** and **9b** gave best data in tubulin and cytotoxicity assays [22].

Chen *et al.* synthesized the 2-acetoxy-4-benzoate analogue, so called "iso-paclitaxel". This compound was totally inactive either in cytotoxicity or tubulin polymerization assays [23]. It is in agreement with previous observations that only small C-4 substituents was tolerated.

Synthesis of a 2α-N substituted analogue 2-debenzoyloxy-2α-benzamido 10-acetyl docetaxel (**10**) was realized in author's lab recently. It is comparably active to paclitaxel against human lung cancer A-549 and less active in other two tumor cell lines [24a]. A series of C-2 *meta*- and *para*-substituted benzamido analogues were also prepared, among which 2-*m*-methoxy and 2-*m*-chloro benzamide are the most active, but not better than paclitaxel and docetaxel [24b]. Besides, a 2*a*-phenylthio analogue of docetaxel exhibited much less cytotoxicity [24c].

1.4. C Ring and its Subtsitutions

6α-OH paclitaxel, the major metabolite of paclitaxel in human, has been known to be less cytotoxic than paclitaxel. 6*a*-F-, Cl- and Br-paclitaxels were designed and prepared as the metabolic site blocked analogues. However, 6*a*-substituents doesn't alter their *in vitro* and *in vivo* efficacies significantly [25]. It was also found that both 6-hydroxylation and 3'-*p*-phenyl-hydroxylation were detected in mice, while 6α-hydroxylation predominates in humans.



Syntheses of both C-6 epimers (**11a-b**) of 7-deoxy-6-hydroxypaclitaxel were realized through the regiospecific reduction of 6, 7-α-thiocarbonate as the key step. They are equipotent to paclitaxel in tubulin assembly assay, and less cytotoxic by about one order of magnitude [26].

Several 7-sulfur analogues were prepared [27]. While 7α-SH and 7β-SH are less toxic, 7β-MeS (**12a**) and 7β-$MeOCH_2S$ (**12b**) analogues are superior to paclitaxel as well as BMS-184476 (7-$MeSCH_2$ ether of paclitaxel, **12c**) which is currently in Phase I clinical trial. BMS-184476 was chosen to be the candidate in systematic evaluation of C-7 ether analogues of paclitaxel with general formula **13**, in which phenyl, 2-furyl and *i*-butenyl were selected as R_1, benzoyl and *t*-Boc as R_2, and MTM, MOM, $CH_2O(CH_2)_2OH$ and Me as R_3 moieties. BMS-184476, although scored behind many competitors *in vitro*, exhibited superior activity in several *in vivo* tumor-bearing animal models, including most paclitaxel-resistant tumor HCC79 model in the company [28].

A 7-{[$^3H_2$]-3-(4-benzoyl)phenylpropanolyl} paclitaxel analogue (**14a**) and its 10-deacetyl derivative (**14b**) were prepared as photoreactive probes in exploration of paclitaxel binding sites on tubulin and P-glycoprotein [29]. However, **14a** is less cytotoxic against either normal or MDR tumor cell lines, while **14b** much more less active than paclitaxel.

A paclitaxel analogue with C4-C6 bridge, built on the connection of carboxyl group of 4-glutarate and the hydroxyl group of 6α-hydroxyacetate, was found almost inactive [30].

1.5. D Ring

Although the oxetane D ring is crucial to cytotoxicity of taxoids, its physiological role was not well understood. Constraint and hydrogen bond acceptor are assumed to be two major roles of D ring in paclitaxel. Recent successful syntheses of aza-, thia-, and selena-substituted, as well as deoxy(cyclopropane) oxetane ring analogues were helpful in clarifying its role.

Low activity or inactivity of azetidine D-ring **15a** and **15b** [31], very poor activity of 4-carbonate-thia-paclitaxel **16** in tubulin polymerization and inactivity in cytotoxicity tests, together with inactivity of 4-deacetyl-selena-paclitaxel **17** in both assays suggested that: (1)oxygen atom may be involved in the interaction with an amino acid residue of tubulin and this interaction may not be replaced by NH; (2)the region surrouding the oxetane ring is very sensitive to steric effects [33]. Very recently, two D-thia analogues, 5(20)-thia-docetaxel **18a** and 7-deoxy-10-acetyl-5(20)-thia-docetaxel **18b** were synthesized [34]. Their poor activity stressed the importance of steric requirement around D-ring. Cyclopropane analogue **19** showed microtubule disassembly inhibitory activity comparable to paclitaxel, but lower than docetaxel. The author thus demonstrated the oxetane ring is not essential for the interaction of paclitaxel analogues with microtubules when C-ring conformation is locked by cyclopropane, but the oxygen atom in D ring may participate in the stabilization of drug-tubulin complex.

Georg's group synthesized some D-seco paclitaxel analogues without 5α-oxygenated group. Although it is disappointing to find these compounds did not exhibit any activities in biological assays, it provided the authors an opportunity to review and revise the "hydrophobic collapse" pharmacophore model [36].

1.6. Macrocyclic Analogues

Ojima *et al.* proposed a common pharmacophore for several anticancer natural products targeting microtubules, including paclitaxel, epothilones, eleutherobin, and discodermolide [37]. It was suggested that macrocyclic taxoids such as **20** may represent hybrid constructs of paclitaxel and epothilone. A group of these macrocyclic taxoids with unsaturated and saturated linkages between C-2 and C-3' were prepared through ring-closure metathesis(RCM) catalysed by Grubb's catalyst and subsequent hydrogenation [38]. Among 30 macrocyclic taxoids, only three of them, including **20**, retained strong cytotoxicity, although less active than paclitaxel by two orders of magnitude. Taxoid **20** was also one of three active taxoids in tubulin polymerization assay with 36% relative activity to paclitaxel.

Georg's group also prepared a group of macrocyclic taxoids, in which C-2 benzoate and C-3' phenyl are tethered by alkenyl, alkyl and ester linkers. The alkenyl linked taxoids were synthesized through Heck reaction, and further hydrogenation formed alkyl linkage. All compounds subjected to tubulin polymerization tests were inactive [39].

Kingston's group reported the synthesis and cytotoxicity of C3'-C4 linked macrocyclic taxoids very recently. The synthetic strategy was also based on RCM. Similar to Ojima's finding, these compounds (**21-22**) are less active than paclitaxel in both cytotoxicity and tubulin assays [40].

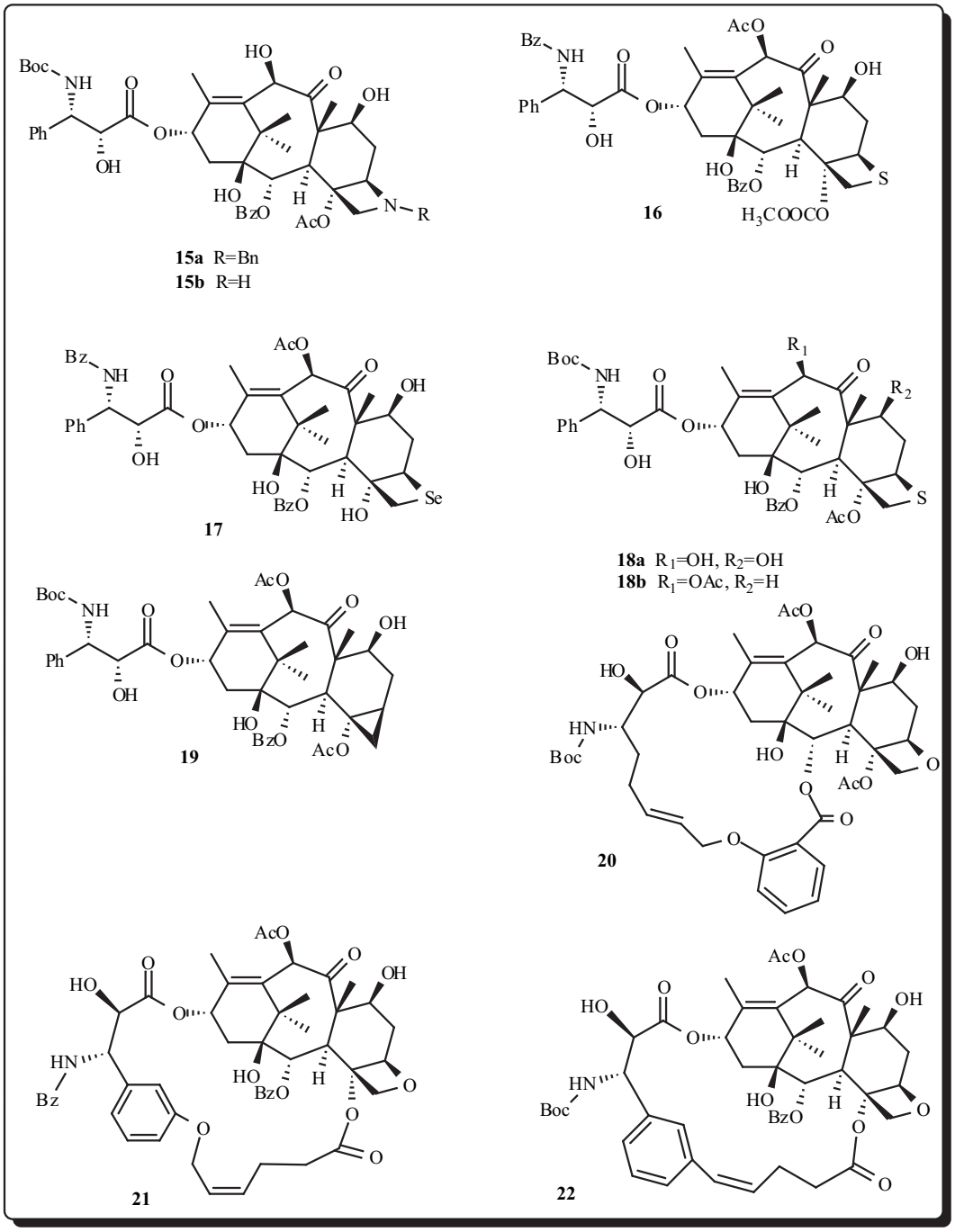
1.7. Miscellaneous

Some dimers consisting of paclitaxel or docetaxel with other taxoids with MDR reverasl activities [41], and with epipodophyllotoxin derivatives were prepared [42]. Some paclitaxel-epipodophyllotoxin dimers showed a little enhancement in cytotoxicity against drug-resistant tumor cells, compared with both precursors. In topoisomerase assays, two paclitaxel conjugates are active topo II inhibitor *in vitro* and also intracellular poisons [42].

An analogue was prepared from a 2(3→20)*abeo*taxane compound deaminoacyltaxine A after incorporation of 2-benzoate and paclitaxel side chain at C-13. It is more active than the parent compound, but still much less potent than paclitaxel [43].

The 9-keto group in 10-DAB was converted into thiosemicarbazone and then complexed with copper ion. The complex was almost as active as 10-DAB below 12.5 μM. Beyond that concentration, cytotoxicity of the complex increased dramatically [44]. Although the complex is still much less active than paclitaxel, one should note there may be alternative ways to enhance the cytotoxicity of inactive taxoids. However, the complex is probably involved in a different cytotoxic mechnism from 10-DAB.

Combinatorial chemistry was also applied to taxoids recent years. However, only very few reports appeared in this



field to date. In 1997, Xiao *et al.* reported the preparation of the first taxane library with 400 compounds by a radiofrequency encoded, solid phase synthesis method. Subsequently, Georg's group utilized a parallel solution phase synthetic method to construct a 26 membered library of C-7 esters [45a] and concluded that modification at C-7 were detrimental to cytotoxicity against MCF-7 cell line. Very recently, this group also constructed a library with C-10 modified paclitaxel analogues [45b]. Although these taxoids showed to be less active in both tubulin assembly and cytotoxicity assays, they are more effective toward drug resistant MCF-7R breast cancer cell lines. Kingston *et al*. also reported a resin-based combinatorial synthesis of 7-acyl, 10-acyl and 7, 10-diacyl analogues. Some more potent and water soluble analogues were found [46].

2. NATURAL AND SEMI-SYNTHETIC TAXOIDS OVERCOMING MULTI-DRUG RESISTANCE (MDR)

2.1. Second-Generation Taxoids with Better Activity Towards MDR Tumors

A series of taxoids with C-2, C-3' and C-10 substituent modifications were synthesized and found to be much more active than paclitaxel in MDR tumors. It was reasoned that these taxoids are not good substrates for P-gP, thus exhibited potent activity in drug-resistant tumors.

In 1996, Ojima's group reported that introduction of carbonate and carbamate at C-10 position and replacement of 3'-phenyl in paclitaxel with an alkenyl or alkyl group provide the taxoids which exhibit 1 to 2 orders of magnitude higher potency against drug-resistant cancer cell lines.

Subsequently, new taxoids bearing 3'-cyclopropane and 3'-epoxide moieties were synthesized. The R/S ratio (ratio of IC_{50} in drug-resistant cell to that in sensitive cell) is 2.48 for 3'-cyclopropane/10-PrCO compound (**3a**) [5]. Later on, they discovered more potent analogues with modifications on C-2 as well as C-3' and C-10 positions, three of them (**23a-c**) showed best R/S ratios at 0.89-1.3 in LCC6 (breast), and 0.92-1.2 in MCF-7 (breast) cell lines, while for paclitaxel and docetaxel 112 and 130 in LCC6, and 300 and 235 in MCF-7 [5].

C-3'-difluoromethyl docetaxel analogues were found 1 to 2 orders of magnitude more potent in MDR LCC6 cell lines [3]. Those C-3'-CF_2H taxoids prepared from 14-β-hydroxyl-10-DAB also exhibited comparable activity in both normal and MDR cell lines, but generally less active than their counterparts prepared from 10-DAB. Several paclitaxel analogues bearing C-3'-(*p*-F)-substitution in combination with 2-*m*-F, 2-difluoro, and 2-*m*-CF_3-*p*-F benzoates were found to be less active in most cases, while C-3'-CF_3 docetaxel analogues found to be more potent in either normal or MDR tumor cell lines [2]. C-3'-thiocarbamate paclitaxel analogues exhibited superior activitiy to that of the parent compound in HCT-116 drug-resistant tumors [8].

Very recently, it was found that a 2-diflurobenzoyl paclitaxel analogue (**24**) exhibited comparable activity to paclitaxel, are best in C-2 mono- and di-substituted benzoyl analogues and better than **9a** and **9b** in paclitaxel-resistant HCT-116/VM46 cancer cell lines [22]. It was also reported that some 2-debenzoyloxy-2α-benzamido docetaxel analogues were comparably cytotoxic to paclitaxel toward some drug-resistant tumor cell lines [24b]

Distefano *et al.* reported the activity of 7, 9-pyrazoline (general formula **25**) and C-seco (general formula **26**) analogues. The pyrazoline analogues of docetaxel were better than paclitaxel but less active than docetaxel. against adariamycin-resistant MCF-7 cells [47]. C-seco analogues were less potent than pyrazoline analogues. Since these pyrazoline analogues were able to arrest cell cycle at G_2/M phase and DNA fragmentation as well, their activities are very probably related to apoptosis.

An interesting finding on taxoid related MDR is the inactivity of orally administered paclitaxel is arisen from P-gP in gastrointestinal tract. Combined use of paclitaxel and a P-gP inhibitor will improve bioavailability to a great extent. Paclitaxel analogue IDN5109 (**27**), a poor substrate of P-gP, showed good oral bioavailability (48% p.o./i.v.), and significant efficacy in clinical trial [48]. 10-Deoxy-10-*C*-morpholinoethyl deocetaxel analogues are also orally active taxoids, and 10-(*C*-morpholinoethyl)-7-MeO docetaxel (**28**) is the best in this series [49].

2.2. Non-Paclitaxel Type Taxoids with MDR Reversal Activities

Some naturally occuring or semisynthetic non-taxol taxoids can restore MDR tumor cells sensitivity toward paclitaxel and other anticancer drugs. These taxoids are usually weakly cytotoxic, thus ideal candidates for combined use with cytotoxic agents.

Ojima's group prepared [50] 23 baccatin III-based taxoids with hydrophobic side chains at different positions of 10-DAB. Taxoids with mono hydrophobic ester substitution could be grouped in two categories. One group including taxoids with C-7 and C-10 modifications showed strong reversal activity (>95%) in most cases at the concentration of 1~3 μM, and another group with C-13 and C-2 modifications exhibited less or no activity. The effects of introducing two or three hydrophobic groups seems to be complicated. Baccatin III-7-(trans-1-naphthanyl-acrylic acid) ester (**29**) is the best among those semi-synthetic taxoids, which does not increase paclitaxel accumulation in sensitive tumor cell MCF-7, but drastically increase the paclitaxel accumulation in drug-resistant cell MCF-7-R with overexpression of P-gP.

Kobayashi's group first reported the effects of non-paclitaxel type taxoids from *Taxus cuspidata* on vincristine (VCR) accumulation in adriamycin-resistant human leukemia K562/ADM cell. Seven taxoids belonging to different sub-types are as potent as Verapamil for MDR reversal, and some of which can competitively bind to P-gP, including taxinine (**30a**) and taxuspine C (**31**) [51]. The derivatives of **30** and **31a** showed promising results [52-56]. A common observation for taxinine and taxuspine C derivatives is that a phenyl containing hydrophobic group attached to C-5 apparently increase the accumulation of paclitaxel in MDR. However, comparison of these results with those obtained from baccatin III derivatives is difficult due to reversed C-5 configuration. Very recently, Kobaysahi *et al.* reviewed their works on taxoids, including MDR reversal and other biological activities of these taxoids [57]. Interested readers can refer to it for a comprehensive description.

In addition, some tricyclic C-aromatic taxoid intermediates also exhibited MDR reversal activity, one of which is comparable to Verapamil. Incorporation of taxol side chain resulted in the reduction of the activity [58].

Some non-taxoid MDR reversal compounds may share common structure features with the above mentioned MDR reversal taxoids. For example, Chibale *et al.* attached hydrophobic moieties to antimalarial drugs chloroquine and primaquine, and found chloroquine derivatives are superior to primaquine derivatives against MDR *in vitro* and *in vivo* when coinjected with paclitaxel. They unexpectedly observed that those chloroquines fit very well to two baccatin III based MDR reversal compounds *in silico* [59].

3. DESIGN, SYNTHESIS AND PHARMACOLOGICAL ACTIVITY OF PRODRUGS OF PACLITAXEL

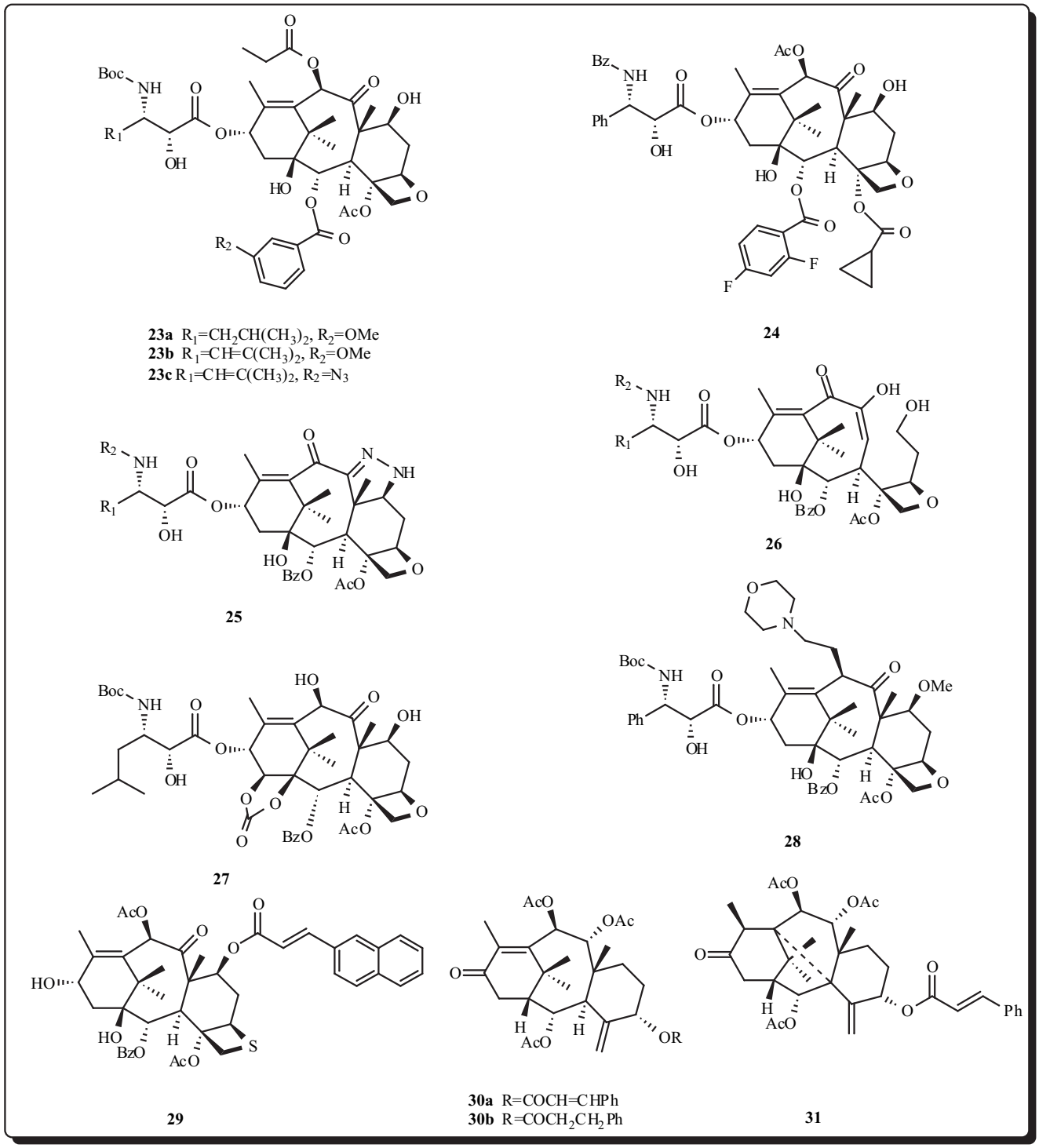
Currently the paclitaxel formulation contains a surfactant, Cremophol EL, to improve the poor water solubility of the drug. Some adverse effects including hypersensitivity have been attributed to Cremophor. Other severe adverse effects, such as neutropenia and dose dependent neurotoxicity, also occur at high dosage of paclitaxel administration. Improved water solubility may lower the dosage of paclitaxel due to effective transportation of the drug to the active sites, thus reduced high dosage related toxicity. Several alternative formulations, such as emulsions and liposomes have been developed to improve efficacy and minimize toxicity of paclitaxel. Another approach, design and preparation of

water-soluble prodrugs will be discussed. Besides, the "smart" prodrugs aiming at specific sites or kinds of tumors emerged in recent years. Some prodrugs with both improved water solubility and enhanced effectiveness and specificity were also documented.

3.1. Prodrugs Prepared to Improve Water Solubility

Most of the water-soluble prodrugs were realized by the derivatization of 2'-OH and 7-OH position, the two most liable positions in paclitaxel molecule. Some prodrugs are as active as the parental drug both *in vitro* and *in vivo*.

A series of amino acids were conjugated to 2'-OH through a glutaryl linker, and the asparagine- and glutamine-glutarylpaclitaxels improved the water solubility as much as 3 orders of magnitude [60]. These two derivatives as well as serine- and glycine- derivatives showed strong cytotoxicity against several sensitive cancer cell lines, and no activity against paclitaxel-resistant cells. Poly(L-glutamic acid)-paclitaxel (PG-TXL) [61.62], a derivative about 5 orders of



23a R_1=$CH_2CH(CH_3)_2$, R_2=OMe
23b R_1=CH=$C(CH_3)_2$, R_2=OMe
23c R_1=CH=$C(CH_3)_2$, R_2=N_3

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25

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30a R=COCH=CHPh
30b R=$COCH_2CH_2Ph$

31

magnitude more soluble than paclitaxel, was active against several kinds of tumors *in vivo* including those not responsive to both paclitaxel and a combination of paclitaxel and polyglutamic acid.

Hydroxy acid esters were also applied to the synthesis of prodrugs. Damen *et al.*[63] prepared 2'- and 7-malic esters of paclitaxel, and found 2'-ester behaved as the prodrug while 7-ester and 2', 7-diesters did not. The prodrug, due to its 2-fold higher maximum tolerance dose (MTD) than its parent drug, exhibited more significant antitumor activity at higher dosage. Wrasidlo and collaborators reported a 7-glycerolyl carbonate of paclitaxel as a prodrug with improved antitumor activity and hydrophilicity [64]. The prodrug, with 50-fold higher solubility in water, possessed 2.5-fold higher MTD, reduced toxicity to stem cells by 100 times, and exhibited almost equal activity *in vitro* as compared with paclitaxel. *In vivo* results are also promising--tumor growth regression in all prodrug treating groups were greater than that in paclitaxel groups. A series of polyol-carbonate prodrug of paclitaxel at 2' and 7 postitions were synthesized, and 7-(2", 3"-dihydroxypropylcarbonato) paclitaxel, named protaxel, was the best in solubilty and stablity assays in human serum [65]. Protaxel is actually the same compound as the one in ref. 64.

Takahashi *et al.* conjugated sialic acid to 7-OH of paclitaxel through a oligo ethylene glycol linker to prepare the potential neuraminidase cleavable, water-soluble prodrug [66]. Like extremely water soluble PEG-paclitaxel [67], the conjugate is also highly soluble in water (*ca*. 28 mg/mL), that is more than 4 orders of magnitude improvement. Other saccharide and PEG-linked prodrugs included conjugates of glucuronide [67], of PEG-glycinate [68], of PEG-HSA [69]. The conjugates with 5KD of PEG and HSA showed comparable cytotoxicity *in vitro* and reduced blood clearance and disposition in liver and spleen [69]. These changes in pharmacokinetics may have positive contribution to its improved *in vivo* antitumor activity.

Contrary to the usual preparation of hydrophilic conjugates, Ali *et al* prepared a series of hydrophobic α-bromoacyl prodrugs of paclitaxel with 6, 8, 10, 12, 14 and 16 carbon chains [70] on the basis of their discovery that the association of paclitaxel prodrugs with lipid bilayers was influenced by the chain length of the bromoacyl paclitaxel analogues [71]. In the absence of α-bromo substitution, the prodrugs were 50- to 250-fold less active, implicating the assistance of bromine in the hydrolysis of 2'-carbonate. Interestingly, it was found that the longer the chain is, the stronger the growth inhibitory activity, probably arising from "the slow hydrolysis of the prodrug followed by sustained delivery of paclitaxel to the tumor", according to the authors.

Non-prodrug water-soluble paclitaxel analogues were also reported [72]. Since their C-10 positions were covalently attached to secondary amines instead of acetate in paclitaxel, they can not convert into paclitaxel under physiological conditions.

3.2. Prodrugs Designed for Enhancing Specificity

Antibody-directed enzyme prodrug therapy (ADEPT) is one of the promising strategies in prodrug design. Rodrigues *et al* [73]. reported for the first time the application of ADEPT method to paclitaxel, using conjugate of β-lactamase and MAb, and cephalosporin-paclitaxel prodrug. To avoid immunogenicity caused by the non-human enzyme, glucuronidase was chosen as the enzyme in ADEPT [74]. Unfortunately, although the prodrug was hydrolyzed by glucoronidase to exhibit similar cytotoxicity to paclitaxel, activation of the prodrug with enzyme-MAb was not realized after 24 hours probably due to insufficient amount of enzyme bound to cells. Schmidt *et al*. prepared another ADEPT candidate very recently [75]. They chose 2'-carbamate instead of 2'-esters, and *para*-nitro group on the benzene ring in the linker can facilitate the attack of phenol ion to 2'-carbamate to liberate the parental drug. However, this prodrug may also suffer from similar problems since 100 μg/mL of enzyme was needed for fast release of the parental drug.

The lower toxicity, improved efficacy and water solubility, as well as tumor specificity make the MAb-paclitaxel conjugate a promising candidate for the treatment of tumors. The MAb for the p75 tyrosine kinase low-affinity receptor has been developed previously and one of them, anti-p75 MAb MC192, was chosen to target p75-overexpressing tumor cells. The *in vivo* efficacy of the conjugate is higher than free paclitaxel and co-injection of paclitaxel and MC192. Besides, "all-purpose" prodrug, which is supposed to target any kind of tumors wanted, were prepared by the conjugation of paclitaxel with anti-immunoglobulin secondary antibody [76].

Attachment of small peptides capable of recognizing tumor cell surface receptors to anticancer drug is another approach to enhance specificity. Safavy *et al*. reported the conjugation of paclitaxel-2'-succinate, PEG linker and a bombesin (BBN) fragment BBN 7-13, a hepta-peptide recognizing binding site on BBN/gastri-releasing peptide (GRP) receptor. The binding ability of BBN in this highly water soluble conjugate is comparable to the free peptide, and the cytotoxicity of the conjugate is stronger than free paclitaxel after 24 and 96 hours administration at dosages of 15 nM and 30 nM against human non-small-cell lung cancer NCI-H1299 cell line with BBN/GRP receptor [77].

Hyaluronic acid (HA) is a linear polysaccharide and one of several glycosaminoglycan components of the extracellular matrix (ECM). Some HA receptors are overexpressed in human breast epithelial cells and other cancer cells. Luo and Prestwich prepared a series of conjugates with different ADH loading from paclitaxel-2'-succinate and adipic dihydrarzide (ADH)-modified HA. Those conjugates showed selective activity toward human ovarian SK-OV-3, colon HCT-116 and breast HBL-100 cell lines, whereas no activity against untransformed murine fibroblast NIH 3T3 cell line. The conjugates with either highest or lowest loading of paclitaxel did not show best activity [78].

Folate-PEG-modified prodrugs of paclitaxel were recently prepared. Although the selected prodrug taxol-7-PEG-folate increased the survival in mice, it was not better than paclitaxel [79a, b].

A group from Bristol-Myers Squibb prepared several cathepsin B cleavable dipeptide (Phe-Lys) conjugate through

p-aminobenzylcarbonyl linker as prodrugs for paclitaxel, and mitomycin C and doxorubicin as well [80]. Unfortunately, the prodrug did not release the parent drug in human plasma.

Scheeren's group reported the synthesis of tumor-associated protease cleavable prodrugs of paclitaxel [81]. The carbamate and carbonate linkers between 2'-OH of paclitaxel and tripeptides D-Ala-Phe-Lys and D-Val-Leu-Lys were utilized instead of ester in these prodrugs to avoid nonspecific hydrolysis of 2'-ester by widely distributed proteases or esterases *in vivo*. These prodrugs are nontoxic, maybe the least toxic paclitaxel prodrugs hitherto reported. They did release the parental drug upon treatment of human plasmin, although at relatively high concentration (100 μg/mL of plasmin and 200 μM of prodrug).

Considering reductive condition in anaerobic environment in the center of tumor tissue, Scheeren's group designed and prepared 2'-carbonate and 3'-N-carbamate prodrugs of paclitaxel which release the parental drug targeting hypoxic tumor tissue. Two of 11 prodrugs are selected for further investigation [82].

4. EXPLORATION OF TUBULIN BINDING MECHANISM OF PACLITAXEL AND QUEST FOR ITS PHARMACOPHORE

4.1. Mechanism of Paclitaxel Related to Tubulin Binding

It has been widely acknowledged that the anticancer mechanism of paclitaxel is promoting tubulin polymerization and stabilizing the polymer since the pioneering work done by Horwitz's group two decades ago. Recent studies stressed the importance of the dynamics for tubulin assembly. It is even proposed that paclitaxel exerts its effect through affecting the dynamic of microtubules rather than its mass [83].

Horwitz's group had reported two photoaffinity labeling experiment results before the crystal structure of αβ-tubulin dimer was solved [84]. Employing [^{3}H]-labeled 3'-(*p*-azidobenzamido)paclitaxel and 2-(*m*-azidobenzoyl)paclitaxel, they found the amino acid residues 1-31 and 217-233 were photolabeled, but the precise position of photoincorporation was not determined. Recently, they used another photoreactive probe, [^{3}H]-7-(*p*-benzoyl)dihydrocinnamate, to explore the binding site of paclitaxel on β-tubulin [85]. Residues 277-293 were attributed to the photolabeling domain, and Arg282 was found to directly crosslink to the probe molecule. Their photoaffinity results are compatible with the electronic crystallographic structure of αβ-tubulin.

Bane's group compared the dynamic properties of paclitaxel and one of its "inactive" analogues baccatin III [86], and concluded that they behave similarly in their interactions with tubulin. These results supported the hypothesis that baccatin, the core structure of paclitaxel, is responsible for the majority of its interaction with tubulin at the binding site. However, one should be aware that the interaction can not be translated into cytotoxicity of taxoids directly. Andreu's group also claimed the importance of baccatin III core in the binding process [87]. They estimated that the C-2 and C-4 substitutions on the core structure account for about 75% of free energy change during taxol binding process. Without the assistance of C-13 side chain, the binding of the core structure is sufficient to initiate those pharmacological events induced by paclitaxel. During an attempt to create a common pharmacophore for paclitaxel and epothilones, Horwitz and coworkers proposed that C-2 benzoate is placed in the pocket formed by His-227 and Asp-224, and C-13 side chain and C-2 benzoate act as "anchors" for the binding of taxane ring to tubulin [88].

Bane's group also explored the binding of paclitaxel to its receptor quantitatively employing a fluorescent paclitaxel analogue [89]. They proposed that there are two types of binding sites, each as a single site on microtubules assembled from different nucleotide-tubulin complex. Before GTP hydrolysis, paclitaxel-tubulin binding has a high affinity with dissociation constant at nM level. The affinity decreased sharply to a μM level after GTP hydrolysis to GDP. Diaz *et al*. probed the binding site of paclitaxel on microtubules using two of its fluorescent derivatives Flutax-1 and -2 [90]. They found that paclitaxel binds very rapidly, a fact difficult to be explained by the current model. So they suggested a rotated or structure modified model, similar to that of Nogales *et al*. [91], in which the binding site may be located between protofilaments, easily accessed from the surface of microtubule.

More recently, several papers revealing microtubule structure with improved resolution were published [92, 93]. In the refined tubulin structure, the binding pocket of paclitaxel was modified slightly [92]. These structures will provide good starting point in the construction for the pharmacophores of paclitaxel and other antitubulin drugs.

While many studies concentrated on β-tubulin, the role of α-tubulin in the binding process was scarcely known. In a recent report, the authors found that assembly of different α-tubulin isoforms differ greatly in the presence of paclitaxel, and thus proposed at least partial involvement of α-tubulin in the binding process [94].

4.2. Quest for Bioactive Conformations and Pharmacophores

What conformation paclitaxel adopts when it binds to its receptor tubulin is an important question to be answered. There are many efforts on construction of common pharmacophore for several other anti-tubulin natural products sharing same binding site with paclitaxel. To simplify the situation, only "active" conformation(s) and pharmacophore for taxoids will be discussed here.

Due to the rigid structure of tetracyclic core of taxane with less change during binding, people focused on the side chain of paclitaxel, especially C-13 isoserine. In 1993, the first hypothesis on "active" conformation, "hydrophobic collapse", was established on the basis of NOESY data of paclitaxel in DMSO-d_6/D_2O solution. It was proposed that 3'-Ph, phenyl rings of 3'-NH and 2-benzoate as well as 4-OAc were close to each other in hydrophilic environment. Major differences in paclitaxel conformations in nonpolar and polar solvents were found, and those conformations assigned as "nonpolar" and "polar" as two representative groups respectively. Despite the difference, both "nonpolar" and "polar" conformations showed some extent of "hydrophobic collapse" property. Until very recently, a non-

collapsed "T-shaped" conformation on the basis of molecular simulation was proposed as the binding conformation of paclitaxel to tubulin [95].

The "nonpolar" conformation was established on the basis of NMR data of paclitaxel and docetaxel in nonpolar solvent such as chloroform, as well as crystallographic data of docetaxel. Snyder's group selected 20 amino acid residues and paclitaxel conformation in $CDCl_3$ to construct a mini-receptor model [96] for both paclitaxel and epothilone, an antimitotic agent with the same tubulin-binding site as paclitaxel. This model has been applied to predict binding of some D-seco analogues. Unfortunately, two D-seco analogues with saturated C ring, which is predicted to be similar to paclitaxel in the free energy of drug binding, were inactive in bioassay [36]. This result prompted the authors to improve their model. They changed the amino acid H-bonding to oxetane ring from Arg to Thr, and incorporated more amino acid residues close to oxetane ring so that the inactivity of those D-seco analogues can be explained [36].

The "polar" conformation was proposed on the basis of NMR data of taxoids in polar solvent and X-ray structure of paclitaxel. Recently, Ojima and collaborators proposed a common pharmcophore for paclitaxel and several other antimotic natural products on the basis of NMR data recorded in DMSO-d_6/D_2O of a macrocyclic pacliaxel analogue, nonataxel, and molecular modeling results [34]. Later on, supportive evidences were collected in photoaffinity labeling experiments [97]. Ojima's group has suggested that two major "collapsed" conformations of paclitaxel, one with H2'-C2'-C3'-H3' dihedral angle of 180º (the characteristics for the "polar" conformation mentioned earlier) and another with the angle of 124º (which is believed to be the third "active" conformation of paclitaxel at that time), are in equilibrium in aqueous environment using "fluorine-probe approach" [98]. They explained the bioactivity of a series of A-seco analogues [16] and fluorine substituted analogues in compliance with "polar" conformation hypothesis. However, "hydrophobic collapse" conformations were questioned to be “active” conformation since some macrocyclic tethered paclitaxel analogues mimicking "hydrophobic collapse" were found inactive [39].

Synder and coworkers proposed that the NMR data is probably the dynamic averages of large sets of conformers rather than one or two major conformers. Ojima's group has recognized the dynamic equlibrium behavior of paclitaxel conformers, but they did not find the “T-shaped” conformer subsets. After reanalyzing paclitaxel ROESY data published earlier with NMR analysis of molecular flexibility in solution (NAMFIS) techniques, Snyder’s group identified 8 energy optimized conformers, among which four representing 33% of the whole conformer mixture belong to neither "non-polar" nor "polar" conformations but what they called "open" conformer subfamily [99]. Further studies led to the discovery of unique "T-shape" ("open") conformation of paclitaxel, which does not resemble either of above mentioned conformations [95]. In this model constructed on the electronic crystallographic data of αβ-tubulin and subsequent molecular simulation, C-2 benzoate and C-3' of paclitaxel can not collapse due to the prevention of His-229 of the receptor protein. NAMFIS revealed that all three major groups of conformers (nonpolar, polar, and T-shape) existed in the solution for a group of C2'-C3'-Ph tethered analogues of paclitaxel [12]. In fact, extended conformations predominate in the mixture of conformers and three T-shape conformers constitutes 59% of the extended conformers.

Some reports focused on conformation of C-13 phenylisoserine side chain of paclitaxel [100]. From the point of view of setting up an “active” conformation model for paclitaxel, a drug-receptor complex rather than a part of the drug, e.g. C-13 side chain, will be more informative and meaningful.

The oxetane D-ring in paclitaxel also attracts a lot of attentions. Previous SAR studies have suggested it plays a critical role in binding, either through taxane skeleton rigidification, and/or a weak hydrogen bonding acceptor. But its essence in binding is not acknowledged anymore [35, 101]. Synder's group has reported [101] their research efforts on the role of the D-ring in paclitaxel recently, showing binding energies of some D-seco analogue of paclitaxel are comparable to that of paclitaxel. They predicted that some analogues without intact oxetane D ring can still bind to tubulin very well. Georg’s group found that conformational changes are relayed from ring C to A in the D-seco analogues [36, 102] Another consequence of SAR study of D-seco analogues [36] is that the second generation of paclitaxel-epothilone minireceptor [96, 101] was revised due to its inconsistence with experimental results.

5. CONCLUDING REMARKS

While most reserach works have focused on the development of paclitaxel analogues or prodrugs with enhanced specificity, MDR reversal and orally effective taxoids were also developed recent years. In the mean time, scientists have gained insights into the mechanism of action of taxoids at molecular level, i.e. binding sites on tubulin and dynamics of tubulin polymerization. It's noteworthy to point out that the SAR results derived from traditional medicinal chemistry [1a-c] have shown the essential role of C-13 side chain, but some pharmacophore studies suggested that C-13 isoserine chain only contributes a small part comparing to the baccatin core structure, to binding and triggering subsequent physiological response [86-88]. On the basis of common pharmacophore established for paclitaxel and several other tubulin-targeting molecules, people tried to apply SAR results of one drug, e.g. paclitaxel, to another molecule sharing the same pharmacophore. But such efforts were usually unsuccessful. Instead, high throughput screening of small molecule libraries with structure diversity may be a good choice in the future discovery of anti-tubulin compounds [103].

In forthcoming years, it is expected that our SAR and mechanistic knowledge will lead to the rational design of the next generation of taxoids with better specificity, lower toxicity as well as better pharmacokinetic properties. The discovery of other mechanisms of taxoids, e.g. apoptosis and stimulation of immune system, will prompt people to find new synergic use of taxoids with other drugs. New techniques such as combinatorial chemistry, genomics, proteomics and pharmacogenetics will reshape pharmaceutical industry in the future and accelerate the R&D of new drugs, and undoubtedly, also benefit taxoid research.

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