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Taccalonolides: Novel Microtubule Stabilizers with Clinical Potential

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Abstract

Nature remains an important source for new anticancer drugs. Numerous microtubule targeting agents currently approved or in clinical development, including paclitaxel, vinblastine, vincristine, colchicine and combretastatin, are plant-derived compounds. The microtubule stabilizing properties of the taccalonolides were discovered as a part of a program to identify new microtubule stabilizers from natural sources. The taccalonolides are unique among all other agents in this class in that they stabilize microtubules through a mechanism of action that does not involve direct tubulin binding. Herein we review the discovery and isolation of the taccalonolides, their biological activities *in vitro* and *in vivo* and their potential advantages over clinically used microtubule stabilizers. We also discuss the challenges in formulation and supply that will need to be solved before the taccalonolides can become candidates for clinical development.

Keywords

Taccalonolides; Microtubule stabilizers; Microtubules; Drug resistance

1. Introduction

Microtubule stabilizers are highly effective drugs used in the treatment of many types of cancers including breast, ovarian, non-small cell lung cancers as well as head and neck tumors. Paclitaxel (TaxolTM) was the first microtubule stabilizer identified and the efforts of many investigators solved its initial problems of low aqueous solubility, hypersensitivity reactions with the Cremophor EL vehicle and limited drug supply [1]. The anticancer efficacy of the taxanes led to efforts to discover new microtubule stabilizers that could potentially circumvent some of the limitations of the taxanes including innate and acquired drug resistance. Several structurally diverse classes of microtubule stabilizers were identified including the epothilones, discodermolide, eleutherobin, sarcodictyins, laulimalide, peloruside, and the taccalonolides [2]. Discodermolide and multiple epothilones have been evaluated in clinical trials with mixed results. Unforeseen lung toxicities halted the further development of discodermolide [3], but the early clinical results with the epothilones were encouraging. The epothilone B analog

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ixabepilone (Ixempra™) was approved by the FDA in 2007 for use in metastatic or locally advanced breast cancer in combination with capecitabine after failure of an anthracycline and a taxane or as monotherapy after failure of an anthracycline, a taxane, and capecitabine. Whether any epothilone will provide better activity than the taxanes or long term clinical benefit in taxane-resistant tumors however has yet to be determined [4].

The taccalonolides stand alone among new microtubule stabilizers in that they appear to have a unique mechanism of action which does not involve direct binding to tubulin [5]. In this review, we will summarize the identification and purification of the taccalonolides, the elucidation of their mechanism of action, potential advantages in their ability to overcome clinically relevant taxane resistance mechanisms, and the challenges ahead for their clinical development.

2. Chemical isolations of the taccalonolides

In the early 1960's Professor Paul Scheuer of the University of Hawaii investigated the "bitter principle" of the tubers of *Tacca leontopetaloides*, a starchy food source. Scheuer and his colleagues purified a compound they named taccalin in 1963 as an intensely bitter, light yellow powder with a probable tetracyclic structure and $C_{18}H_{26}O_7$ chemical composition [6]. Although the actual structure of the taccalonolides was later found to be much larger, $C_{36}H_{46}O_{14}$, this pioneering work laid the groundwork for the elucidation of their structures twenty-five years later.

Chen and colleagues first elucidated the complete structures of taccalonolides A and B (Figure 2), the steroidal bitter principles from *Tacca plantaginea*, in 1987 using modern chemical techniques [7]. These compounds showed spectral properties consistent with a structurally complex pentacyclic steroidal-like structure. Since the initial isolation and structure elucidation of taccalonolides A and B, a total of 23 additional taccalonolides have been isolated from many species of *Tacca* with the latest group, taccalonolides W, X, and Y, isolated in 2008 [8]. Each taccalonolide molecule contains a C_2 – C_3 epoxide and all except taccalonolide C have a C_{23} – C_{26} lactone ring. Taccalonolide B differs from taccalonolide A in the replacement of a C_{15} acetyl group with a C_{15} hydroxyl. Other taccalonolides of note include E and N, which differ from A and B by the loss of an acetyl group at the C_{11} position (Figure 2). The taccalonolides present a significant synthetic challenge and no total or partial syntheses of the taccalonolides have been reported to date.

3. Biological activities of the taccalonolides

In the original paper describing the isolation of taccalonolide A, the purified compound was noted to have cytotoxic activity against P-388 leukemia cells in culture as well as antimalarial activity [7]. The first hint of the mechanism by which extracts and purified compounds from the *Tacca* genus inhibit cellular proliferation came from the finding that the crude lipophilic extract of the roots of *Tacca chantrieri* (Figure 1) caused paclitaxel-like microtubule bundling in a cell-based phenotypic assay. Bioassay guided fractionation of the active constituents led to the identification of taccalonolides A and E as microtubule stabilizing compounds [9].

3.1. Microtubule stabilizing activities of the taccalonolides

The effects of the taccalonolides A and E on cellular microtubule structures were evaluated by immunofluorescence techniques in numerous cancer cell lines and in A-10 embryonic aortic smooth muscle cells. The A-10 cell line is useful in the analysis of microtubule-targeting agents because they are large, relatively flat cells with an easily visualized cytoskeleton. Like other smooth muscle cells, A-10 cells arrest in G_1 in response to microtubule disrupting drugs, facilitating the analysis of drug effects on interphase microtubule density and structure [10].

The taccalonolides cause a notable increase in the cellular density of interphase microtubules and they initiate bundling of interphase microtubules in A-10 smooth muscle cells at low micromolar concentrations, comparable to the effects of paclitaxel (Figure 3). Consistent with the effects noted in phenotypic immunofluorescence assays, a shift in cellular tubulin polymer from the soluble to the polymerized state was quantified by Western blotting techniques in cancer cell lines treated for one hour with either taccalonolide E or paclitaxel [9].

The structures of the microtubule bundles initiated by the taccalonolides or paclitaxel differ to some degree as does the concentration of these drugs required to initiate bundling. The first noticeable effect of these drugs on interphase microtubules is an increased density of interphase microtubules focused around the nucleus. This is first observed with 500 nM taccalonolide A (Figure 3B) or 100 nM paclitaxel (Figure 3D). Treatment of cells with slightly higher concentrations of taccalonolide A (2.5 μ M) initiates the formation of short, compact microtubule tufts that constitute the entire microtubule mass of the cell. In contrast, paclitaxel-induced microtubule bundles remain long, loose, and consist of only half of cellular microtubule mass even at concentrations up to 10 μ M (Figure 3 and [9]). In paclitaxel-treated cells the microtubules are nucleated primarily from the microtubule organizing center, but taccalonolide-treated cells have a greater propensity to nucleate microtubules independent of the microtubule organizing center. Although the potencies of the taccalonolides for antiproliferative and cytotoxic effects in cancer cells are over 100 fold lower than paclitaxel (see below) the fact that they are only slightly (5-fold) less potent than paclitaxel at initiating cellular microtubule stabilization supports the hypothesis that the taccalonolides work through a different mechanism of action.

In addition to disruption of interphase microtubules in smooth muscle cells (Figure 3), the taccalonolides also initiate mitotic arrest and the formation of aberrant β -tubulin spindles in cancer cell lines (Figure 4) [5,9]. There is a concentration dependent increase in the number mitotic spindle asters, with most cells containing 2 to 4 spindles at concentrations near the IC₅₀ for inhibition of proliferation. The number of spindles increases to an average of five per cell at 10 times the IC₅₀ value [9]. Regardless of the number of these mitotic spindles present, normal centrosomal markers, including γ -tubulin, are found at only 2 of these spindles. Ongoing efforts to further characterize the centrosomal and microtubule associated proteins that are present at these aberrant mitotic spindles should provide additional insight into the mechanism of taccalonolide action and their resulting antitumor activity. As expected from the formation of abnormal mitotic spindles, the taccalonolides inhibit cell cycle progression and lead to mitotic arrest of cancer cells [5,9]. Consistent with the effects of the taxanes, the taccalonolides cause the formation of micronuclei and cause Bcl-2 phosphorylation and initiation of apoptosis [9].

3.2. Interaction of the taccalonolides with purified tubulin

The most striking and surprising difference between the taccalonolides and all other microtubule stabilizing drugs is their inability to bind to or polymerize purified tubulin. In a comprehensive analysis of all drugs that have been reported to have microtubule stabilizing activity, the taccalonolides were the only compounds that were able to cause paclitaxel-like cellular effects but were unable to induce the assembly of purified tubulin even when the drug was present at equimolar concentrations with tubulin [5]. Additional experiments performed under conditions sufficient for spontaneous tubulin polymerization demonstrated that the taccalonolides were not capable of lowering the critical concentration of tubulin required for microtubule assembly, even in the presence of purified microtubule-associated proteins (MAPs) [5]. These data suggest that the taccalonolides stabilize microtubules through a novel mechanism that requires more than purified tubulin and MAPs.

To further test the nature of the interaction of the taccalonolides with tubulin, the ability of the taccalonolides to impact taxane binding to microtubules was evaluated by anisotropy using the fluorescent paclitaxel analog, Flutax-2 [5]. Although initial studies suggested that the taccalonolides caused a weak and slow displacement of Flutax-2 from microtubules, further studies showed this to be an experimental artifact. Centrifugation studies were used to study the interaction and the results showed that the taccalonolides were unable to displace Flutax-2. Additionally, preincubation of the microtubules with the taccalonolides did not inhibit Flutax-2 binding. Collectively the centrifugation experiments demonstrate that the taccalonolides do not displace or inhibit the binding of Flutax-2 to microtubules, consistent with lack of overlap between the taccalonolides and the taxane binding site [5]. Direct binding of the taccalonolides to purified microtubules with and without the addition of a crosslinking agent was measured by extraction and HPLC detection of the drug in microtubule pellets and the supernatant. No taccalonolides were detected in the microtubule pellet, confirming that the taccalonolides are unique among microtubule stabilizers in that they do not directly bind to purified tubulin or microtubules [5].

3.3. Antiproliferative actions of the taccalonolides against drug sensitive and drug resistant cell lines *in vitro*

Another major difference between the taccalonolides and other microtubule stabilizers is *in vitro* potency. The taccalonolides are substantially less potent antiproliferative agents than the taxanes. *In vitro*, the taccalonolides are at best 100-fold less potent than paclitaxel, with IC₅₀ values ranging from 200 nM to 2 μ M depending on the taccalonolide and cell line [9,11]. The taccalonolides A, B, E, and N have cytotoxic potency in the high nanomolar range against cervical, ovarian, breast, and lung cancer cell lines [9,11]. Although clinically approved microtubule-stabilizing agents including paclitaxel, docetaxel and the epothilone ixabepilone are approved for the above indications, innate and acquired resistance to these drugs limits their utility.

The two clinically documented forms of drug resistance to taxanes include overexpression of the P-glycoprotein (Pgp) multidrug transporter and expression of the β III isotype of tubulin [12]. The taccalonolides were initially shown to be effective *in vitro* against NCI/ADR cells (previously known as MCF7/ADR)[9], a cell line that has intrinsically high levels of Pgp. More recent studies with an isogenic cell line pair of SK-OV-3 cells and a single cell clone transduced with the *MDR-1* gene, SK-OV-3 M6/6, demonstrated relative resistance values (Rr) for the taccalonolides of 4.1–12.2 [11]. This relative resistance between the Pgp-expressing and parental cell lines is within the range measured for epothilone B (Rr 6.8), a compound known to circumvent Pgp-mediated drug resistance [2]. In comparison paclitaxel, a documented Pgp substrate, had a Rr of 860 in this pair of cell lines [11]. The taccalonolides are also able to overcome drug resistance mediated by the expression of the β III isotype of tubulin. β III expressing HeLa cells were more sensitive to the taccalonolides than the parental HeLa cells, which do not contain measurable amounts of β III-tubulin [11]. The ability to circumvent β III-tubulin-mediated resistance gives the taccalonolides a distinct efficacy profile as compared to any clinically utilized class of microtubule targeting agents, including epothilone B [11].

Other mechanisms implicated in taxane resistance include mutations in the paclitaxel-binding site [13] and expression of the ABC transporter multidrug resistance protein 7 (MRP7) [14]. Although most studies have not demonstrated a link between mutations in the paclitaxel binding site on tubulin and clinical drug resistance, the taccalonolides retain efficacy in cell lines harboring these mutations [9]. This finding is not surprising given the preponderance of data suggesting that the taccalonolides do not bind directly to tubulin. Cell lines expressing the MRP7 transporter are equally sensitive to the taccalonolides as compared to parental cell lines [11]. Interestingly, cells expressing MRP7 exhibit resistance to epothilone B, providing an

additional advantage of the taccalonolides in circumventing multiple drug resistance mechanisms [15].

3.4. In vivo antitumor efficacy of the taccalonolides

Although the taccalonolides are considerably less potent than other microtubule stabilizers *in vitro*, initial *in vivo* dose range finding studies with a taccalonolide A-enriched fraction showed that it was surprisingly potent. The first antitumor efficacy trial conducted with taccalonolides A and E showed that each was very potent *in vivo* with optimal efficacy at a total dose of 38 mg/kg for taccalonolide A and 86 mg/kg total for taccalonolide E against a Pgp-expressing Mam17/ADR syngeneic murine adenocarcinoma model [11]. These doses were similar to the standard maximal tolerated total dose of paclitaxel, 60–90 mg/kg, used in this model.

The antitumor efficacy results of this trial demonstrated that the taccalonolides were effective antitumor agents and that they were highly active against a doxorubicin and paclitaxel insensitive tumor [11]. The finding that taccalonolides are equally, if not more, potent than paclitaxel *in vivo* in spite of the fact that they are hundreds of times less potent *in vitro* suggest that the potency of the taccalonolides will not be a limiting factor for consideration of therapeutic relevance. These results also highlight the caution of forming conclusions about *in vivo* potency of novel therapeutics based solely on *in vitro* potency. The pharmacokinetic parameters of taccalonolide metabolism and clearance rates will be interesting to establish because they might explain their relatively high *in vivo* potency.

4. Future Directions

It is clear that the taccalonolides represent a novel class of microtubule stabilizers that retain efficacy in taxane and epothilone-resistant models due in part to a unique mechanism of action. However, supply and formulation issues must be solved before the clinical usefulness of the taccalonolides can be evaluated.

One of the first questions surrounding the development of the taccalonolides is that of supply. Currently, the complete chemical synthesis of the taccalonolides is not a viable option, and the structural complexity of these compounds suggests that the total synthesis as a mechanism for large scale supply could be cost prohibitive even if it were achieved. The process of purifying the taccalonolides from the roots and rhizomes of *Tacca chantrieri* plants requires a significant investment of time and resources, yet the yield from roots and rhizomes is 10-fold higher than the yield of paclitaxel from the bark of the Pacific Yew [16,17] and the *taccas* are relatively fast growing compared to the Yew tree. *Tacca chantrieri* are tropical plants that grow well in a greenhouse or tropical environment with rich, well-drained soil, high humidity and protection from cold, sun and wind. *Tacca chantrieri* can be easily propagated by seed or by dividing the rhizomes. Field and greenhouse grown plants have provided us a renewable resource of root material.

Although the plant resources required to produce sufficient quantities of taccalonolides for clinical testing appear daunting, it is valuable to keep in mind the strategies employed in the past to produce other widely used anticancer drugs derived from natural sources [18]. The most dramatic example of such a drug is paclitaxel. Paclitaxel was originally purified from the bark of the Pacific Yew, *Taxus brevifolia* in a laborious process that required the bark of thousands of trees to produce one kg of purified compound [18]. This process led to major debates within the scientific and environmental communities, leading to speculation that paclitaxel, as a natural product, would never be commercialized regardless of its clinical efficacy [19]. Although paclitaxel purified from Yew species was used clinically for over a decade, efforts to produce taxanes in plant tissue culture resulted in the commercialization of plant cell culture-derived paclitaxel [20]. Due to similar impediments to total synthesis and challenges in

procurement of the natural product, clinical development of the taccalonolides may require an analogous strategy.

Another challenge for the clinical development of the taccalonolides will be the development of a non-toxic, readily bioavailable formulation. The *in vivo* studies of the taccalonolides involved their formulation in a concentrated 50/50 mixture of Cremophor EL with DMSO that was then diluted with water shortly before injection, providing a final Cremophor EL/DMSO concentration of less than 5%. This formulation is similar to the formulation of clinical grade paclitaxel with the notable substitution of ethanol for DMSO because the taccalonolides are even less water soluble than paclitaxel. Given the undesirable effects of Cremophor EL in patients [19], it is clear that the taccalonolides will require a new formulation for clinical evaluation.

One attractive possibility for taccalonolide formulation involves encapsulation of the drug into a pharmaceutically acceptable carrier molecule. Incorporation into microparticles has proven successful to increase the solubility and bioavailability of several hydrophobic drugs, including paclitaxel [21]. The matrix of these polymers can be made from organic or non-organic materials, including ceramics, lipids, and sugars, which effectively encapsulate the drug to improve solubility and bioavailability. Other advantages of microparticle-based formulations include the ability to regulate drug targeting and the rate of drug release through careful selection of matrix properties [21].

The final hurdle in the clinical development of the taccalonolides is, ironically, the same issue that makes them such exciting new drugs, their lack of direct interaction with tubulin. Although the taccalonolides are microtubule stabilizers at the cellular level, the multiple lines of evidence outlined above suggest they work through a novel mechanism of action that is independent of the direct tubulin binding exhibited by all other microtubule stabilizers. While questions of supply and formulation are being addressed, it will also be imperative to define the cellular taccalonolide binding site and resulting mechanism of action that leads to microtubule stabilization, mitotic arrest and initiation of apoptosis.

Mechanism-blind strategies, including co-immunoprecipitation of taccalonolide associated proteins or screening for proteins whose activity modulates taccalonolide-induced phenotypes, are approaches that may be employed to identify the taccalonolide binding site. However, biochemical identification of taccalonolide binding partners will be difficult without a process by which the taccalonolides can themselves be visualized or isolated from intact cells. In the absence of such a tool, candidate-based approaches to determine the effect of the taccalonolides on processes known to affect microtubule stabilization might provide insight into their mechanism of action. However, the list of candidate proteins that may be targets of taccalonolide binding, including microtubule associated proteins as well as those that indirectly affect microtubule dynamics, is quite extensive. While it is clear that the taccalonolides themselves are unable to directly bind to tubulin, the possibility remains that a cellular metabolite of the taccalonolides is the true active agent. Additional studies are warranted to determine whether the taccalonolides are metabolized intracellularly and, if so, whether the metabolites are active and bind directly to tubulin.

In summary, the taccalonolides are a novel class of microtubule stabilizers that retain efficacy in drug-resistant models *in vitro* and *in vivo*. In spite of their unique mechanism of action, issues of supply and formulation need to be addressed before serious consideration can be given to their clinical development; however, as has been shown with the clinical development of the taxanes, there is good reason to believe that these challenges can be met. Even if the taccalonolides do not pass the rigorous gauntlet of criteria required for clinical development, identification of the taccalonolide binding site will uncover a unique mechanism of

pharmacological microtubule stabilization that could potentially lead to the identification of other molecules capable of producing taccalonolide-like effects with improved drug properties.

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Figure 1.
Tacca chantrieri, commonly referred to as the “bat flower” or “cat’s whiskers”.

	R1	R2
Taccalonolide A	OAc	OAc
Taccalonolide B	OH	OAc
Taccalonolide E	OAc	H
Taccalonolide N	OH	H

Figure 2.
Chemical structures of selected taccalonolides.

Figure 3.

The effects of microtubule stabilizers on interphase microtubules. A-10 smooth muscle cells were treated with vehicle (A), 500 nM taccalonolide A (B), 2.5 μ M taccalonolide A (C), 100 nM paclitaxel (D), or 5 μ M paclitaxel (E) for 18 hours. Microtubules were visualized by indirect immunofluorescence using an antibody to β -tubulin.

Figure 4. The effect of microtubule stabilizers on mitotic spindles. HeLa cells were treated with vehicle (A), 10 nM paclitaxel (B), or 500 nM taccalonolide A (C). Microtubules were visualized by indirect immunofluorescence using an antibody to β -tubulin.