

# Taccalonolide Microtubule Stabilizers



Samantha S. Yee, Lin Du, and April L. Risinger

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## 1 Introduction

The taccalonolides are a unique class of microtubule stabilizers that are produced by several *Tacca* species (Fig. 1). What we now know as the taccalonolides were first identified in the early 1960s by Dr. Paul Scheuer as 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 proposed tetracyclic structure [1]. While the actual structure of the taccalonolides was later found to be much larger than originally proposed, it laid the groundwork for the future study of this class of compounds. In 1987, Chen et al. [2] elucidated the structures of taccalonolides A (**1**) and B (**2**) isolated from the rhizomes of *Tacca plantaginea*. Taccalonolide A (**1**), the most abundant taccalonolide, demonstrated cytotoxic activity against P-388 leukemia in cell culture as well as antimalarial activity against *Plasmodium berghei*.

Over the following decades, two dozen additional taccalonolides were purified from *Tacca* species. Taccalonolides C–M (**3–13**) and W–Y (**23–25**) were first isolated from *Tacca plantaginea* [3–7], taccalonolides O–Q (**15–17**) from *Tacca subflabellata* [8], taccalonolides R–V (**18–22**) from *Tacca paxiana* [9], and taccalonolides Z (**26**) and AA (**27**) from *Tacca integrifolia* and *Tacca chantrieri*, respectively (Table 1) [10]. The discovery of the microtubule-stabilizing effects of the taccalonolides has sustained interest in these compounds as potential anticancer agents and will be the primary focus of this contribution. The taccalonolides and extracts from the leaves and tubers of *Tacca leontopetaloides* have also demonstrated antitrypanosomal activity [11] and the ability to control against snails [12] leading to a patent for their use as anthelmintic and molluscicidal agents [13].

Fig. 1 *Tacca chantrieri*

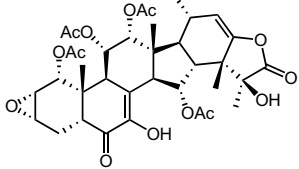
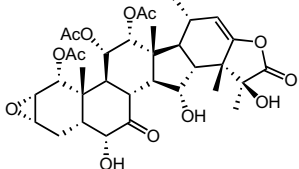
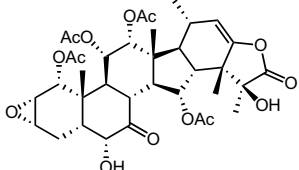
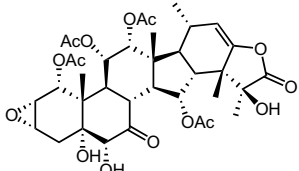
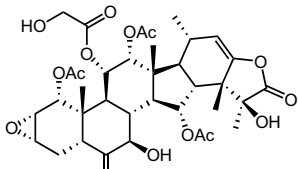
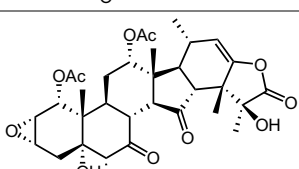
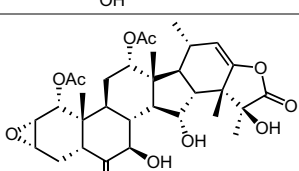


**Table 1** The structures and sources of taccalonolides

Structure	Name	Source	Ref.
	Taccalonolide A (1)	<i>Tacca plantaginea</i> <i>Tacca paxiana</i> <i>Tacca chantrieri</i>	[2] [9] [10]
	Taccalonolide B (2)	<i>Tacca plantaginea</i> <i>Tacca paxiana</i>	[2] [9]
	Taccalonolide C (3)	<i>Tacca plantaginea</i>	[3]
	Taccalonolide D (4)	<i>Tacca plantaginea</i>	[3]
	Taccalonolide E (5)	<i>Tacca plantaginea</i> <i>Tacca paxiana</i> <i>Tacca chantrieri</i>	[4] [9] [10]
	Taccalonolide F (6)	<i>Tacca plantaginea</i>	[4]
	Taccalonolide G (7)	<i>Tacca plantaginea</i>	[5]

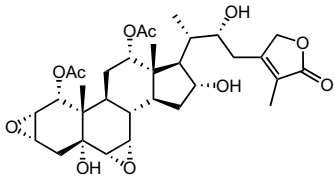
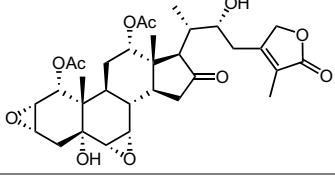
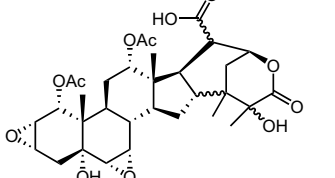
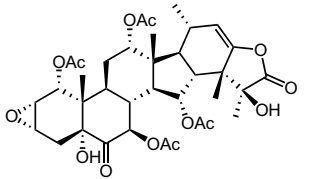
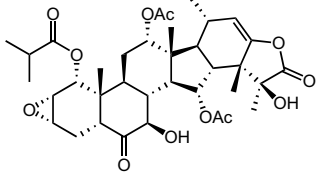
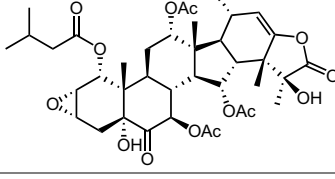
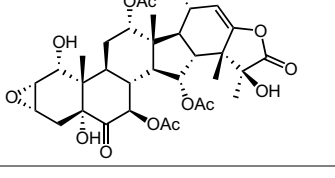
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**Table 1** (continued)

Structure	Name	Source	Ref.
	Taccalonolide H ( <b>8</b> )	<i>Tacca plantaginea</i>	[5]
	Taccalonolide I ( <b>9</b> )	<i>Tacca plantaginea</i>	[5]
	Taccalonolide J ( <b>10</b> )	<i>Tacca plantaginea</i>	[5]
	Taccalonolide K ( <b>11</b> )	<i>Tacca plantaginea</i> <i>Tacca paxiana</i>	[5] [9]
	Taccalonolide L ( <b>12</b> )	<i>Tacca plantaginea</i>	[6]
	Taccalonolide M ( <b>13</b> )	<i>Tacca plantaginea</i>	[6]
	Taccalonolide N ( <b>14</b> )	<i>Tacca paxiana</i>	[9]

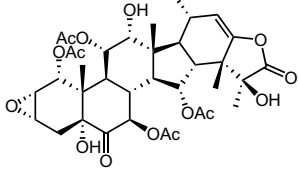
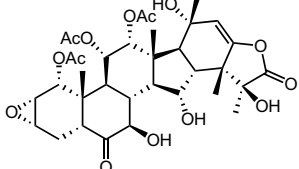
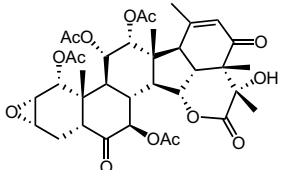
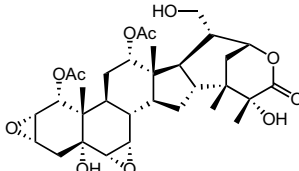
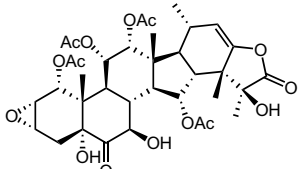
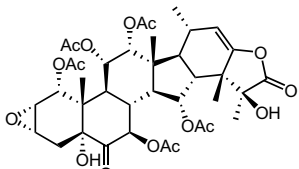
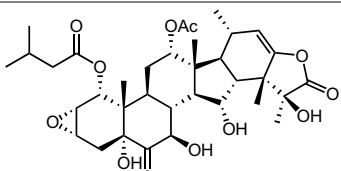
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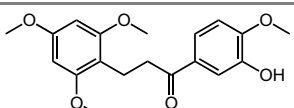
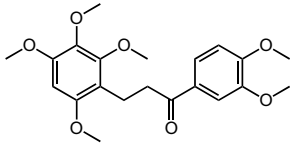
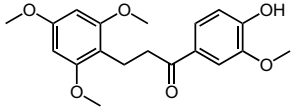
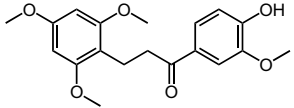
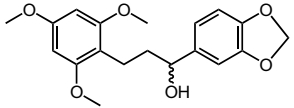
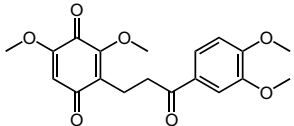
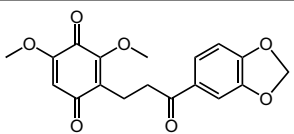
Structure	Name	Source	Ref.
	Taccalonolide O (15)	<i>Tacca subflabellata</i>	[8]
	Taccalonolide P (16)	<i>Tacca subflabellata</i>	[8]
	Taccalonolide Q (17)	<i>Tacca subflabellata</i>	[8]
	Taccalonolide R (18)	<i>Tacca paxiana</i> <i>Tacca chantrieri</i>	[9] [10]
	Taccalonolide S (19)	<i>Tacca paxiana</i>	[9]
	Taccalonolide T (20)	<i>Tacca paxiana</i> <i>Tacca chantrieri</i>	[9] [10]
	Taccalonolide U (21)	<i>Tacca paxiana</i>	[9]

(continued)

**Table 1** (continued)

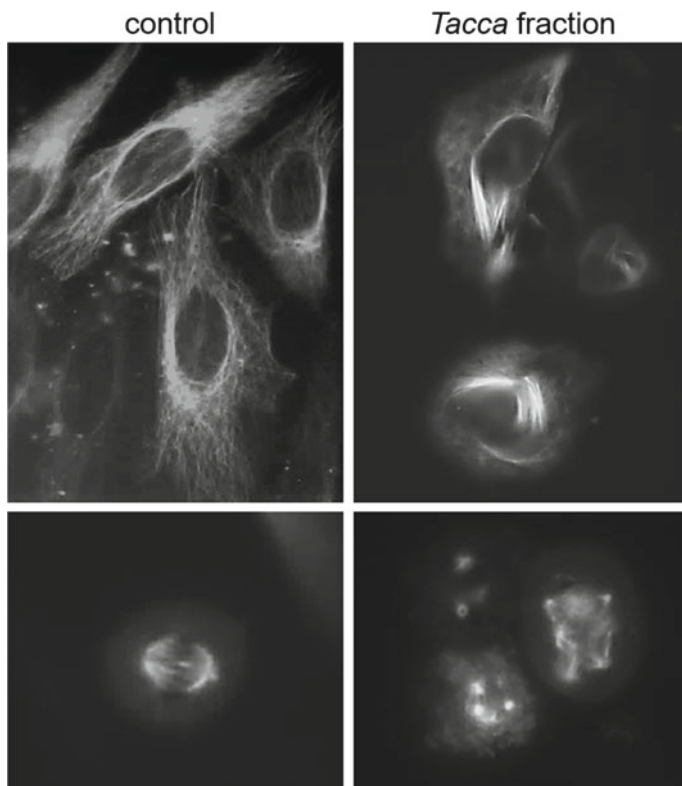
Structure	Name	Source	Ref.
	Taccalonolide V ( <b>22</b> )	<i>Tacca paxiana</i>	[9]
	Taccalonolide W ( <b>23</b> )	<i>Tacca plantaginea</i>	[7]
	Taccalonolide X ( <b>24</b> )	<i>Tacca plantaginea</i>	[7]
	Taccalonolide Y ( <b>25</b> )	<i>Tacca plantaginea</i>	[7]
	Taccalonolide Z ( <b>26</b> )	<i>Tacca integrifolia</i>	[10]
	Taccalonolide AA ( <b>27</b> )	<i>Tacca chantrieri</i>	[10]
	Taccalonolide AI ( <b>30</b> )	<i>Tacca chantrieri</i>	[33]

**Table 2** The structures of bioactive retro-dihydrochalcones isolated from *Tacca* sp.

Structure	Common name	Source	Ref.
	Taccabulin A ( <b>31</b> )	<i>Tacca chantrieri</i> <i>Tacca integrifolia</i>	[36]
	Taccabulin B ( <b>32</b> )	<i>Tacca chantrieri</i> <i>Tacca integrifolia</i>	[37]
	Taccabulin C ( <b>33</b> )	<i>Tacca chantrieri</i> <i>Tacca integrifolia</i>	[37]
	Taccabulin D ( <b>34</b> )	<i>Tacca chantrieri</i> <i>Tacca integrifolia</i>	[37]
	Taccabulin E ( <b>35</b> )	<i>Tacca chantrieri</i> <i>Tacca integrifolia</i>	[37]
	Evelynin A ( <b>36</b> )	<i>Tacca chantrieri</i> <i>Tacca integrifolia</i>	[37] [38]
	Evelynin B ( <b>37</b> )	<i>Tacca chantrieri</i> <i>Tacca integrifolia</i>	[37]

## 2 Taccalonolides as Microtubule Stabilizers

The initial bioassays performed with purified taccalonolides were crude measures of cancer cell toxicity *in vitro* or antiparasitic and nematocidal activities that were not attributable to a specific mechanism of action. However, in 2003, the taccalonolides A (**1**) and E (**5**) (for structures see Table 1) were isolated as the bioactive components from *Tacca chantrieri* extracts that led to paclitaxel-like microtubule bundling and mitotic arrest with the formation of multiple spindle asters in cellular assays (Fig. 2) [14]. The antiproliferative potency of the taccalonolides A (**1**) and E (**5**) was found to be in the low micromolar range against human ovarian cancer (SK-OV-3, 1A9) cervical cancer (HeLa), and melanoma (MDA-MB-435 [15]) cell



**Fig. 2** The effect of taccalonolide-enriched *Tacca* fractions on microtubule structures in HeLa cervical cancer cells expressing GFP-tagged tubulin. The taccalonolides promote bundling of interphase microtubules (top panels) as well as the formation of multiple asters in mitotic cells that are markedly distinct from the microtubule spindle in normal mitotic cells (bottom panels)

lines, approximately 1000 times less potent than paclitaxel. However, the taccalonolides retained potency in the NCI/ADR-RES paclitaxel-resistant model that expresses high levels of the P-glycoprotein drug efflux pump, a major mechanism of taxane resistance in the clinic. The taccalonolides also retained potency in the 1A9 ovarian cancer cell line that contains mutations in paclitaxel (PTX 10 and PTX 22) binding sites in the human M40  $\beta$ -tubulin isotype [16, 17].

The ability to circumvent these taxane resistance mechanisms was the first indication that their mechanism of action could be distinct from this other plant-derived class of microtubule stabilizers. Although the taccalonolides caused cellular microtubule bundling, mitotic arrest with multipolar spindles, and subsequent apoptosis similar to paclitaxel, the taccalonolides were also distinct in that they promoted the formation of spindle poles greater in number than paclitaxel, further suggesting the possibility of a distinct mechanism of action. Together, these findings first demonstrated that the taccalonolides were a structurally novel class of



microtubule stabilizers produced from a plant source with micromolar potency that were able to circumvent mechanisms of drug resistance to the taxanes potentially through a distinct mechanism.

The cellular microtubule-stabilizing activity of taccalonolides A (**1**) and E (**5**) was confirmed by Buey et al. [18] who demonstrated that 5  $\mu\text{M}$  taccalonolide A (**1**) and 10  $\mu\text{M}$  taccalonolide E (**5**) induced microtubule bundling, multipolar spindles, and multiple micronuclei in A549 adenocarcinomic human alveolar basal epithelial cells. However, these cellular microtubule effects could not be recapitulated in biochemical tubulin binding or polymerization assays. Furthermore, taccalonolides A (**1**) and E (**5**) did not promote tubulin assembly at concentrations as high as 66  $\mu\text{M}$  with 60  $\mu\text{M}$  GTP-tubulin when analyzed by either centrifugation or electron microscopy, and there was no evidence of taccalonolide binding to cross-linked or native microtubules. The taccalonolides only weakly displaced the paclitaxel-site probe Flutax-2, and the effect was not concentration dependent or observed in preincubation experiments leading to the conclusion that any observed Flutax-2 displacement was artifactual. Taccalonolide A (**1**) was also unable to promote microtubule polymerization even in non-denatured cytosolic extracts [19], further suggesting that the cellular microtubule-stabilizing activity of this taccalonolide was not the result of direct binding to microtubules or interactions with other soluble cellular factors that regulate microtubule polymer mass.

In spite of the inability to detect a direct interaction with microtubules and in being less potent than other classes of microtubule stabilizers, there was a continued interest in the taccalonolides due to their potential inability to interact with tubulin in biochemical preparations and their efficacy against taxane-resistant cancer cells. These studies were expanded by the evaluation of taccalonolides A (**1**), B (**2**), E (**5**), and N (**14**) as compared to other classes of microtubule-targeted drugs in cell lines representing clinical mechanisms of taxane resistance, including overexpression of P-glycoprotein, MRP7, and  $\beta\text{III-tubulin}$  [20]. All four taccalonolides retained *in vitro* efficacy in taxane-resistant human ovarian cancer cell lines expressing P-glycoprotein, human embryonic kidney cell lines overexpressing MRP7, and HeLa cervical cancer cell lines expressing  $\beta\text{III-tubulin}$ . Taccalonolides A (**1**) and E (**5**) were also found to be effective *in vivo* in a P-glycoprotein-expressing multidrug-resistant syngeneic murine mammary adenocarcinoma model Mam17/ADR that is resistant to both paclitaxel and doxorubicin [20].

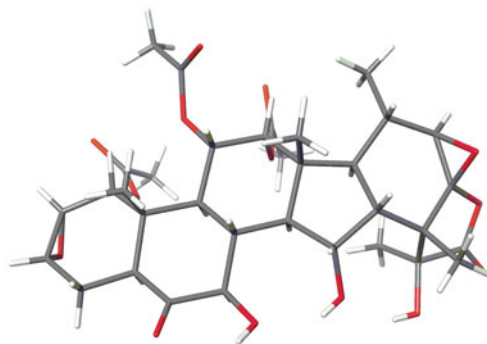
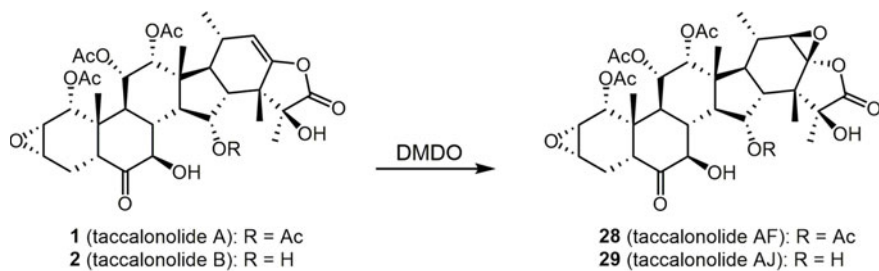
Surprisingly, although the taccalonolides were on average over 100-fold less potent than the taxanes *in vitro*, they demonstrated *in vivo* efficacy at concentrations comparable to or even lower than those used for paclitaxel. Further studies also demonstrated *in vivo* efficacy of taccalonolides A (**1**), E (**5**), N (**14**), and B (**2**) in the mammary 16/c syngeneic tumor model at total doses of 20–90 mg/kg, which were comparable to a total dose of 74 mg/kg paclitaxel [10]. These data not only confirmed that the taccalonolides were a novel class of microtubule stabilizers that can circumvent clinically relevant forms of drug resistance, but also demonstrated *in vivo* antitumor efficacy in paclitaxel-sensitive and -resistant tumor models at doses much lower than expected from studies based on their *in vitro* potency.

The lack of biochemical tubulin-polymerizing activity of the taccalonolides prompted additional cellular studies to elucidate the mechanism of cellular microtubule stabilization and how these effects were distinct from those of the taxanes. One intriguing finding was that gross bundling of interphase microtubules occurred at concentrations of taccalonolide A (**1**) that were equal to or less than those that promoted antiproliferative effects, whereas the concentration of paclitaxel required to observe cellular microtubule bundling was over 30-fold greater than its antiproliferative  $IC_{50}$  value, further suggesting a mechanistic difference between these two microtubule stabilizers [19]. This was particularly significant as it coincided with reports suggesting that the interphase effects of microtubule-targeting agents contribute to their antitumor efficacy in the clinic [21, 22]. It was also found that the cellular effects of the taccalonolides were highly persistent, providing long-term antiproliferative and cytotoxic efficacy even after only short periods of drug exposure and subsequent removal from the culture medium. This cellular persistence was not observed for other classes of microtubule stabilizers, including paclitaxel, further highlighting mechanistic differences between the taccalonolides and the taxanes. A high degree of cellular persistence has been associated with potent *in vivo* efficacy of the clinically approved microtubule destabilizer eribulin [23], providing a rationale for the unexpected *in vivo* potency of the taccalonolides.

### 3 Identification of Epoxidized Taccalonolides

Although early experiments demonstrated that taccalonolides A and E enriched preparations had microtubule stabilizing activity that was distinct from that of the taxanes, there were two issues that confounded a full understanding of their mechanism of action. One is the aforementioned lack of interaction with purified tubulin and the second was the inconsistent potency of different preparations of taccalonolides A (**1**) and E (**5**).

The original characterization of the microtubule-stabilizing effects of taccalonolide A (**1**) in 2004 demonstrated low micromolar potency, but follow-up studies by our same group in 2008 using a newly purified batch of taccalonolide A (**1**) were approximately 10-fold more potent. This inconsistency in the potency of our taccalonolide A (**1**) batches from preparation to preparation led to a rigorous evaluation of the chemical and biological properties of each of our HPLC fractions, including those before and after the prominent taccalonolide peak. To our surprise, we found that the microtubule-stabilizing potency did not comigrate perfectly with fractions that contained the highest taccalonolide A (**1**) levels. A careful chemical interrogation of the most potent HPLC fractions identified a sample containing compounds with the taccalonolide backbone possessing an unanticipated epoxide at positions C-22 and C-23 as opposed to the double bond in taccalonolide A (**1**). While this was a trace product that was not in sufficient quantity to purify fully from the natural product, the identification of the presence of this product led to its efficient semisynthesis from abundant 22,23-alkene taccalonolides (Scheme 1).



X-ray structure of **29**  
(CCDC ID: 1907790)

**Scheme 1** Synthesis of taccalonolides AF (**28**) and AJ (**29**) via epoxidation of taccalonolides A (**1**) and B (**2**), respectively

Remarkably, the 22,23-epoxidation of taccalonolides A (**1**) and B (**2**) to generate taccalonolides AF (**28**) and AJ (**29**), respectively, resulted in taccalonolide microtubule stabilizers with low nanomolar potency in cells. Additionally, these potent taccalonolides were able to effectively bind and polymerize tubulin in biochemical preparations, a property that was never observed for the non-22,23-epoxidized taccalonolides [24]. With this knowledge in hand, our group has revisited the activity of taccalonolides A (**1**) and B (**2**), in particular, and found that the microtubule-stabilizing activity of these compounds can be diminished by additional rounds of purification with highly purified material being completely devoid of any antiproliferative, cytotoxic, or cellular microtubule-stabilizing effects (unpublished observations).

Together, these data demonstrate that the previously reported micromolar potency biological activities of 22,23-alkene taccalonolides, including A (**1**), E (**5**), B (**2**), and N (**14**) among others, are likely a result of small amounts of material that was oxidized to generate a 22,23-epoxide. These formerly undetected trace amounts of nanomolar potency epoxidized taccalonolide were sufficient to promote cellular

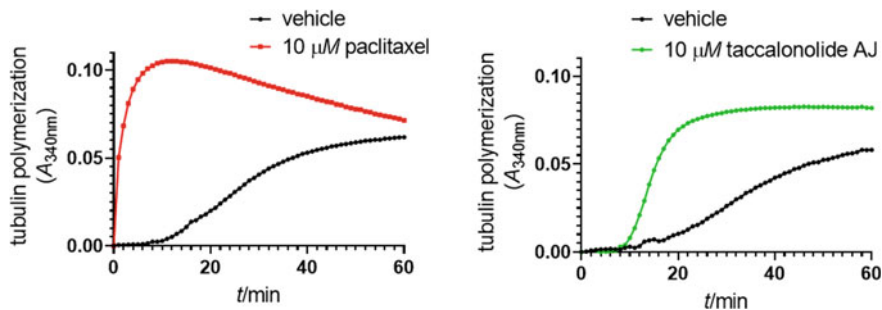
efficacy in the micromolar range that was attributed to the more abundant 22,23-alkene taccalonolides. In contrast, the low quantities of epoxidized taccalonolides in these preparations were insufficient to promote biochemical tubulin polymerization, which requires near equimolar concentrations to tubulin, providing a rationale for why cellular but not biochemical microtubule polymerization could be observed in the early evaluations of taccalonolides A (**1**) and E (**5**). To provide further evidence for this rationale, Peng et al. demonstrated that the semisynthetic introduction of a 22,23-epoxide to ten additional purified taccalonolides was sufficient to improve their antiproliferative potency, some into the sub-nanomolar range. Furthermore, there has been minimal to no batch-to-batch variation in potency among preparations of the 22,23-epoxidized taccalonolides AF (**28**) and AJ (**29**) isolated from different plant sources by different laboratory groups, providing confidence that these are indeed the bioactive component of *Tacca* species that have been investigated for decades. Importantly, the potent taccalonolides, AF (**28**) and AJ (**29**), retain many of the same biological properties that were previously ascribed to taccalonolides A (**1**) and B (**2**), including the ability to circumvent clinically relevant drug resistance mechanisms, a high degree of cellular persistence, and in vivo antitumor efficacy [25, 26].

#### 4 Identification of a Direct Interaction Between the Taccalonolides and Tubulin

Equipped with an understanding of the role of the 22,23-epoxide in the microtubule-stabilizing activity of the taccalonolides and semisynthetic strategies to convert the naturally abundant 22,23-alkene into the potent 22,23-epoxy-taccalonolides, there was a renewed approach in understanding the molecular interactions between the taccalonolides and tubulin/microtubules. Unlike the 22,23-alkene taccalonolides A (**1**) and E (**5**), the 22,23-epoxy-taccalonolides were indeed sufficient to polymerize purified tubulin in a similar manner to other microtubule-stabilizing agents. However, there was a significant lag time associated with taccalonolide-induced tubulin polymerization in contrast to the almost immediate polymerization induced by the taxanes (Fig. 3) [26].

Additionally, the microtubules formed in the presence of taccalonolide AJ (**29**) were highly resistant to cold-induced depolymerization as determined both turbidometrically and by electron microscopy [26]. This was markedly distinct from microtubules induced by other stabilizers, including paclitaxel and laulimalide (also named fijianolide B), which were subject to cold-induced depolymerization. Together, these results suggested that the taccalonolides promoted a distinct mechanism of microtubule polymerization from other classes of microtubule stabilizers.

To address whether the taccalonolides bind to the same site as paclitaxel and laulimalide on microtubules, synergism and displacement studies were employed.

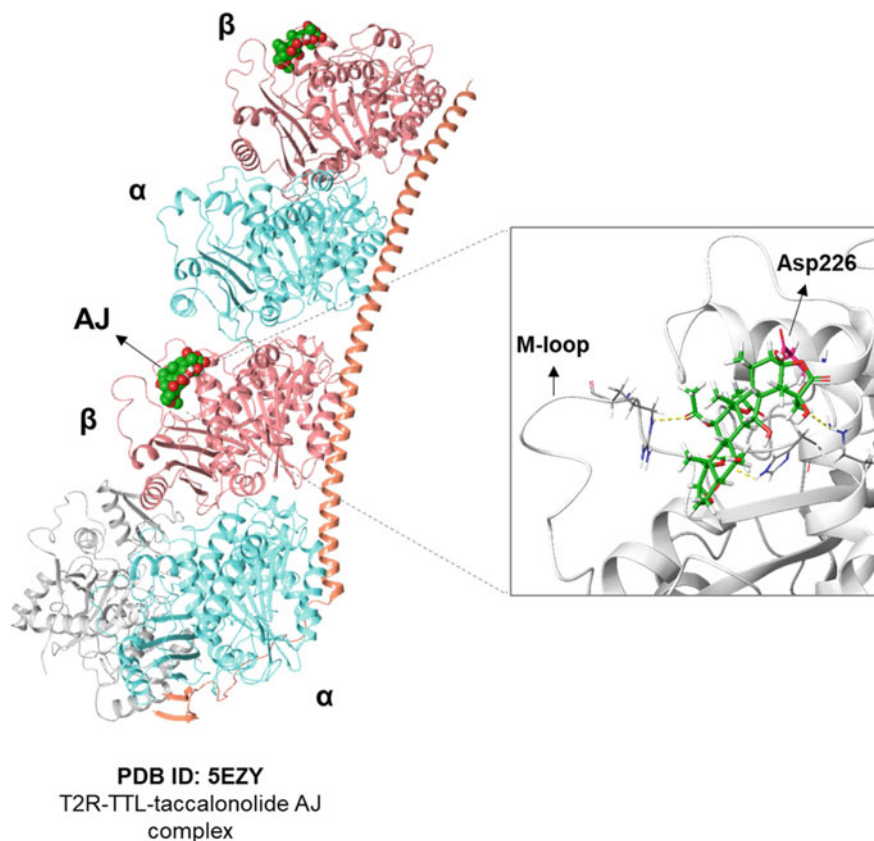


**Fig. 3** Comparison of the biochemical tubulin polymerization activities of paclitaxel and taccalonolide AJ. Left: paclitaxel ( $10\ \mu\text{M}$ ) promotes the immediate polymerization of purified tubulin ( $20\ \mu\text{M}$ ) as compared to a vehicle control. Right: in contrast, taccalonolide AJ ( $10\ \mu\text{M}$ ) dependent polymerization of purified tubulin ( $20\ \mu\text{M}$ ) is associated with a lag time of 8–10 min

Synergistic effects were observed between taccalonolide AF (**28**) and either paclitaxel or laulimalide [26], further indicating that the taccalonolides bind to a site pharmacologically distinct from the two major stabilizer-binding sites on tubulin. Displacement studies using equimolar concentrations of taccalonolide AJ (**29**) with either laulimalide or paclitaxel with purified tubulin demonstrated some competition between taccalonolide AJ (**29**) and paclitaxel [26]. However, it was notable that prior addition of taccalonolide AJ (**29**) before paclitaxel was required to observe decreased paclitaxel binding. This temporal effect on taxane displacement was the first indication that the taccalonolides might be interacting in an irreversible manner with tubulin. Indeed, after interaction with purified tubulin, the taccalonolides could not be extracted from either the supernatant or the microtubule pellet [26].

Mass spectrometric analysis confirmed that the  $m/z$  212–230 peptic fragment of  $\beta$ -tubulin was lost after incubation with taccalonolide AJ (**29**) and replaced by a peptide that was increased by the molecular weight of **29** [26]. Together, these results demonstrated that the 22,23-epoxy-taccalonolides covalently bound  $\beta$ -tubulin within the  $\beta$ 212–230 region, which includes the  $\beta$ His229 residue that is the covalent binding site of the cyclostreptin and zampanolide microtubule stabilizers. Additional hydrogen–deuterium exchange mass spectrometry was employed to determine that taccalonolide AJ-induced microtubule stabilization did not involve profound stabilization of the M-loop of tubulin, which is associated with taxane and zampanolide-induced microtubule stabilization, but instead promoted dramatic inter-protofilament stability as a mechanism of microtubule stabilization [26].

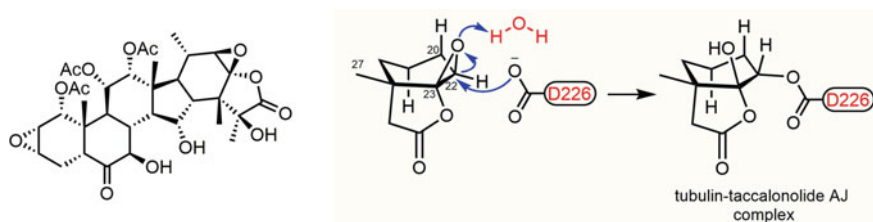
These biochemical studies were confirmed by Wang et al., who reported the first crystal structure of tubulin complexed with taccalonolide AJ (**29**) (Fig. 4), demonstrating that the 22,23-epoxide of **29** binds covalently to the Asp226 residue on  $\beta$ -tubulin [27]. Their data suggested that this covalent interaction promotes a conformation shift in the M-loop of tubulin that favors the binding of GTP in the E-site of tubulin. While the authors suggested in the supplemental data that the previously assigned stereochemistry of the 22,23-epoxide may need to be revisited, they did not ultimately promote this adjustment in configuration. However,



**Fig. 4** Taccalonolide AJ binding covalently to Asp226 on  $\beta$ -tubulin as determined by X-ray crystallography of the T2R-TTL-taccalonolide AJ complex

additional crystallographic data of taccalonolide AJ (**29**) in the absence of tubulin and elucidation of the reaction mechanism (Scheme 2) confirmed that the stereochemistry of the 22,23-epoxide originally described by Li et al. needed to be revised [28, 29].

Based on these data, Sanchez-Murcia et al. undertook extensive *in silico* modeling and molecular dynamics simulations to elucidate further the unique interaction between the 22,23-epoxy-taccalonolides and  $\beta$ -tubulin [30]. They proposed that the nucleophilic attack on C-22 by the OD1/OD2 carboxylate of  $\beta$ Asp226 and opening of the 22,23-epoxide is facilitated by the long-lived hydrogen bond interaction of the carboxylate with the side-chain hydroxy of Thr223 and enhanced stabilization mediated via water-bridged hydrogen bonds. The C-22 carbon was suggested to undergo initial addition, indicating that the epoxide is non-protonated prior to nucleophilic attack. They further emphasize that the 22,23-epoxide is essential for covalent bond formation between the taccalonolide and tubulin. Their detailed molecular analysis of the putative interactions provides an important framework for



**Scheme 2** Reaction mechanism of the covalent binding of taccalonolide AJ to  $\beta$ -tubulin Asp226

additional biochemical and molecular biological studies exploring their functional importance.

The discrepancy between the lack of taccalonolide-induced M-loop stabilization detected by hydrogen–deuterium exchange mass spectrometry and the observed conformational shift of the M-loop in the taccalonolide AJ-tubulin crystal structure [27] was clarified by Balaguer et al. [25]. This elegant study compared the binding, allosteric effects, and tubulin polymerization dynamics of the three known covalent microtubule stabilizers: zampanolide, cyclostreptin, and the potent 22,23-epoxy-taccanolides.

The crystal structure of cyclostreptin bound to  $\beta$ -tubulin corrected previous literature suggesting covalent interactions with  $\beta$ Thr220 and  $\beta$ Asn228 and instead confirmed that cyclostreptin and zampanolide both bind covalently to  $\beta$ His229 [25]. Furthermore, they demonstrated that cyclostreptin-dependent tubulin polymerization was associated with a similar lag period that had been observed with taccalonolide AJ (29). In contrast, zampanolide rapidly induced tubulin polymerization in a manner similar to taxane microtubule stabilizers. Superimposition of the crystal structures of each of these compounds with tubulin demonstrated that extensive M-loop interactions and helical stabilization were correlated with stabilizers that promoted a strong initial rate of assembly [25]. In contrast, stabilizers such as taccalonolide AJ (29) that only promote partial M-loop structuring without inducing a helical confirmation were associated with a significant lag time prior to the initiation of tubulin polymerization. Together, these data demonstrate that while the taccalonolides do promote some structuring of the M-loop of  $\beta$ -tubulin, these interactions are not as significant as those promoted by the taxanes, which result in a delay in the initiation of microtubule stabilization by the taccalonolides.

## 5 Cellular Effects of Taccalonolide-Induced Microtubule Stabilization

Given the distinct biochemical interaction of the taccalonolides with tubulin, studies were undertaken to evaluate the effects of the 22,23-epoxy-taccanolides as compared to taxanes on microtubule dynamics in biochemical preparations and in live cells [31]. While paclitaxel and taccalonolide AJ (29) had similar overall effects on the microtubule dynamics of purified tubulin that promoted overall stabilization,

taccalonolide AJ (**29**) demonstrated a greater suppression of catastrophe frequency likely as a result of its irreversible binding. In contrast, paclitaxel had a greater effect on microtubule rescue frequency than taccalonolide AJ (**29**). Similar effects were observed when microtubule dynamicity was evaluated in live cells with **29** having a greater impact on microtubule catastrophe and paclitaxel affecting rescue frequency to a larger extent. These differences in cellular microtubule dynamics were found to underlie the distinct microtubule aster morphology observed in cells treated with the taccalonolides as compared to the taxanes [14, 32].

Real-time spindle formation was evaluated in live cells expressing GFP-tagged tubulin upon treatment with the taccalonolides or paclitaxel compared to vehicle controls. While cells entered mitosis at similar rates with similar effects on aster formation, differences were noted in the consolidation of these asters by paclitaxel but not the taccalonolides during extended mitotic arrest. This aster consolidation in paclitaxel-treated cells led to the previously described phenotype of 2–3 asters per cell in contrast to the taccalonolides that result in an average over five asters per cell (Fig. 5). The finding that the taccalonolides suppress microtubule catastrophe and inhibit aster consolidation to a greater extent than paclitaxel demonstrates that these distinct effects on microtubule dynamicity between the test compounds can lead to the formation of different cellular microtubule structures that could contribute toward distinct biological readouts.

Rohena et al. [32] investigated the microtubule-associated mitotic effects initiated by three structurally and functionally diverse microtubule-stabilizing agents: taccalonolide AJ (**29**), laulilamide, and paclitaxel. Each microtubule stabilizer initiated distinct mitotic defects and differentially dysregulated the expression of key mitotic kinases. Taccalonolide AJ (**29**) produced the most profound defects in centrosome maturation, separation, and disjunction as observed by indirect immunofluorescence of the centrosomal-associated proteins rootletin, Nek2, and  $\gamma$ -tubulin [32]. However, taccalonolide AJ-treated cells also contained the more peripheral centrosomal protein pericentrin at every spindle aster, suggesting these structures facilitated the maintenance and stability of the multiple, highly focused asters observed in taccalonolide-treated cells as compared to the other two microtubule stabilizers, which only contained two pericentrin foci [32]. Not surprisingly, these defects in centrosomal structures were accompanied by mitotic signaling



**Fig. 5** Distinct mitotic spindle structures in normal cells (left), paclitaxel-treated cells (middle), and taccalonolide-treated cells (right)



defects, including enhanced Eg5 phosphorylation by taccalonolide AJ-treated cells as compared to those treated with the other stabilizers [32].

## 6 In Vivo Antitumor Efficacy of 22,23-Epoxy-taccalonolides

The antitumor efficacy of the epoxy-taccalonolides AF (**28**) and AJ (**29**) was evaluated initially in a MDA-MB-231 flank triple-negative breast cancer xenograft murine model. Taccalonolide AF (**28**) exhibited antitumor efficacy at a total dose of 5 mg/kg that produced a greater degree of tumor regression than 40 mg/kg paclitaxel [26]. Additional antitumor studies with the potent 22,23-epoxidation products of taccalonolides T (**20**) and AI (**30**) also demonstrated antitumor efficacy in a MDA-MB-231 xenograft model [33]. However, taccalonolide AJ (**29**) did not demonstrate antitumor effects even at the  $LD_{40}$  dose of 2 mg/kg [26]. These results suggested that taccalonolides AF and AJ, with similar biochemical and cellular microtubule-stabilizing activities, may have distinct pharmacokinetic properties.

Initial efforts to characterize differences in the chemical stability of taccalonolides AF (**28**) and AJ (**29**) demonstrated that the C-15 acetoxy group of taccalonolide AF (**28**) was hydrolyzed in aqueous solutions to generate AJ (**29**) [26]. In vivo pharmacokinetic properties were evaluated for both taccalonolides AJ (**29**) and AF (**29**) in the same strain of nude mice that were utilized for xenograft studies. AJ (**29**) was demonstrated to have an elimination half-life of 8.1 min, when administered systemically, while the half time of AF (**28**) was 44.1 min [34]. AJ (**29**) had excellent and persistent antitumor efficacy when administered directly into the tumor, suggesting that the lack of antitumor efficacy demonstrated with systemic administration of AJ (**29**) was likely due to its short half-life in vivo [34].

Given the fact that the C-15 acetyl group on taccalonolide AF (**28**), which demonstrated in vivo efficacy, was effectively hydrolyzed in aqueous solution to generate taccalonolide AJ (**29**), which does not have a therapeutic window for systemic administration in vivo, we hypothesized that semisynthesis of taccalonolides with C-15 substitutions could provide increased stability of an active antitumor drug to provide an increased therapeutic window. The in vitro biological activities of 28 novel taccalonolides with mono substitutions at C-7 or C-15 or disubstitutions at C-7 and C-25 ranged in antiproliferative potency from 2.4 nM to >20  $\mu$ M [29]. However, no improved stability or therapeutic window was observed with isovalerate, cyclopropyl, isobutyrate, or formate substituents at C-7 or C-15. Additionally, substitutions at C-25 completely abrogated in vitro activity, likely due to interference with the covalent binding of the 22,23-epoxide to  $\beta$ 226 of tubulin. The two most potent taccalonolides in vitro, isovalerate modifications at C-7 or C-15, were evaluated for in vivo antitumor efficacy by intratumoral injection in the drug-resistant human NCI/ADR-RES xenograft murine model. Similarly to taccalonolide AJ (**29**), the isovalerate-modified taccalonolides demonstrated potent in vivo efficacy when directly administered to the tumor and notably caused long-term antineoplastic efficacy for over a month after the final

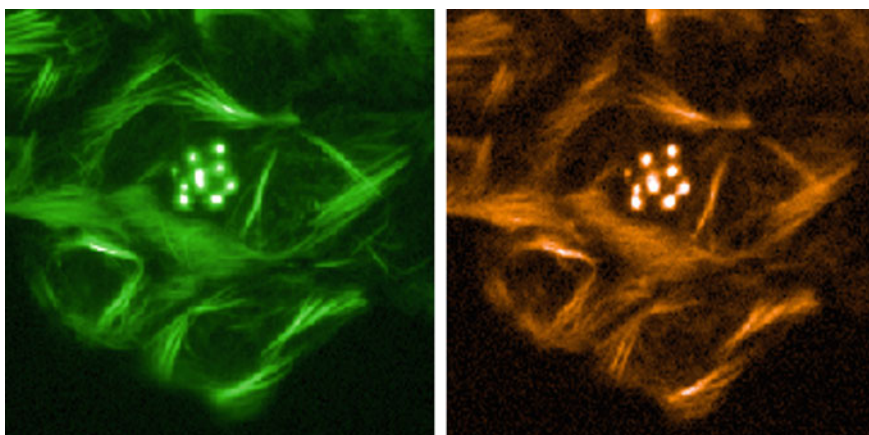
dose was administered [29]. These results demonstrate that targeted delivery of the taccalonolides provides for long-term efficacy in drug-resistant tumor models and led to studies to identify a handle on the taccalonolides that could be used for tumor targeting strategies.

## 7 Taccalonolide Conjugates Provide Evidence of Specificity and a Handle for the Generation of Targeted Agents

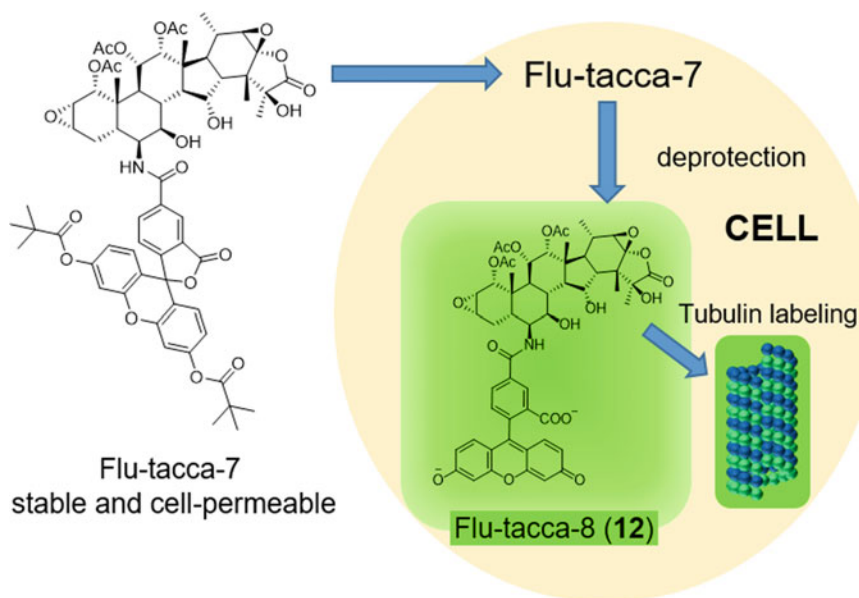
Data from previous semisynthetic efforts as well as an interrogation of the taccalonolide AJ-tubulin crystal structure led to the identification of C-6 as a possible handle amenable to functionalization. Indeed, modification of this site provided a stable fluorescein-tagged taccalonolide that retained microtubule-stabilizing activity and could be visualized colocalizing with microtubules (Fig. 6) [35].

These efforts were expanded to eventually generate a C-6-fluorescein taccalonolide conjugate that retained *in vitro* potency in the low nanomolar range and provided the stability to perform detailed imaging and cellular binding studies [28]. Optimization of the taccalonolide–fluorescein probe included the addition of pivaloyl-protected groups on fluorescein to quench fluorescence of the probe prior to cellular import, which provided the ability to monitor uptake and binding in live cells with no need to remove excess probe from the surrounding medium (Fig. 7).

Serendipitously, the C-6 fluorescein modification actually improved the biochemical tubulin-polymerizing activity of the taccalonolides by making additional contacts with tubulin [28]. However, the pivalate protective groups prevented a direct interaction with tubulin in biochemical assays, demonstrating that the pivalate



**Fig. 6** Fluorescein-tagged taccalonolide (green) colocalized with  $\beta$ -tubulin immunofluorescence (orange) in fixed HCC1937 triple-negative breast cancer cells after 24 h treatment



**Fig. 7** Flu-tacca-7 is a cell-permeable fluorescent taccalonolide containing pivalate protective groups on the fluorophore that prevent fluorescence as well as target engagement prior to intracellular esterase cleavage. Upon cellular entry and pivalate deprotection to generate flu-tacca-8, the probe can directly bind tubulin and fluorescently label intracellular microtubules. The quenching provided by the pivalate groups permits live cellular imaging without the need to remove excess probe from the medium, providing a no-wash, irreversible fluorogenic labeling system for cellular microtubules

modification simultaneously prevented fluorescence and target engagement prior to cellular hydrolysis. The taccalonolide–fluorescein probe was found to be superior to commercially available taxane probes with regard to its microtubule staining without the addition of carrier molecules or removing excess probe from the medium [28]. It also provided microtubule staining under conditions that are not amenable to visualization with reversible taxane-based probes, including chilled conditions where microtubules are sensitive to depolymerization or in cells with high expression of drug efflux transporters [28].

The fluorescent taccalonolide probe strikingly colocalized with  $\beta$ -tubulin by immunofluorescence in human cancer cells, and the interaction was retained throughout immunoblotting to demonstrate a specific interaction between tubulin and the labeled taccalonolide [28]. A taccalonolide probe lacking the 22,23-epoxide completely abrogated this colocalization and binding, providing the first direct evidence of the exquisite specificity of the covalent interaction between the 22,23-epoxide of the taccalonolides and tubulin. This provided an unprecedented opportunity to use mutational analysis of an ectopically expressed form of tubulin to systematically evaluate the relative contribution of  $\beta$ -tubulin residues to taccalonolide binding with a focus on those that would be predicted to facilitate this

interaction based on the crystallographic and modeling data [27, 30]. Consistent with these data,  $\beta$ Asp226 was critical for the covalent interaction between the taccalonolides and tubulin [28]. Additionally,  $\beta$ Lys19 and  $\beta$ Leu219 were also critical for taccalonolide binding,  $\beta$ His229 and  $\beta$ Thr223A had a moderate effect on binding, and  $\beta$ Arg278 did not influence binding [28]. These data provide critical insight into the taccalonolide pharmacophore that will be highly valuable in strategies to optimize target binding and, potentially, to facilitate the synthesis of new classes of taccalonolide-like small molecules. Overall, this study provided insight into the target specificity and detailed drug–target interactions of the taccalonolides and strategies to further develop targeted taccalonolides.

## 8 Other Bioactive Compounds Isolated from *Tacca* Species

In addition to the taccalonolides, other bioactive compounds have been isolated from *Tacca* species. Most intriguingly, a microtubule destabilizer, taccabulin A (**31**) (Table 2), was isolated from the roots and rhizomes of *Tacca* species [36], which was the first study reporting the isolation of both a microtubule stabilizer and microtubule destabilizer from the same natural product source. Taccabulin A (**31**) effectively displaced colchicine binding to tubulin, suggesting that it binds within the colchicine pharmacophore, and demonstrated synergistic effects when combined with the taccalonolides [36]. Similar to the taccalonolides and other colchicine site-binding agents, taccabulin A (**31**) retained efficacy in drug-resistant models, including those that express elevated levels of the P-glycoprotein drug efflux pump or the  $\beta$ -III isotype of tubulin [36]. Six additional retro-chalcones, taccabulins B–E (**32–35**) and evelynins A (**36**) and B (**37**), were also isolated from *Tacca* extracts. Evelynin A (**36**) and B (**37**), as well as taccabulin D (**34**) demonstrated some cytotoxic activity toward cancer cells *in vitro* but with no evidence of microtubule stabilizing or destabilizing activities [37, 38]. Other classes of compounds isolated from *Tacca* species include withanolides, glucosides, steroidal glycosides, diarylheptanoids, and diarylheptanoid glycosides [39–49].

## 9 Conclusion

In the 60 years since the taccalonolides were first identified as the bitter principle of *Tacca* tubers, they have continually provided interesting and often unanticipated discoveries. These include the finding in 1987 that the structure of the taccalonolides was more complicated than initially proposed, the elucidation of their mechanism of action as microtubule stabilizers in 2003, and the critical nature of the 22,23-epoxide for direct tubulin binding in 2013. This last finding is somewhat

of a cautionary tale in natural products research that describes how a potent minor constituent, in this case 22,23-epoxy-taccanolides, could be responsible for the activity originally ascribed to more naturally abundant compounds. We now know that taccalonolides without a 22,23-epoxide lack the ability to bind and polymerize tubulin and have no detectable antiproliferative activity against cancer cell lines in culture. In contrast, potent 22,23-epoxy-taccanolides, including AJ (**29**) and AF (**28**), have the ability to covalently and irreversibly bind the Asp226 residue of  $\beta$ -tubulin to promote a distinct profile of microtubule stability as compared to other classes of clinically approved microtubule stabilizers. Most notably, some of these taccalonolides have demonstrated in vivo antitumor efficacy in drug-resistant breast and ovarian cancer models that persists for extended periods after drug treatment due to their covalent binding. Continued efforts to improve the therapeutic window for systemic administration and/or promote localized drug delivery based on the recent identification of a drug handle on the taccalonolide backbone may provide for their development as novel anticancer agents for the treatment of drug-resistant disease.

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