Potent Antitumor Activity of Novel Taxoids in Anaplastic Thyroid Cancer

Meichen Wang  
Xi'an Jiaotong University

Changwei Wang  
Stony Brook University

Chao Feng  
Xi'an Jiaotong University

Wanrong Guo  
Guangzhou Institutes of Biomedicine and Health

Huan Chen  
Xi'an Jiaotong University

Bing Liu  
Xi'an Jiaotong University

Enxiao Li  
Xi'an Jiaotong University

Wei Liu  
Shaanxi Provincial People's Hospital

Adam Taouil  
Stony Brook University

Iwao Ojima  
Stony Brook University

Peng Hou  (phou@xjtu.edu.cn)  
Xi'an Jiaotong University  https://orcid.org/0000-0001-7010-7944

Research Article

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Abstract

**Purpose:** Anaplastic thyroid cancer (ATC) is the most aggressive form of thyroid cancers and it is rapidly fatal without any effective therapeutic regimens. There are some clinical trials showing that paclitaxel-based chemotherapy for ATC can achieve a relatively high response rate and low incidence of adverse reaction. The aim of this study was to evaluate potential therapeutic activity of novel taxoids in ATC cells.

**Methods:** We evaluated antitumor activity of five novel 3′-difluorovinyltaxoids (DFV-taxoids) in anaplastic thyroid cancer cells by a series of in vitro and in vivo experiments. Besides, we also explored the potential mechanism underlying the difference among the taxoids and paclitaxel by molecular docking and tubulin polymerization assays.

**Results:** Our data showed that these novel DFV-taxoids were more effective than paclitaxel in ATC cell lines and xenografts, as reflected by the inhibition of cell proliferation, colony formation and tumorigenic potential in nude mice, and the induction of G₂/M phase arrest and cell apoptosis. Using tubulin polymerization assays and molecular docking analysis, we found that these DFV-taxoids promoted more rapid polymerization of β-tubulin than paclitaxel.

**Conclusions:** Our data demonstrate that these novel taxoids exhibit stronger antitumor activity in ATC cells than paclitaxel, thereby providing a promising therapeutic strategy for the patients with ATC.

Introduction

From 2000 to 2011, the incidence rate of thyroid cancers has risen the fastest, and it is the fourth most common cancer in China based on the latest statistics [1]. Anaplastic thyroid cancer (ATC) accounts for 1–2 % of all thyroid cancers. Although rare, it is a major cause of death in thyroid cancer patients due to its aggressive behavior and resistance to treatment [2]. Thus, there is an urgent need to develop effective therapeutic strategies against ATC.

Paclitaxel is one of the most active single chemotherapeutic agents used clinically for the treatment of head and neck cancers, including thyroid cancer [3]. Paclitaxel promotes tubulin polymerization, causing cell cycle arrest and apoptotic death by disrupting normal microtubule dynamics required for cell division and vital interphase processes [4]. Despite their potent antitumor activity, paclitaxel can cause undesirable side effects and drug resistance [5]. Thus, it was apparent in the early 1990s that it would be essential to develop new taxoids with fewer side effects, enhanced activity, improved solubility and superior pharmacological properties [6].

Due to the limited availability and side effects of paclitaxel, numerous taxoids were designed and synthesized based on the structure of paclitaxel in the past decades [6, 7]. Ojima group developed a series of novel generation taxoids with systematic modifications at the C2, C10, C3’, and C3’ N positions of the tetracyclic diterpene skeleton, 10-deacetylbaccatin (DAB) [7, 8]. There was also a study to develop taxoids based on 14 β-hydroxy-10-deacetylbaccatin (14-OH-DAB) [9]. A key feature of the second-
generation taxoids is their possession of an isobutenyl or isobutyl group at the C3’ position in place of a phenyl group, which has been demonstrated to enhance potency, especially the enhancement of tubulin polymerization [7, 9]. In recent years, novel taxanes have been developed, presenting a new generation of taxoids [10]. Some of them have been found to be more effective than paclitaxel in the treatment of different types of cancer [11].

In this study, we systematically evaluated therapeutic potential of five novel 3’-difluorovinyltaxoids (DFV-taxoids) in ATC cells by a series of in vitro and in vivo experiments.

Materials And Methods

Chemical compounds

Paclitaxel was purchased from MedChemExpress (Monmouth Junction, NJ, USA). Five novel 3rd-generation 3’-difluorovinyltaxoids (DFV-taxoids) were kindly provided by Dr. Changwei Wang (Stony Brook University, USA), and their chemical structure was shown in Fig. 1a.

ATC cell lines

Human thyroid cancer cell lines 8505C and 8305C were kindly provided from Dr. Haixia Guan (The First Affiliated Hospital of China Medical University, Shenyang, China) and routinely cultured at 37°C in RPMI 1640 medium with 10% fetal bovine serum (FBS). Paclitaxel and novel taxoids were dissolved in dimethyl sulfoxide (DMSO), aliquoted and stored at -80°C until use. The concentration of DMSO was 0.1% as vehicle control.

Cell proliferation assay

Cells were treated with different concentrations of paclitaxel or taxoids and vehicle control (0.1% DMSO) for 72 h. The MTT assay was then performed to assess their effect on cell proliferation, and IC<sub>50</sub> values were calculated as described previously [12].

Colony formation assay

Cells were treated with various concentrations of paclitaxel or taxoids for 48 h, followed by culturing in RPMI-1640 medium with 10 % FBS for 5–6 days. Colonies were then fixed with methanol and stained with crystal violet. Each experiment was performed in triplicate.

Cell cycle assay

Cells were serum starved for 12 h, and then treated with 50 nM paclitaxel or taxoids and vehicle control for the indicated time. Next, cells were harvested and fixed in ice-cold 70% ethanol for at least 4 h. After washing twice with PBS, propidium iodide (PI) solution (50 µg/mL propidium iodide, 50 µg/mL RNase A, 0.1% Triton-X, 0.1 mM EDTA) was added to stain the cells. Cells were then subjected to flow cytometer analysis to determine cell cycle distributions.
Cell apoptosis assay

Cells were treated with 50 nM paclitaxel or taxoids and vehicle control for 24 h and then stained with Annexin V-FITC/PI Apoptosis Detection Kit according to the manufacturer’s protocol. Apoptotic cells were measured by flow cytometry. Each experiment was run in triplicate.

Western blot analysis

Cells were treated with 50 nM paclitaxel or taxoids and vehicle control for the indicated times. Next, cells were lysed, and the lysates were subjected to western blot analysis. Antibody information was shown in Table S1.

Protein expression and purification

Human TUBB2B gene was cloned to pET-28a plasmid and transformed into E. coli BL21 strain. After induced by 0.2 mM IPTG for 6 h, bacterial cells were harvested and lysed by ultrasonication. The fusion protein TUBB2B was expressed in inclusion bodies. After complete denaturation by 8 M urea, the protein was rapidly diluted by 7-fold Tris-buffer followed by dialysis with the same buffer for 4 times. After folding, the protein was purified by His-tag with a Ni-NTA column, desalting and concentrated by a Millipore ultrafiltration centrifuge tube. The expressed and purified proteins were analyzed by SDS-Page and RP-HPLC. RP-HPLC was performed at 40°C on a Waters XBridge C18 column (4.6 × 150 mm, 3.5 µm) running a 45 min, 5 – 95% linear gradient of acetonitrile in water containing 0.1% TFA at a flow rate of 1 mL/min.

Tubulin polymerization assay

Tubulin polymerization assay was carried out with 10 µM paclitaxel, taxoids and vehicle control in MES buffer containing 1 mM EGTA, 0.5 mM MgCl₂, 1 mM GTP, and 10% glycerol. The reaction system was kept at room temperature before addition of tubulin and shifting to 37°C [13]. Assembly of TUBB2B proteins was monitored spectrophotometrically by recording changes in turbidity at 350 nm at 37°C [14, 15].

Molecular docking

Molecular docking of the DFV 3rd generation taxoids was carried out using AutoDock Vina [16] and the available cocystal structure of paclitaxel in β-tubulin (PDB: 1JFF). Structures of the new taxoids were adapted from the coordinates of paclitaxel in 1JFF, which conserves the baccatin core and relevant ring conformations. Modified groups were minimized using the Avogadro molecule builder [17] and relaxed under the MMFF94 forcefield [18–21]. Partial charges of each atoms were calculated using the Gastieger method [22, 23] and non-polar hydrogen atoms were merged to their respective heavy atoms. Calculations were done using a grid box size of 40 Å³ and the default scoring function for AutoDock Vina. Visualization of the docked poses were done using the UCSF Chimera [24], where the T-taxol-type [25] structure was identified from a total of 20 docking solutions for each molecule.

Animal studies
Five- to six-week-old female athymic nude mice were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China), and housed in a specific pathogen-free (SPF) environment. Tumor xenografts were established by subcutaneously injecting 8505C \( (5 \times 10^6) \) cells into right iliac fossa region of nude mice. When the tumors grew to \( \sim 5 \) mm in diameter, the mice were randomly divided into three groups (five mice per group). Vehicle control (< 1%), 5 mg/kg paclitaxel or taxoid SB-T-1285406 were then administered by intraperitoneal injection three times a week. Tumor volumes were measured by a Vernier Caliper every other day and calculated by the following formula: Tumor volume \( (\text{mm}^3) = \text{length} \times \text{width}^2 \times 0.5 \). All mice were sacrificed 4 h after the last treatment, and tumors were harvested and weighted.

Xenograft tumors were fixed in 10% formalin for 48 h, embedded in paraffin, sectioned at 5 µm, deparaffinized and rehydrated. Immunohistochemistry (IHC) assay was performed to evaluate Ki-67 levels in these tumors. Hematoxylin-Eosin (H&E) staining was used to observe histologic structure and cell morphology of hepatic and renal tissues. The serum levels of alkaline phosphatase (AKP), aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN), creatinine (CRE) were measured by spectrophotometry according to the manufacturer's protocol. The TUNEL assay was used to determine cell apoptosis in tumor tissues by using the TUNEL Andy Fluor™ 488 Apoptosis Detection Kit according to the manufacturer’s protocol. All animal experiments were conducted and approved by the Laboratory Animal Center of Xi’an Jiaotong University.

### Statistical analysis

Student’s \( t \)-test, two-way ANOVA with Bonferroni post-test and one-way ANOVA with Dunnett’s post-test were used to compare the data between two groups. The data were expressed as mean ± standard deviation (SD). \( P < 0.05 \) was considered statistically significant. All statistical analyses were conducted using the SPSS statistical package (16.0, SPSS Inc. Chicago, IL).

### Results

#### Taxoids inhibit the proliferation and colony formation of ATC cells

To determine \textit{in vitro} growth response of ATC cells to these novel taxoids (Fig. 1a) and paclitaxel, we first treated 8505C and 8305C cells with increasing concentrations of these 5 taxoids and paclitaxel from 0.01 nM to 100 nM for 48 h to calculate \( \text{IC}_{50} \) values. The results showed that these taxoids and paclitaxel significantly inhibited the proliferation of ATC cells in a dose-dependent manner, and the taxoids were more effective than paclitaxel with a 2-9-fold increase in sensitivity (Fig. 1b). This was also supported by the MTT assay. As shown in Fig. 2, we found that these taxoids more significantly inhibited cell proliferation than paclitaxel when 8505C and 8305C cells were treated with the same concentration (5 nM) of taxoids and paclitaxel. Besides, we treated these cells with different concentrations of taxoids and paclitaxel, and performed colony formation assay to further confirm the above conclusion. The results showed that these taxoids exhibited more potent inhibitory effect on colony formation of ATC cells than
paclitaxel (Fig. 3). Altogether, our data indicate that these novel taxoids are more effective cytotoxic antitumor agents than paclitaxel against ATC cells.

**Taxoids induce G2/M phase arrest in ATC cells**

Paclitaxel, one of the broadest-spectrum antitumor agents, performs its anti-mitotic and anti-cancer activity by targeting microtubule [26]. The consequent arrest of cell cycle at mitotic phase has been considered the cause of paclitaxel-induced cytotoxicity [27]. In this study, we treated 8505C and 8305C cells with 50 nM paclitaxel or 5 taxoids and vehicle control for 48 h, and analyzed their cell cycle distributions. As shown in Fig. 4a and Fig. S1, both paclitaxel and the taxoids caused a significant G2/M phase arrest compared to the control. The percentage of G2/M phase cells increased from 23.66 ± 2.01 % to 38.99 ± 1.10 % in paclitaxel-treated 8505C cells, while the percentage of G2/M phase cells increased from 23.66 ± 2.01 % to 65.02 ± 1.62 %, 66.02 ± 3.07 %, 59.77 ± 1.28 %, 62.59 ± 2.29 % and 62.14 ± 1.13 % in taxoids-treated 8505C cells relative to DMSO-treated 8505C cells. Similarly, paclitaxel treatment increased the percentage of G2/M phase cells from 21.58 ± 1.59 % to 26.27 ± 1.19 % in 8305C cells, while taxoids treatment increased the percentage of G2/M phase cells from 21.58 ± 1.59 % to 46.29 ± 0.80 %, 46.23 ± 4.53 %, 41.51 ± 1.31 %, 46.68 ± 0.20 % and 45.59 ± 0.94 % compared to the control. These results indicate that these novel taxoids have a more prominent advantage in inducing cell cycle arrest than paclitaxel, further supporting the above conclusion.

Considering that paclitaxel and taxoids can induce G2/M phase arrest of ATC cells, thus we determined their effect on the expression of cell cycle regulatory components at the G2/M boundary, such as p-Rb, Weel, Cyclin B1, Cyclin E, CDK1 and p21. First, we treated 8505C and 8305C cells with 50 nM paclitaxel or SB-T-1285406, one representative of these 5 taxoids, for 24 h and 48 h, respectively. Cells were lysed and subjected to western blot analysis. The results showed that paclitaxel or SB-T-1285406 treatment resulted in a marked decrease in the expression of p-Rb, Weel, Cyclin B1, Cyclin E, CDK1 and p21 both in 8505C and 8305C compared to the control, particularly after 48 h treatment (Fig. 4b). These results indicate the G2/M arrest induced by paclitaxel or SB-T-1285406 is closely associated with a marked alteration in the expression of G2/M cell cycle regulatory proteins.

**Taxoids induce the apoptosis of ATC cells**

To evaluate the effect of these taxoids on cell apoptosis, we treated 8505C and 8305C cells with 50 nM of paclitaxel, taxoids and vehicle control for 24 h. The results showed that, compared to the control, the percentage of apoptotic cells increased from 2.60 ± 0.14 % to 21.62 ± 0.10 % in paclitaxel-treated 8505C cells, and from 2.60 ± 0.14 % to 12.57 ± 0.40 %, 20.17 ± 0.38 %, 23.28 ± 0.47 %, 21.71 ± 0.15 % and 14.66 ± 0.83 % in taxoids-treated cells (Fig. 5a). Similarly, paclitaxel treatment increased the percentage of apoptotic cells from 3.54 ± 0.06 % to 7.54 ± 0.24 % in 8305C cells, while taxoids treatment increased the percentage of apoptotic cells from 3.54 ± 0.06 % to 11.24 ± 0.19 %, 10.86 ± 0.02 %, 8.25 ± 0.50 %, 8.42 ± 0.16 % and 6.32 ± 0.30 % compared to the control (Fig. 5b).
Taxoid SB-T-1285406 inhibits tumor growth in nude mice

To determine in vivo antitumor activity of 1285406, we established 8505C cell-derived tumor xenografts in nude mice and treated these mice with vehicle control, 5 mg/kg paclitaxel or SB-T-1285406 for every other day for 2 weeks. As shown in Fig. 6a, both SB-T-1285406 and paclitaxel significantly inhibited the growth of xenograft tumors compared to the control, and the former showed better effect than the latter. At the end of the experiments, the tumors were isolated and weighted. The results showed that mean tumor weight was significantly lower in SB-T-1285406- and paclitaxel-treated groups compared to control group, and SB-T-1285406 treatment exhibited better inhibitory effect than paclitaxel treatment (Fig. 6b).

Ki-67 is present in all proliferating cells, but absent in resting cells, thus it serves as a proliferation marker [28]. In this study we evaluated Ki-67 levels in xenograft tumors by IHC assay. As shown in Fig. 6c, the percentage of Ki-67 positive cells was significantly decreased in SB-T-1285406- and paclitaxel-treated xenograft tumors compared to control tumors. Similarly, SB-T-1285406 showed more potent inhibitory effect than paclitaxel. Next, we also evaluated cell apoptosis in xenograft tumors by TUNEL assay. As shown in Fig. 6d, the number of apoptotic cells was significantly increased in SB-T-1285406- and paclitaxel-treated tumors compared to control tumors. Similar to in vitro results, we did not find significant difference between SB-T-1285406- and paclitaxel-treated tumors. Importantly, there was no significant difference with respect to serum levels of AKP, ALT, AST, BUN, CRE and H&E staining of hepatic and renal tissues among three treatment groups (Fig. 6e and Fig. S2). Besides, SB-T-1285406 and paclitaxel treatment almost did not affect body weight of mice compared to the control (Fig. S3). Collectively, our data indicate that novel taxoids such as SB-T-1285406 have more potent antitumor activity than paclitaxel in ATC cells, and exhibit sufficient safety like paclitaxel.

Taxoids promote more rapid polymerization of tubulin than paclitaxel

We next attempted to reveal potential mechanism by which novel taxoids are superior to paclitaxel. It is well-known that paclitaxel binds at the interface between the nucleotide binding and intermediate domains on the luminal face of β-tubulin [29]. Thus, we first cloned human TUBB2B gene to pET-28a plasmid and transformed it into E. coli BL21 strains to express TUBB2B proteins (Fig. S4). After purifying these proteins, we evaluated the activities of these 5 taxoids and paclitaxel by in vitro tubulin polymerization assay. This assay will provide a direct measure of turbidity, indicating the speed and extent of tubulin polymerization. As shown in Fig. 7a, these taxoids universally promoted rapid polymerization of β-tubulin at a faster rate than paclitaxel. The turbidity of tubulin solution treated by these taxoids quickly reached a plateau relative to paclitaxel. Briefly, it took about 10 mins for paclitaxel to reach the plateau, while for taxoids, it took only 3–5 mins to reach the peak. These observations imply that there is a difference in structure between microtubules formed with these taxoids and those with paclitaxel.
Paclitaxel exerts potent antitumor activity by promoting tubulin polymerization and inhibiting depolymerization of formed microtubules via its binding to β-tubulin components of microtubules [30, 31]. To explain why the ability of these taxoids binding to microtubules are better than paclitaxel, we performed molecular docking analysis using paclitaxel-bound α, β-tubulin crystal structure 1JFF from protein database (PDB) (http://www.rcsb.org/) [30]. In all cases, the “T-taxol”-type binding mode was found as a reasonable solution. For example, an excellent overlay of paclitaxel and SB-T-1285406 shown in Fig. 7b (1) exhibited a hydrogen bond between the NH of Gly362 and the C2’-OH group of paclitaxel and the DFV-taxoid with distances of 2.69 Å and 2.39 Å, respectively. A previous study has indicated the expected binding orientation of the C2 benzoate substituents [32]. The unique property of the difluoromethoxy group results in the orientation that is placed in a hydrophobic binding site, which reduces solvent exposure and bolsters the protein-drug contact interface. Moreover, Fig. 7b (2) showed the interactions of the difluoromethoxy group of SB-T-1285406 with His229 and Phe272. The difluoromethoxy group is oriented to make van der Waals contacts with the proximal surface of the protein consisting of Phe272 while simultaneously forming a hydrogen bond (OCF$_2$H—N) with the delta nitrogen of His229.

In the case of trifluoromethoxy-taxoids, one of the fluorine atoms can form a hydrogen bond with the amine hydrogen of His229 (NH–F-CF$_2$O) (SB-T-1285205 and SB-T-1285605). As shown in Fig. S5, three difluoromethoxy-taxoids SB-T-1285106, SB-T-1285406 and SB-T-1285506, the hydrogen bond distances were 3.57 Å, 3.48 Å and 3.57 Å, respectively. For the two trifluoromethoxy-taxoids, SB-T-1285205 and SB-T-1285605, the hydrogen bond distances were 2.64 Å and 2.66 Å, respectively. The hydrophobic interaction distances between DFV taxoids and Phe272 were 3.43 ~ 4.14 Å (Fig. S5). The non-polar/polar duality of the difluoromethoxy group presents a unique binding motif with considerably stronger complementarity than paclitaxel.

The introduction of the DFV group did not change the overall “T-taxol”-type conformation. However, there were DFV specific interactions of the two fluorine atoms with surrounding amino acid residues, Ser236, Ala233 and Phe272, which paclitaxel did not have (Fig. 7b (3)). The introduced fluorine atoms exhibited a broader interaction profile than the phenyl group presents in paclitaxel, allowing it to act as a hydrogen bond acceptor with the hydroxyl group of Ser236 and also interacting with nearby hydrophobic amino acid residues such as Ala233 and Phe272. Since the two fluorine atoms of the 3’-DFV group was located substantially closer to Phe272, as compared to the 3’-phenyl group of paclitaxel, this van der Waals interaction would make the binding of the DFV-taxoid tighter than paclitaxel. Heavy atom distances (i.e., C–F) of the DFV group of SB-T-1285406 with Ser236, Ala233, and Phe272 were 2.87 Å, 3.36 Å, and 3.51/3.88 Å, respectively (Fig. 7b (3)).

In general, both organofluorine groups are observed to interact with the deep binding pocket behind the protruding residue His229, to which all of DFV-taxoids wrap around. The electrostatic surface representation (Fig. 7b (4)) showed the hydrophobicity of this pocket composed of Phe272 and Ala236 to which these organofluorine moieties can more tightly and favorably bind, as compared to paclitaxel. The
These findings suggest that these unique interactions may be essential for enhancing the ability of these novel taxoids binding to β-tubulin, thereby contributing to their better anti-cancer effect than paclitaxel.

**Discussion**

Although rare, ATC is the most lethal histotype of thyroid cancer [33]. ATC patients generally have a palpable, size variable and irregular rigid mass in the thyroid with or without hoarseness, dysphagia and dispomes. Clinical pathology of ATC is characterized by rapid cell growth and the ability to metastasize to other organs of the body, especially lungs, bones and brain. These features contribute to its high mortality. At present, the principle of ATC treatment is total thyroidectomy combined with postoperatively systemic chemotherapy, radiotherapy and other therapeutic strategies. Although multimodality strategies have been demonstrated to be better than monotherapy, the vast majority of ATC patients still have a very poor survival because they are unresponsive to conventional antitumor agents. Thus, it is urgent to develop more effective therapeutic drugs for ATC treatment.

For the last decades, paclitaxel emerged as one of the most important compounds broadly used in the therapy of breast, ovarian, lung, head and neck cancers [4]. A phase 2 clinical trial first proposed the efficacy of continuous infusion paclitaxel every 3 weeks for 1 to 6 cycles in 20 patients with ATC [34]. Besides, another study analyzed the clinical data of 13 patients undergoing paclitaxel treatment every week, and finally observed 1 patient as complete response (CR) [35]. These observations indicate that paclitaxel treatment may be beneficial to prolong the survival of ATC patients. Meanwhile, there are studies demonstrating that antitumor effect of paclitaxel in ATC can be further enhanced when combined with lenvatinib, sorafenib or radiotherapy [36, 37]. However, traditional paclitaxel often causes serious side-effects besides drug resistance. Thus, it is essential to develop new taxoids with better pharmacological properties and improved activity in order to overcome the problems with traditional paclitaxel in cancer therapy. Evidently, new taxanes can effectively control advanced cancers including ATC [35–39], indicating that it may be a promising therapeutic strategy to prolong the survival of ATC patients without serious toxic side-effects.

In this study, we evaluated 5 novel DFV-taxoids (SB-T-1285106, SB-T-1285205, SB-T-1285406, SB-T-1285506 and SB-T-1285605), which were modified at the C2, C10 positions based on chemo-structure of paclitaxel. We next compared their antitumor activity with paclitaxel by a series of *in vitro* and *in vivo* experiments. The results showed that all of these taxoids exhibited exceptionally high potency than paclitaxel against ATC cells, as reflected by their lower IC$_{50}$ values and more potent inhibition of cell proliferation, colony formation and tumorigenic potential in nude mice. Besides, these DFV-taxoids also exhibited more apparently G$_2$/M phase arrest in ATC cells compared to paclitaxel. More importantly, we did not observe serious toxic side-effects, indicating that these DFV-taxoids such as SB-T-1285406 is a safe and effective agent for ATC therapy.

To clarify the mechanism of these taxoids showing better antitumor effect than paclitaxel, we performed *in vitro* tubulin polymerization assay, and found that these taxoids induced GTP-independent tubulin
polymerization faster than paclitaxel, implying that there exists difference in structure between microtubules formed with these taxoids and paclitaxel. These results were in consistent with our previous study [8], besides the difference in binding rates, our previous study also mentioned rapid tubulin polymerization of new generation taxoids producing numerous short microtubules compared with paclitaxel, which may also be another difference between the DFV-taxoids and paclitaxel. This may be the main reason why these taxoids exhibit potent antitumor effect than paclitaxel.

To further look into the mechanism behind this difference, we performed molecular docking to analyze their interaction with β-tubulin. In fact, we have found that the DFV-taxoids possess unique and specific interactions with β-tubulin through organofluorine groups in the molecule, which do not exist in paclitaxel. The difluoromethoxy group of SB-T-1285406 interacts with His229 and Phe272 (Fig. 7b (2)). The difluoromethoxy group makes van der Waals interactions with the proximal surface of the protein consisting of Phe272, while simultaneously forming a hydrogen bond (OCF₂H–N) with the delta nitrogen of His229. In the case of trifluoromethoxy-taxoids, one of the fluorine atoms can form a hydrogen bond with the amine hydrogen of His229 (NH–F-CF₂CO) (Fig. S5). The introduction of the DFV group did not change the overall “T-taxol”-type conformation, while there are DFV-specific interactions of the two fluorine atoms with surrounding residues Ser236, Ala233 and Phe272, which paclitaxel does not have (Fig. 7b (3)). Since the 3’-DFV group is located substantially closer to Phe272, as compared to the 3’-phenyl group of paclitaxel, this van der Waals contact would make the binding of the DFV-taxoid tighter than paclitaxel. As the electrostatic surface representation in Fig. 7b (4) indicates, the hydrophobicity of this pocket allows the tighter and more favorable binding of these organofluorine moieties, as compared to paclitaxel.

In summary, by a series of extensive in vitro and in vivo experiments, we demonstrate that these five novel DFV-taxoids exhibit promising antitumor effect in ATC cells. They exhibit better antitumor activity than paclitaxel, thereby causing shorten therapeutic periods and less side-effects during several cycles of chemotherapy. Thus, our data indicate that these taxoids as monotherapy or in combination with other antitumor agents will provide a relatively safe and effective strategy for the treatment of ATC patients.

Declarations

Data availability

The data included in this study are available on request from the corresponding authors.

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Compliance with ethical standards

Conflicts of interest The authors have no potential conflict of interest to disclose.

Ethical approval All animal experiments were conducted and approved by the Laboratory Animal Center of Xi’an Jiaotong University.

Author contributions

PH and IO conceived and designed the experiments. MCW, CWW and CF performed the experiments, WRG, EXL and WL analyzed the data. HC, BL, AT performed the docking analysis. PH and CWW contributed reagents and materials. MCW, IO and PH wrote the manuscript. All the authors read and approved the final manuscript.

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**Figures**

### Structures of DVF-taxoids

|        | R₁     | R₂     | R₃     |
|--------|--------|--------|--------|
| SB-T-1285106 | OCF₂H  | MeCO   | H      |
| SB-T-1285205 | OCF₃   | c-PrCO | H      |
| SB-T-1285406 | OCF₂H  | N(Me)₂CO | H |
| SB-T-1285506 | OCF₂H  | Me     | H      |
| SB-T-1285605 | OCF₃   | Me     | Me     |

### IC₅₀ values of paclitaxel and DFV-taxoids in ATC cells

| Taxanes   | 8505C (Mean ± SD) nM | 8305C (Mean ± SD) nM |
|-----------|-----------------------|-----------------------|
| Paclitaxel| 5.06 ± 0.89           | 2.51 ± 0.43           |
| SB-T-1285106 | 0.57 ± 0.09            | 0.52 ± 0.10           |
| SB-T-1285205 | 1.43 ± 0.22            | 1.15 ± 0.19           |
| SB-T-1285406 | 1.09 ± 0.21            | 0.36 ± 0.09           |
| SB-T-1285506 | 1.61 ± 0.56            | 0.55 ± 0.18           |
| SB-T-1285605 | 1.41 ± 0.35            | 0.50 ± 0.10           |

Figure 1
3rd-generation DFV-taxoids. (a) Structures of DVF-taxoids. (b) IC50 values of paclitaxel and DFV-taxoids in ATC cells.

**Figure 2**

Inhibitory effect of taxoids and paclitaxel on the proliferation of ATC cells. ATC cell lines 8505C (a) and 8305C (b) were treated with 5 nM of paclitaxel or taxoids and vehicle control for the indicated times, and the MTT assay was performed to evaluate cell proliferation. ***, P <0.001.**
Figure 3

Inhibitory effect of DFV-taxoids and paclitaxel on colony formation of ATC cells. (a) Representative images of colony formation of 8505C and 8305C cells treated with indicated concentrations of paclitaxel or DFV-taxoids and vehicle control for 48 h. (b) Quantitative analysis of colony numbers from three independent experiments. *, P <0.05; **, P <0.01; ***, P <0.001.
Figure 4

Induction of cell cycle arrest by DFV-taxoids and paclitaxel in ATC cells. (a) The indicated cells were treated with 50 nM paclitaxel or DFV-taxoids and vehicle control for 48 h, then stained with propidium iodide to assess DNA content by flow cytometry analysis. The percentage of each cell cycle phase is presented. Data are presented as mean ± SD. *, P <0.05; ***, P <0.001. (b) The antibodies against p-Rb, Rb, Weel, Cyclin B1, Cyclin E, CDK1 and p21 were used to determine the effect of SB-T-1285406 or
paclitaxel on the expression of cell cycle-related proteins at indicated time points. GAPDH was used as a loading control.

Figure 5

Induction of cell apoptosis by DFV-taxoids and paclitaxel in ATC cells. ATC cell lines 8505C (a) and 8305C (b) were treated with 50 nM of DFV-taxoids or paclitaxel for 24 h, and the apoptotic cells were stained by Annexin V-FITC/ PI and then measured by flow cytometry. Representative flow cytometry
profiles of 8505C and 8305C cells with indicated treatments from three independent experiments are shown in right panels. The percentage of apoptotic cells is presented at left bottom of representative profiles of each cell line. Data are presented as mean ± SD. ***, P < 0.001.

Figure 6

Anti-tumor effect of SB-T-1285406 and paclitaxel in xenograft tumors. (a) Time course of tumor growth, measured as tumor volume in each group at the indicated time points with vehicle control, SB-T-1285406 and paclitaxel, individually, 5 mg/kg i.p. Data are presented as mean ± SD (n =5 per group). (b)
Representative images of xenograft tumors in mice treated with vehicle control, SB-T-1285406 or paclitaxel are shown in left panel. Bar graphs represent tumor weight in each group (right panel). Data are presented as mean ± SD (n =5 per group). (c) Representative Ki-67 staining of 8505C-derived xenograft tumors from different groups are shown in left panels. Bar graphs represent the number of Ki-67-positive cells from 5 microscopic fields in each group (right panel). Data are presented as means ± SD. Scale bars, 200 μm. (d) TUNEL assay was performed to evaluate cell apoptosis in xenograft tumors from DMSO-, SB-T-1285406- and paclitaxel-treated mice. Green color represents target TUNEL staining, and blue color represents Hoechst33342 staining for nuclei. Scale bars, 100 μm. (e) Serum activity of alkaline phosphatase (AKP), aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN) and creatinine (CRE) were measured by spectrophotometry in the indicated tumors. *, P <0.05; **, P <0.01;***, P <0.001.
The effect of DFV-taxoids and paclitaxel on tubulin polymerization in vitro. (a) Tubulin polymerization assay was carried out in a system containing 1 mg/mL β-tubulin, 1 mM GTP, 10 µM taxoids or paclitaxel, the assembly of human microtubule protein TUBB2B was monitored spectrophotometrically by recording changes in turbidity at 350 nm every min. (b) Molecular docking of paclitaxel and DFV-taxoids with β-tubulin. (1) Overlay of SB-T-1285406 (cyan) and paclitaxel (light grey) in β-tubulin, showing a conserved T-taxol binding mode. (2) The van der Waals contacts of the CHF2O group in the C2 benzoate with nearby hydrophobic amino acid residues, Ala233 and Phe272. (3) The van der Waals contacts of the C3'-DFV group with nearby amino acid residues, Ala233, Ser236, and Phe272. (4) Electrostatic surface model, illustrating the hydrophobic interface of β-tubulin where the DFV and tri/difluoromethoxy groups are located.

**Supplementary Files**

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