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2013

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Recommended Citation

Rohena, Cristina C; Peng, Jiangnan; Johnson, Tyler A.; Crews, Phillip; and Mooberry, Susan L, "Chemically diverse microtubule stabilizing agents initiate distinct mitotic defects and dysregulated expression of key mitotic kinases." (2013). *Natural Sciences and Mathematics | Faculty Scholarship*. 1.
https://scholar.dominican.edu/natural-sciences-and-mathematics-faculty-scholarship/1

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NIH Public Access
Author Manuscript
Biochem Pharmacol. Author manuscript; available in PMC 2014 April 15.

Published in final edited form as:
Biochem Pharmacol. 2013 April 15; 85(8): 1104–1114. doi:10.1016/j.bcp.2013.01.030.

Chemically Diverse Microtubule Stabilizing Agents Initiate Distinct Mitotic Defects and Dysregulated Expression of Key Mitotic Kinases

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Abstract

Microtubule stabilizers are some of the most successful drugs used in the treatment of adult solid tumors and yet the molecular events responsible for their antimitotic actions are not well defined. The mitotic events initiated by three structurally and biologically diverse microtubule stabilizers; taccalonolide AJ, laulimalide/fijianolide B and paclitaxel were studied. These microtubule stabilizers cause the formation of aberrant, but structurally distinct mitotic spindles leading to the hypothesis that they differentially affect mitotic signaling. Each microtubule stabilizer initiated different patterns of expression of key mitotic signaling proteins. Taccalonolide AJ causes centrosome separation and disjunction failure to a much greater extent than paclitaxel or laulimalide, which is consistent with the distinct defects in expression and activation of Plk1 and Eg5 caused by each stabilizer. Localization studies revealed that TPX2 and Aurora A are associated with each spindle aster formed by each stabilizer. This suggests a common mechanism of aster formation. However, taccalonolide AJ also causes pericentrin accumulation on every spindle aster. The presence of pericentrin at every spindle aster initiated by taccalonolide AJ might facilitate the maintenance and stability of the highly focused asters formed by this stabilizer. Laulimalide and paclitaxel cause completely different patterns of expression and activation of these proteins, as well as phenotypically different spindle phenotypes. Delineating how diverse microtubule stabilizers affect mitotic signaling pathways could identify key proteins involved in modulating sensitivity and resistance to the antimitotic actions of these compounds.

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Conflict of Interest Statement:
J. Peng and S.L. Mooberry are inventors of a pending patent application that includes composition and use of taccalonolide AJ. S.L. Mooberry is an inventor on a patent for the use of laulimalide in hyperproliferative diseases including cancer.

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1. Introduction

Microtubules are key components of the cytoskeleton and are important for numerous cellular processes including intracellular transport, maintenance of cell shape and separation of the sister chromatids. Because of their roles in mitosis and other cellular processes, microtubules are an attractive and proven target for anticancer drugs. Microtubule targeting agents continue to be useful in the treatment of adult and pediatric cancers. These agents are classified as microtubule stabilizers or destabilizers based on their effects on interphase microtubules at relatively high concentrations. Paclitaxel, the first microtubule stabilizer identified, binds within the taxane site on the interior surface of the microtubule, where it strengthens protofilament interactions leading to microtubule stabilization (1, 2). In addition to the taxanes, the epothilones, discodermolide, and dictyostatin bind within the taxane site but with subtly different orientations (3). Laulimalide and peloruside A, bind to a second microtubule stabilizer site that is located on the exterior surface of microtubules (3–7). All microtubule stabilizers suppress microtubule dynamics at low antiproliferative concentrations (8).

The taccalonolides are a class of chemically and mechanistically distinct microtubule stabilizing agents (9, 10). Consistent with the effects of all other microtubule stabilizers, the taccalonolides cause the appearance of thick bundles of microtubules in interphase cells and the formation of aberrant mitotic spindles that lead to mitotic arrest and initiation of apoptosis (10). The taccalonolides are potent antitumor agents *in vivo* with efficacy superior or equal to paclitaxel in multiple murine models (10, 11). A direct interaction of taccalonolides A and E with microtubules was not detected (12) but the newly identified, highly potent taccalonolides, AF and AJ, bind directly to tubulin/microtubules and stimulate tubulin polymerization (13). Their binding site is under investigation.

While the phenotypic effects of the taccalonolides are similar to other microtubule stabilizers, they are also subtly different. The taccalonolides initiate short, thick microtubule bundles throughout the cytoplasm, while paclitaxel initiates long bundles of microtubules that appear to nucleate from the centrosome (10). Additionally, the taccalonolides initiate microtubule bundling at much lower concentrations relative to the IC_{50} for inhibition of proliferation as compared to paclitaxel (10). Together, these data support the hypothesis that the taccalonolides have cellular effects somewhat different than other microtubule stabilizers.

Mitosis involves a series of highly coordinated events that are temporally and spatially regulated leading to the formation of a bipolar mitotic spindle. Extensive research has examined the effects of paclitaxel on cellular stress and apoptotic pathways. However, the molecular events leading from stabilizer-initiated suppression of microtubule dynamics to aberrant mitotic spindle assembly and mitotic arrest have not been defined. The aim of this study was to begin to identify how structurally diverse microtubule stabilizers alter mitotic signaling to cause the severe mitotic defects that precede mitotic arrest and ultimately, cell death. Identification of these signaling pathways will help identify key partners in the response to microtubule stabilizers that might be altered in some cancers precluding a robust response to microtubule stabilizers and potentially clinical drug resistance. Our studies demonstrate that chemically diverse microtubule stabilizers initiate substantially different effects on mitotic signaling pathways and begin to identify potential biomarkers that might predict sensitivity or resistance to microtubule stabilizing agents.

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2. Materials and methods

2.1 Materials

Paclitaxel was purchased from Sigma-Aldrich (St. Louis, MO). Taccalonolide AJ was semi-synthesized as previously described (13). Ethanol (Sigma-Aldrich, St. Louis, MO) was used to solubilize each drug and as the vehicle control. Laulimalide/fijianolide B was isolated as previously described (7).

2.2 Cell culture

HeLa cells were obtained directly from American Type Culture Collection (Manassas, VA). They were maintained in Basal Medium Eagle (Sigma-Aldrich, St. Louis, MO) with Earle's salts supplemented with 10% fetal bovine serum (Hyclone, South Logan, Utah) and 50 μg/ml gentamicin (Invitrogen, Grand Island, NY). Cells were maintained at 37°C in a humidified incubator with 5% CO_2.

2.3 Whole cell lysate preparation and Western blotting

HeLa cells were synchronized by double thymidine block as described previously (14). Immediately after release, drug or vehicle was added. The cells were treated with the lowest concentration that caused maximal G_2/M accumulation; for paclitaxel, 12.5 nM, for taccalonolide AJ, 16 nM, and for laulimalide, 17.5 nM. At specific time points after drug addition, cells were harvested by scraping and centrifugation and divided into aliquots. One aliquot was used to make whole cell lysates and the second was used for evaluating cell cycle distribution. Cells were lysed using cell extraction buffer (Invitrogen, Grand Island, NY) supplemented with protease inhibitors. Protein concentrations were measured and equal amounts of proteins were separated by SDS-PAGE, transferred to a PVDF membrane and probed overnight at 4°C for specific proteins using antibodies for: Aurora A, P-Aurora A Thr 288, P-Lamin A/C, P-Histone H3 Ser 10, and P-Tacc3 Ser 558 (Cell Signaling, Danvers, MA); Actin (Sigma-Aldrich, St. Louis, MO); TPX2 and P-Eg5 Thr 927 (Biolegend, San Diego, CA); Tacc3, Plk1, and P-Plk1 Thr 210 (Epitomics, Burlingame, CA). The signal was visualized with Amersham ECL Plus (GE Health Care, Piscataway, NJ) in a Geliance (Perkin Elmer, Waltham, MA) imaging system. The signal intensity was quantitated using GeneTools (Syngene, Frederick, MD) by normalizing the signal to the time zero lysate, which was the same for all treatment groups.

2.4 Flow cytometry

The second aliquot of cells harvested was spun at 2000 rpm for 10 min. The supernatant was discarded and the pellet resuspended in Krishan's reagent (Sigma-Aldrich, St. Louis, MO) containing propidium iodide and RNAse A. Cells were analyzed for DNA content using a FACSCalibur (BD Biosciences, San Jose, CA).

2.5 Indirect immunofluorescence

HeLa cells were plated on glass coverslips, allowed to grow for 24 h and then treated with the compounds for 18 h and fixed with cold methanol. The pertinent structures were visualized with the following antibodies: Rabbit β-tubulin (Abcam, Cambridge, MA); mouse β-tubulin (Sigma-Aldrich, St. Louis, MO); γ-tubulin (Sigma-Aldrich, St. Louis, MO), pericentrin (Abcam, Cambridge, MA) Aurora A (Cell Signaling, Danvers, MA), TPX2 (Biolegend, San Diego, CA) Nek2 and Rootletin (Novus Biologicals, Littleton, CO) and the DNA was visualized using DAPI. The centrin antibody was a generous gift from Dr. Salisbury, Mayo Clinic, Rochester, MN. For centrosome separation or disjunction, a minimum of 100 mitotic cells were counted per coverslip and the numbers from a minimum

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of three independent experiments were averaged. Cells were classified as having centrosomes that failed to separate if individual centrosomes were touching.

3. Results

3.1 Mitotic spindle phenotypes

Initial studies indicated that the mitotic spindles formed in the presence of the taccalonolides were different from those seen with other microtubule stabilizers (10, 13, 15). The effects of taccalonolide AJ were evaluated in HeLa cells and compared to the effects of paclitaxel and laulimalide. The chemical structures of these microtubule stabilizers are shown in Fig 1A. The relative potencies of these compounds are similar, with IC_{50} values in HeLa of 2–4 nM and G_2/M arrest concentrations of 12.5–17.5 nM. The cellular effects of microtubule stabilizers depend on concentration and thus the concentration used for each stabilizer was the lowest concentration that caused maximal G_2/M arrest. This concentration was carefully defined for each compound using flow cytometry. These concentrations were used throughout the current studies to compare the effects of these microtubule stabilizers on mitotic spindle structures and on mitotic signaling.

Normal bipolar mitotic spindles were observed in vehicle-treated cells (Fig 1B). In contrast, taccalonolide AJ initiated the formation of multiple mitotic spindle asters, typically 5–9 per cell (Fig 1B) that were compact and located throughout the cytoplasm. Phenotypically different mitotic spindle asters were formed in laulimalide-treated cells, with a distinguishing feature of circular mitotic spindles with a central region devoid of β-tubulin (Fig 1B). This distinctive laulimalide spindle phenotype has been noted in other cell types with laulimalide (6) or laulimalide analogs (16). Other mitotic spindle structures are also observed in laulimalide-treated cells, and these consisted of 2–5 distinct, yet slightly diffuse spindle asters (Fig 1B). Paclitaxel initiated abnormal mitotic spindles containing 2–3 diffuse spindle asters that appeared more poorly organized (Fig 1B). The differences in both spindle morphology and the number of mitotic spindle asters in cells treated with taccalonolide AJ in comparison to laulimalide or paclitaxel led us to hypothesize that the mechanisms of spindle formation might be different among these microtubule stabilizers, possibly in response to different mitotic signaling defects.

3.2 Taccalonolide AJ leads to severe and distinct centrosomal defects

The numerous and focused nature of the spindle asters formed by taccalonolide AJ suggested the possibility that these aberrant asters could be induced by centrosome amplification. Taccalonolide AJ-treated cells contained only two γ-tubulin foci, associated with only two of the many spindle asters (Fig 2A), suggesting that there was no centrosome amplification or fragmentation. This was also true for the asters formed in the presence of laulimalide or paclitaxel where two γ-tubulin foci were seen. Other components of the centrosome including centrin and pericentrin were also evaluated. In vehicle-treated cells the normal co-localization of pericentrin to each of the centrioles was observed (data not shown). Taccalonolide AJ-treated cells also contained two pairs of centrin signals, similar to γ-tubulin. This same phenotype was also seen in laulimalide and paclitaxel-treated cells (Fig 2C). In contrast, multiple pericentrin foci (Fig 2C) were observed in taccalonolide AJ-treated cells but not in paclitaxel or laulimalide-treated cells. Two large pericentrin foci always co-localized with γ-tubulin at the centrosomes (Fig 2E), while less intense pericentrin staining was found at every other aberrant spindle aster (Fig 2D). Laulimalide and paclitaxel did not cause extra pericentrin foci as evidenced by the presence of only two pericentrin foci that co-localized with γ-tubulin (Fig 2E) and only two of the spindle asters (Fig 2D).

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In the process of evaluating γ-tubulin, centrin and pericentrin localization, we observed a significant population of taccalonolide AJ-treated cells that had entered mitosis with unseparated centrosomes. Unlike vehicle-treated cells, 64% of taccalonolide AJ-treated cells had centrosomes that had not separated prior to mitosis (Fig 2A). It is important to note that none of these cells with unseparated centrosomes had monopolar spindles (Fig 2B). In contrast, only 21% of paclitaxel- treated cells showed centrosome separation defects (Fig 2B). Laulimalide was intermediate with 41% of cells having failed centrosome separation. These results highlight major differences among the stabilizers with regard to the organization of mitotic spindle aster formation and suggest functional defects in centrosome maturation and disjunction, especially with taccalonolide AJ.

The taccalonolides have a higher propensity for microtubule bundling at low antiproliferative concentrations as compared to paclitaxel (9). It was possible that the centrosome defects observed represent a continuum and that the more severe defects seen with taccalonolide AJ could occur with higher concentrations of laulimalide or paclitaxel. The concentration dependent effects of the stabilizers on centrosomal defects were evaluated. A dose dependent increase in the percentage of cells that failed to separate their centrosomes, similar to the effects of taccalonolide AJ (Fig 2F), was observed with higher concentrations of laulimalide and paclitaxel. At a concentration of 500 nM paclitaxel or laulimalide, the majority of cells failed to separate their centrosomes. In contrast, the centrosome separation defects were maximal with taccalonolide AJ at the G_2/M arrest concentration (16 nM) (Fig 2B and F). Consistent with the centrosome separation defects, higher concentrations of laulimalide or paclitaxel increased the number of spindle asters observed in mitotic cells. Surprisingly, although both centrosome separation defects and mitotic spindle asters increased with higher concentrations of laulimalide or paclitaxel, the localization of pericentrin did not change with higher concentrations of these stabilizers and remained different from that seen with taccalonolide AJ (data not shown).

3.3 Taccalonolide AJ causes centrosome disjunction defects

Further studies were conducted to evaluate the mechanisms by which taccalonolide AJ inhibited centrosome separation. Prior to mitosis, the centrosomes are held together by linker proteins, C-Nap-1 and Rootletin. During centrosome disjunction, Nek2 kinase phosphorylates these proteins resulting in their displacement from the centrosome, allowing for separation. Centrosome disjunction defects in drug-treated cells were investigated by evaluating the localization of Nek2 and Rootletin in interphase cells since centrosomes normally separate prior to mitotic entry. Vehicle-treated cells positive for Nek2 showed two patterns (Fig 3A). A total of 57% of the cells had Nek2 foci that were separate and 43% had Nek2 foci that appeared to be touching (Fig 3A and B). This same pattern was seen in cells treated with laulimalide (47% together, 53% apart) or paclitaxel (44% together, 56% apart). In contrast, 92% of taccalonolide AJ-treated cells had Nek2 foci that were touching (Fig 3A and B). This was corroborated by Rootletin localization. 60% of vehicle-treated cells had Rootletin positive centrosomes that were still together and 40% were separate. With taccalonolide AJ, 97% of cells had Rootletin foci that were touching (Fig 3C and D). In contrast, laulimalide or paclitaxel-treated cells showed Rootletin localization (Fig 3C and D) that matched that of Nek2 and did not differ substantially from the vehicle control. These data suggest that taccalonolide AJ-initiated defects in centrosome separation are due in part to inhibition of centrosome disjunction and that these effects are unique to the taccalonolides as they were not observed with paclitaxel or laulimalide.

3.4 Microtubule stabilizers lead to changes in the expression of Plk1 and Eg5

The centrosomal defects initiated by taccalonolide AJ and to a lesser extent laulimalide suggested the possibility that they disrupt Plk1. This kinase plays important roles in

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centrosome maturation and separation and it regulates centrosome disjunction by indirectly activating Nek2. Additionally, Plk1 targets the microtubule motor Eg5 to the centrosome where Eg5 facilitates centrosome separation. As vehicle-treated cells moved into mitosis Plk1 expression increased and was maximal at 6 h. Plk1 is activated by phosphorylation, which began 5 h after release and was maintained until the vehicle-treated cells moved back into G_1. In cells treated with taccalonolide AJ, the levels of total Plk1 were diminished and expression was delayed with maximal accumulation at 8 h. Phosphorylated Plk1 was also delayed in taccalonolide AJ-treated cells. The initial increase in Plk1 activation was not apparent until 8 h where it increased further during mitotic arrest (Fig 4 A and B). In stark contrast, laulimalide caused early Plk1 expression as compared to control, and an early increase in Plk1 activation with phosphorylation levels rising 3 h earlier than that seen in vehicle-treated cells. Paclitaxel-treated cells exhibited lower expression of Plk1 as cells moved into mitosis with lower levels of Plk1 than was seen with the other microtubule stabilizers or with vehicle. Additionally, the phosphorylation of Plk1 in paclitaxel-treated cells was substantially delayed. These data indicate substantial differences among the stabilizers in both the timing and levels of Plk1 expression and activation. Laulimalide initiated early expression and activation and paclitaxel and taccalonolide AJ caused a delay in expression and activation as cells moved into mitosis. Later during the prolonged mitotic arrest, the levels of phospho-Plk1 were highest with paclitaxel and taccalonolide AJ.

Inhibition of Eg5 causes the formation of monopolar mitotic spindles due to centrosome separation failure. Additionally, phosphorylation of Eg5 on Thr 927 by Cdk1 supports Eg5's association with microtubules, thereby facilitating centrosome separation. Taccalonolide AJ initiated higher levels of phospho-Eg5 than were observed with vehicle, paclitaxel or laulimalide (Fig 4A and D). In contrast, laulimalide and paclitaxel, caused a slight delay in Eg5 phosphorylation, and the levels were much lower than those seen with taccalonolide AJ and resemble vehicle (Fig 4A and D). With paclitaxel, a late increase in levels of phospho-Eg5 was observed at 9–10 h similar to the levels that occurred with taccalonolide AJ (Fig 4 and D), but higher than was obtained with laulimalide. These results again show distinct molecular differences among the microtubule stabilizers.

3.5 Effects of microtubule stabilizers on Aurora A expression

The microtubule stabilizer induced changes in Plk1 suggested possible defects in Aurora A signaling since Plk1 targets Aurora A to the centrosome. In taccalonolide AJ-treated cells, the expression of Aurora A increased at the same time as in vehicle-treated cells (Fig 5A and B). However, 8 h after release, Aurora A levels in taccalonolide AJ-treated cells were higher than was observed with vehicle and protein levels accumulated further 8–10 h after release. In contrast, laulimalide initiated premature expression of Aurora A beginning 2 h after release and these higher levels were maintained through 9 h. (Fig 5 A and B). Surprisingly, with paclitaxel, the levels of Aurora A did not increase as the cells moved into G_2/M at 5–6 h, and did not begin to increase until 8 h after release. The cumulative levels of Aurora A in paclitaxel-treated cells were much lower 8–10 h after release as compared to cells treated with laulimalide or taccalonolide AJ.

Aurora A is phosphorylated on multiple sites, including Thr 288 which controls its kinase activity (17, 18). Consistent with the protein levels of Aurora A, phosphorylation of Thr 288 increased as vehicle-treated cells entered mitosis and decreased as they exited into G_1 (Fig 5A and C). Levels of phospho-Aurora A in taccalonolide AJ-treated cells were similar to control. Laulimalide initiated early and higher levels of activated Aurora A, consistent with total protein accumulation. Although the levels of Aurora A were lower in paclitaxel-treated cells, phospho-Aurora A levels were similar to the controls as the cells moved into mitosis. In comparing the drugs, the total levels of phospho-Aurora A were highest in laulimalide-treated cells throughout the time course.

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Aurora A is targeted to the spindle poles by TPX2 where it facilitates Aurora A autophosphorylation and protects it from dephosphorylation by PP1, thereby preventing the proteosomal degradation of Aurora A (19). TPX2 expression increased in vehicle-treated cells as cells moved into mitosis at 5 h and then diminished as cells exited mitosis (Fig 5A and D). Taccalonolide AJ caused a similar pattern of TPX2 expression with levels slightly higher than control at all time points with a time-dependent accumulation 8–10 h during mitotic arrest. In laulimalide-treated cells, the same pattern of expression seen with Aurora A was observed; the levels of TPX2 increased early, 2 h after release, were maintained from 2–9 h, and began to diminish slightly by 10 h. This differs substantially from the pattern seen with vehicle or taccalonolide AJ (Fig 5A and D). Consistent with the effects of paclitaxel on Aurora A levels, paclitaxel also initiated different effects on TPX2 expression. Paclitaxel did not cause a major change in the levels of TPX2 as cells entered mitosis, and only a small increase was seen at 8 h, indicating slower accumulation of TPX2 as compared with vehicle, taccalonolide AJ or laulimalide. Additionally, levels of TPX2 were lower in paclitaxel-treated cells as compared to vehicle from 5–9 h, especially when compared to TPX2 expression in the presence of the other microtubule stabilizers.

The effects of the microtubule stabilizers on Tacc3, a downstream substrate of Aurora A were evaluated as a measure of Aurora A activity. In vehicle-treated cells, the levels of Tacc3 increased as the cells moved into mitosis and levels of phosphorylated Tacc3 increased at 8 h with maximal levels obtained at 9 h. Taccalonolide AJ had no major effects on total Tacc3, but higher levels of phospho-Tacc3 were observed as compared to control as cells moved into mitosis. In laulimalide-treated cells the levels of total Tacc3 increased as the cells moved into mitosis and total protein levels diminished slightly by 10 h from the maximal levels that occurred at 5–9 h. Laulimalide initiated an increase in the levels of phospho-Tacc3 occurring within 4 h, substantially earlier than was observed with vehicle. Paclitaxel caused a different pattern of Tacc3 and phospho-Tacc3 expression. There was an early accumulation of total Tacc3 at 4 h, but by 9 and 10 h higher levels than any other treatment group were maintained. Phospho-Tacc3 was not observed until 8 h post release, in contrast to the earlier accumulation that occurred in vehicle, taccalonolide AJ and laulimalide-treated cells. Overall, these changes are consistent with our findings on the effects of these drugs on Plk1 expression and activation and further validate our hypothesis that these stabilizers cause distinct and specific defects in these mitotic pathways.

3.6 Differential localization of TPX2 and Aurora A

Due to the defects caused by these microtubule stabilizers on the expression of Aurora A and associated proteins; we evaluated their effects on the localization of Aurora A and its activating partner TPX2. In vehicle-treated cells (Fig 6A) Aurora A was localized to both spindle poles, with staining observed along the microtubules close to the poles. Aurora A staining was similar after treatment with any of the microtubule stabilizers. Aurora A was associated with each of the spindle asters with brighter staining apparent at 2 asters (Fig 6A), which were shown to co-localize with λ-tubulin at the centrosome (Fig 6B). While each aster contained Aurora A, there was more Aurora A associated with the λ-tubulin containing spindle asters (Fig 6B).

The effects of the drugs on the localization of TPX2, which targets Aurora A to microtubules, were evaluated. In vehicle-treated cells, TPX2 was localized along the microtubules of the mitotic spindle (Fig 6C). Aurora A was also co-localized with TPX2 only close to the centrosome. Surprisingly, in taccalonolide AJ, laulimalide and paclitaxel-treated cells, unlike Aurora A localization, TPX2 was equally localized to each mitotic aster (Fig 6C). This was confirmed by co-staining TPX2 and Aurora A (Fig 6D). The differential localization of TPX2 and Aurora A in response to the microtubule stabilizers highlights some of the similarities shared among these agents. These results also suggest a constant

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difference between centrosomal nucleated asters and those that lack normal centrosomal components. Even though the expression and activation of central mitotic proteins were differentially affected the localization patterns did not differ between the stabilizers.

4. Discussion

The ability of microtubule stabilizers to inhibit mitosis leading to initiation of apoptosis has been known for decades. However, the molecular mechanisms leading from microtubule stabilizer-initiated suppression of microtubule dynamics to initiation of apoptosis are still not well defined. In this study we begin to elucidate the signaling defects initiated by 3 chemically diverse microtubule stabilizers. This is early work highlighting the similarities and differences in the ability of taccalonolide AJ, laulimalide or paclitaxel to alter the expression, activation and appropriate localization of central mitotic kinases including Plk1, Aurora A and TPX2. Additionally, our data show that these compounds cause very different effects on the mitotic signaling cascades that we suggest contribute to the distinct aberrant mitotic spindle structures.

Unlike paclitaxel and laulimalide, the majority of cells treated with taccalonolide AJ failed to complete centrosome separation. It is possible that the lower levels of Plk1 and the 3 h delay in Plk1 phosphorylation caused by taccalonolide AJ contributes to centrosome separation and disjunction failures. This is consistent with studies that have shown that inhibition of Plk1 prevents centrosomal targeting of Eg5 and activation of Nek2 (20, 21). Nek2 activation is necessary for the phosphorylation of its target linker proteins, C-Nap1 and Rootletin, which causes their displacement from the centrosome thus allowing centrosome disjunction (22–25). Plk1 and Eg5 regulate centrosome separation and either pathway can compensate for the other (20). The high Eg5 activity observed in the taccalonolide AJ-treated cells may be a mechanism by which cells are attempting to overcome the lack of normal Plk1 activity. However, the increased levels of phosphorylated Eg5 caused by taccalonolide AJ were unexpected considering the centrosomal phenotypes we observed, because high Eg5 activity can support centrosome separation even in the absence of Plk1 activity (20). Our results suggest that the multiple defects initiated by taccalonolide AJ cannot be overcome to allow centrosome separation and disjunction. Taccalonolide AJ is the only stabilizer of the three that causes dramatic interphase microtubule bundling at the minimum concentration that causes full G_2/M arrest (Fig 3A, C). A role for microtubules in centrosome separation has been established (20) and it is possible that the microtubule stabilization initiated by taccalonolide AJ contributes to inhibition of centrosome separation. We have evidence of this because higher concentrations of paclitaxel or laulimalide that initiate microtubule bundling also cause more centrosome separation defects (Fig 2F).

Diverse mitotic phenotypes have been observed with different concentrations of paclitaxel (26). An important question to address was whether the severe defects in centrosome separation observed with low concentrations of taccalonolide AJ could also be obtained with higher concentrations of laulimalide and paclitaxel. Our results with laulimalide and paclitaxel, at concentrations from 50–500 nM, show a dose dependent increase in centrosome separation failure (Fig 2F) and a shift in mitotic aster morphology to closely resemble the phenotypes seen with the lower, G_2/M arresting concentrations of taccalonolide AJ. In contrast, higher concentrations of taccalonolide AJ did not change the incidence of centrosome separation defects, highlighting the fact that the taccalonolides produce these effects at much lower relative concentrations than paclitaxel or laulimalide. It is interesting to speculate that these concentration dependent differences represent a continuum of effects related to the ability of these microtubule stabilizers to disrupt normal microtubule structures. Our previous studies showed that the taccalonolides caused

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microtubule bundling defects at low, antiproliferative concentrations and that higher relative concentrations of paclitaxel are needed to initiate these effects (9). Therefore, the differences in centrosome separation and spindle aster defects observed among the drugs at the G_2/M arrest concentrations might relate to whether they cause microtubule reorganization and are not unique to one class of microtubule stabilizers. In contrast, no change in pericentrin localization to the additional asters was seen with higher concentrations of either paclitaxel or laulimalide even though the spindle asters resembled taccalonolide AJ induced asters. This suggests that the taccalonolides are unique in their ability to alter centrosome integrity.

One mechanism by which multiple spindle asters form is through centrosome fragmentation. If this were the case the multipolar asters would be expected to contain normal centrosomal components. This was not the case in cells treated with taccalonolide AJ, paclitaxel or laulimalide, suggesting that these asters are not formed by centrosome fragmentation. However, taccalonolide AJ was unique among the stabilizers evaluated in that it caused aberrant spindle asters that all contained pericentrin but lacked additional centrosomal components. Phosphorylation of pericentrin by Plk1 leads to the recruitment of γ-tubulin ring complexes to the centrosomes (27). Our data suggest that while pericentrin localizes to each aberrant aster, it is not likely that Plk1 is phosphorylating it because of the lack of additional centrosomal components at these aberrant asters. This is consistent with the delayed Plk1 expression and activation caused by taccalonolide AJ.

Our results show that taccalonolide AJ, paclitaxel and laulimalide cause distinct changes in the expression of Aurora A and are consistent with their effects on Plk1 and TPX2 expression. In addition to being mitotic cofactors, studies show that Aurora A can directly phosphorylate and activate Plk1 (28). The pattern of Aurora A expression in laulimalide-treated cells matches that seen with Plk1 and TPX2. Moreover, the early activation of Aurora A caused by laulimalide is consistent with the early phosphorylation of Plk1 observed, suggesting appropriate co-localization of these kinases. The activation of Plk1 was most delayed in paclitaxel-treated cells, consistent with late expression and activation of Aurora A, and also the possibility of inappropriate co-localization. In contrast, taccalonolide AJ did not dramatically change the timing of Aurora A expression. Not unexpectedly, with taccalonolide AJ the levels and timing of Plk1 phosphorylation mirrors the activation of Aurora A. The coordinated timing of Aurora A expression and Plk1 activation suggests that they are localized together.

Aurora A amplification has been shown to confer resistance to paclitaxel and studies have shown that its down regulation can sensitize cells to paclitaxel treatment (29). Our data suggest that one of the mechanisms by which paclitaxel inhibits mitotic progression is mediated by inhibition of Aurora A expression and activation. This provides a mechanism by which overexpression of Aurora A can confer resistance to paclitaxel. Based on our results it is interesting to speculate that Aurora A amplification might not lead to resistance to laulimalide or taccalonolide AJ, but further studies will be needed to evaluate this possibility.

Tacc3 recruits XMAP215 to the spindle, which aids in the stabilization and focusing of the mitotic spindle (30, 31). Consistent with a reduction in Aurora A levels, a delay and reduction in the levels of phospho-Tacc3 was also observed with paclitaxel. This could result in an inability to focus paclitaxel-induced aberrant asters, leading to diffuse asters. In contrast, both taccalonolide AJ and laulimalide caused much higher levels of phospho-Tacc3 and mitotic spindle asters that are much more focused and compact. It is possible that the increased phospho-Tacc3 facilitates maintenance and focusing of mitotic asters. Additionally, recent studies demonstrated a kinase-independent role for Aurora A in the formation and stabilization of centrosome-independent spindle asters (32). It is possible that

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the laulimalide-induced increase in total Aurora A levels contributes to the distinct mitotic structures observed with laulimalide. In taccalonolide AJ-treated cells, and more so with paclitaxel, the delay in expression of Aurora A might contribute to the formation of different mitotic aster structures.

Various studies have shown that in the absence of centrosomes TPX2, Aurora A and another microtubule associated protein, NuMA, can induce the formation of mitotic spindle poles in the vicinity of the chromosomes (33, 34). Our data show that TPX2 and Aurora A are present in each spindle aster formed by all three of the microtubule stabilizers (Fig 6). This is consistent with their established role in acentrosomal spindle formation and suggests a shared mechanism among these microtubule stabilizers. Taccalonolide AJ is unique, however, in that pericentrin is also seen localized to each taccalonolide AJ-induced spindle aster. We propose that the presence of the combination of TPX2, Aurora A and pericentrin leads to the maintenance and stability of the highly focused spindle asters that occur only with taccalonolide AJ.

Another possibility for the differences observed in both number and morphology of the mitotic spindle asters generated in response to laulimalide and paclitaxel at the G2/M arrest concentration may be related to their distinct and non-overlapping binding sites on tubulin. Recent studies show that while paclitaxel stabilizes longitudinal interactions between α- and β-tubulin dimers, laulimalide can strongly stabilize the lateral interactions between adjacent protofilaments (3). Additionally this study showed that overall, microtubule stabilizers strongly stabilize specific sites on tubulin that have been shown to overlap with the binding sites of a variety of microtubule associated proteins and motors (3). The potential for a distinct binding site for taccalonolide AJ could also lead to differential effects on microtubules, leading ultimately to distinct spindle phenotypes at the G2/M arrest concentration.

In this study we begin to lay a framework that shows that chemically diverse microtubule stabilizers alter mitotic signaling pathways in different ways. While microtubule stabilizers have been used in the clinic for over two decades, it is not yet possible to predict which patients will respond. In fact, studies have shown that there is variability not only among different cancer cell types but also within cells of the cell line or tumor (35, 36). While it is not yet possible to identify definitive biomarkers for patient response to these agents, this work begins to outline which pathways are differentially affected by these compounds. Further work on these findings could identify proteins that confer sensitivity or resistance and potentially new combinations of agents that could provide synergistic anticancer effects.

Acknowledgments

Grant support from the National Institutes of Health, National Cancer Institute CA121138 and CA121138S and the CTRC Cancer Center Support grant CA054174 are gratefully acknowledged. We would also like to thank Dr. April Risinger for her thoughtful suggestions and critical discussions of the data.

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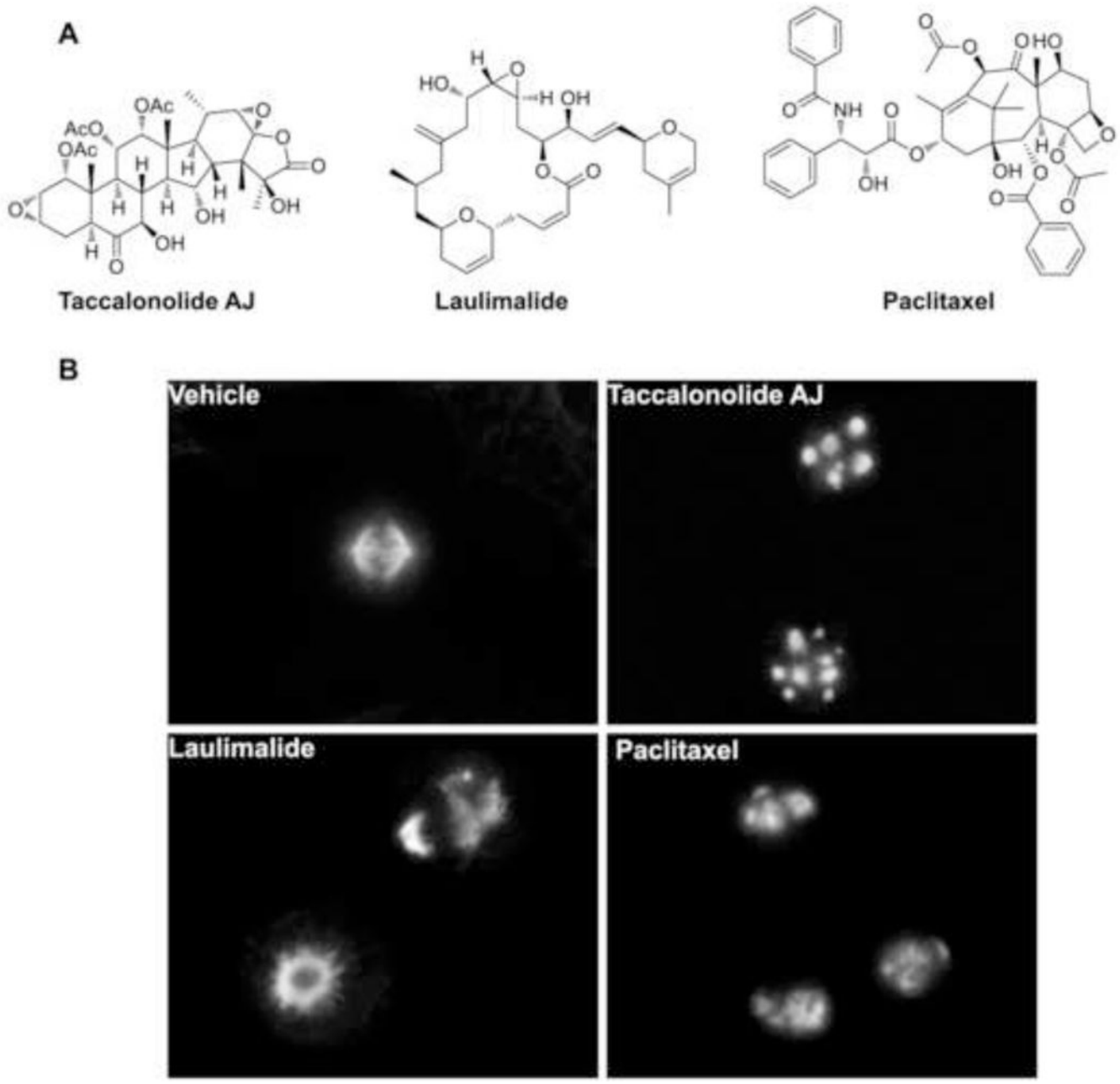

Figure 1. Mitotic spindle defects caused by chemically distinct microtubule stabilizers

(A) Chemical structures of microtubule stabilizers used in this study. (B) HeLa cells were treated with vehicle (ethanol), taccalonolide AJ, laulimalide, or paclitaxel for 18 hours.

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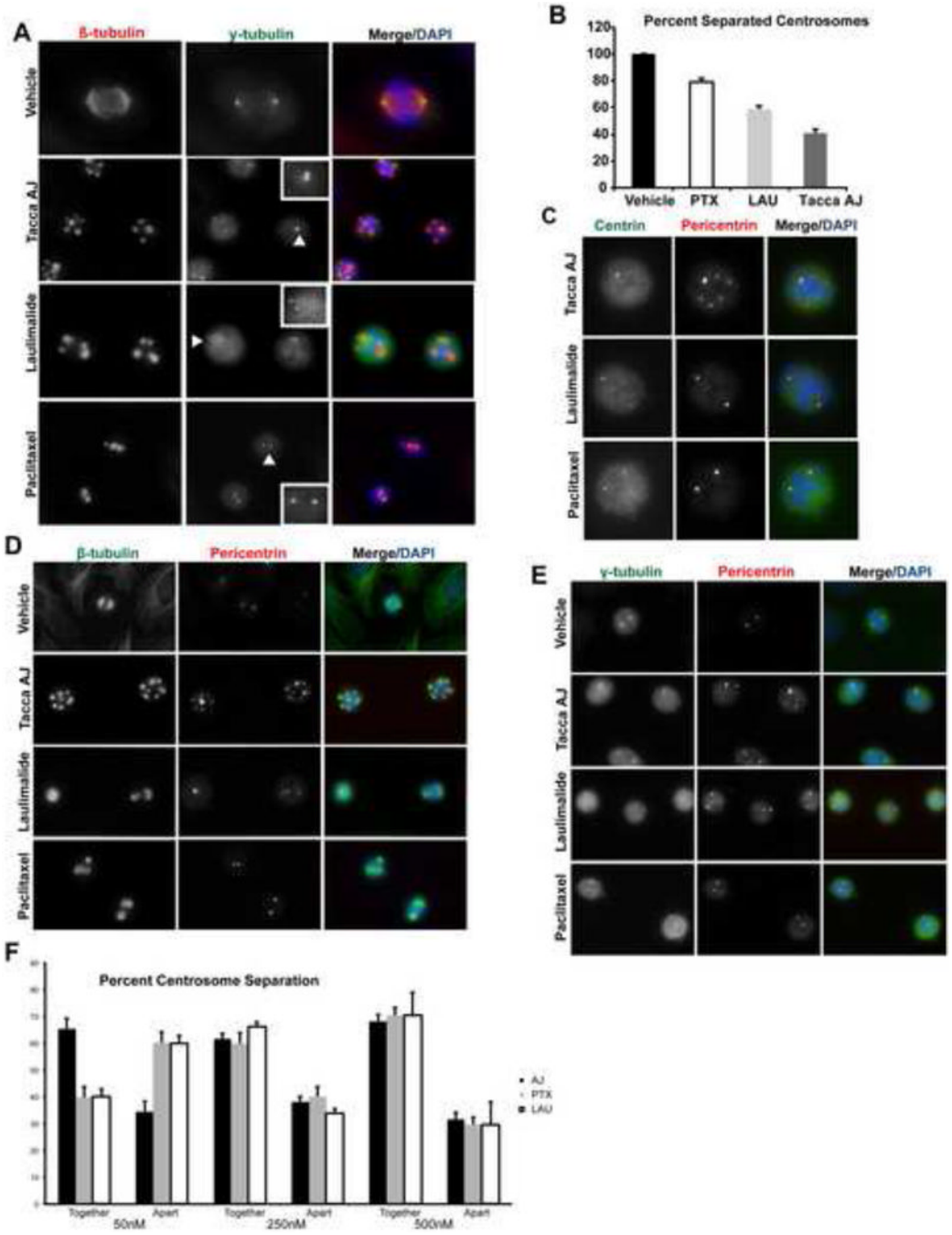

Figure 2. Taccalonolide AJ causes centrosomal defects

HeLa cells were treated with vehicle, taccalonolide AJ, laulimalide or paclitaxel for 18 h. (A) Co-localization of β-tubulin, γ-tubulin and pericentrin. White arrows indicate cells expanded in the inset. (B) Quantification of (A). Mitotic cells were counted and scored as containing either separated or unseparated centrosomes. Each bar graph represents the mean from an average of at least 3 independent experiments with standard error of the mean. (C) Co-localization of pericentrin and centrin. (D) Co-localization of pericentrin and mitotic asters. (E) Co-localization of pericentrin and γ-tubulin. (F) Quantification as in (B) of centrosome separation in cells treated with a range of concentrations of taccalonolide AJ, paclitaxel or laulimalide.

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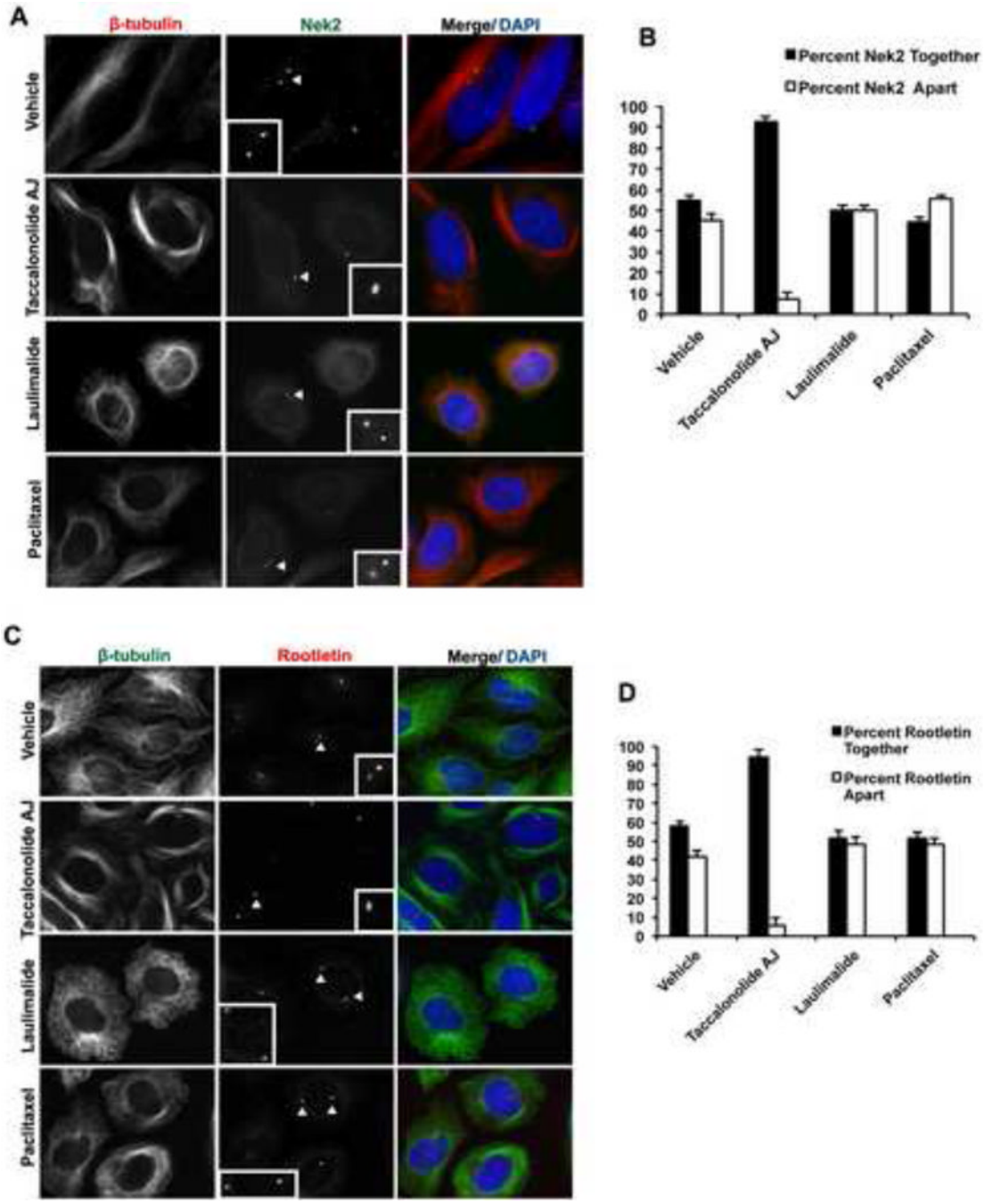

Figure 3. Taccalonolide AJ causes defects in centrosome disjunction

HeLa cells were treated with vehicle, taccalonolide AJ, laulimalide or paclitaxel for 18 h and pertinent structures visualized by immunofluorescence. (A) Localization of microtubules and Nek2. (B) Localization of microtubules and Rootletin (C and D). Quantitation of data shown in (A and B). Data is from an average of at least 3 independent experiments.

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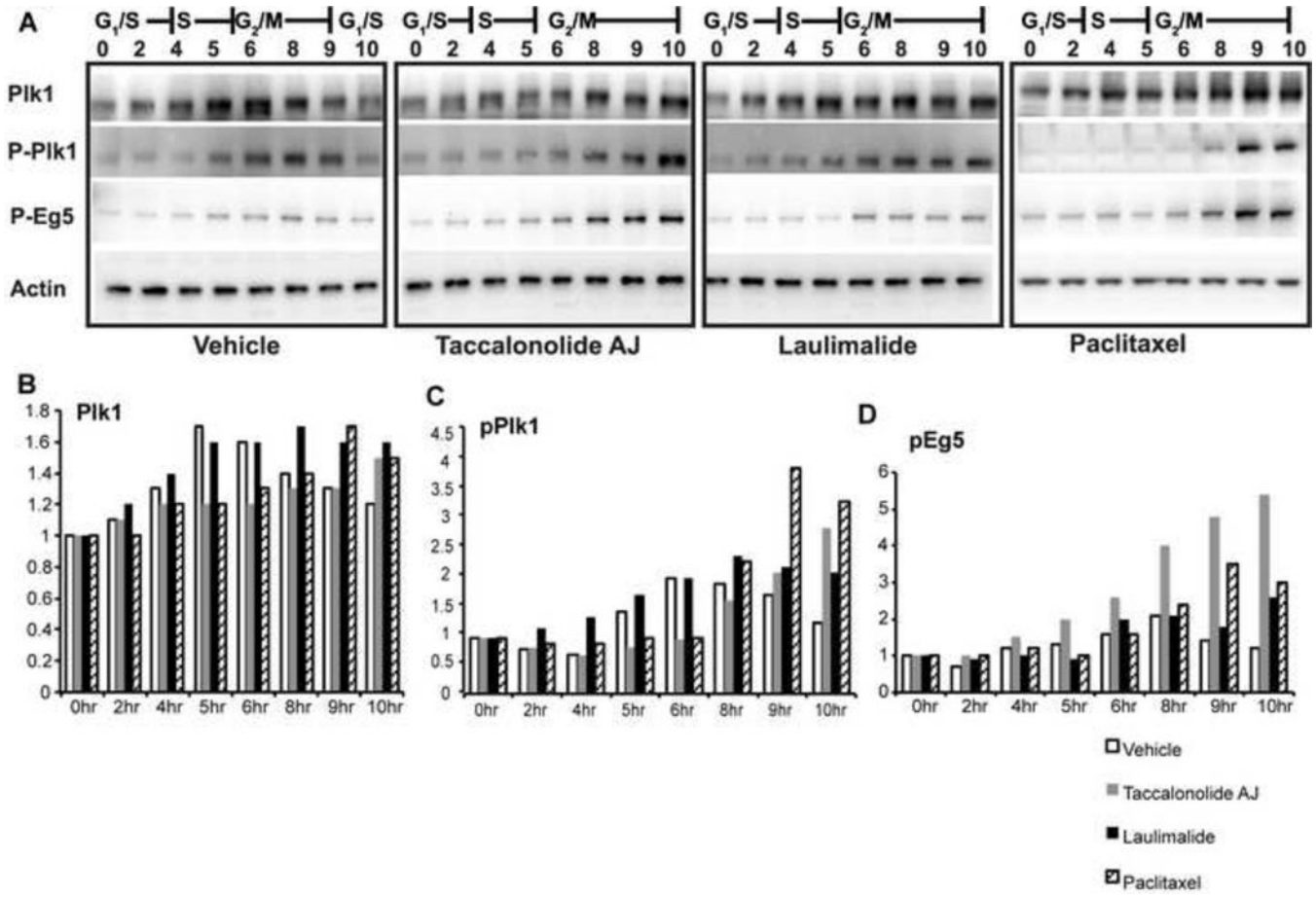

Figure 4. Microtubule stabilizers cause aberrant expression and activation of Plk1 and Eg5

(A) The effects of taccalonolide AJ, laulimalide and paclitaxel on the expression and phosphorylation of Plk1 and Eg5 were evaluated by preparing whole cell lysates of synchronized HeLa cells harvested 2–10 h after release. Cell cycle phase as determined by DNA content by flow cytometry is also depicted. (B) Quantitation of western blots in (A). Proteins were quantified by densitometry using GeneTools software. Relative values were obtained by dividing each value by the time 0 time point.

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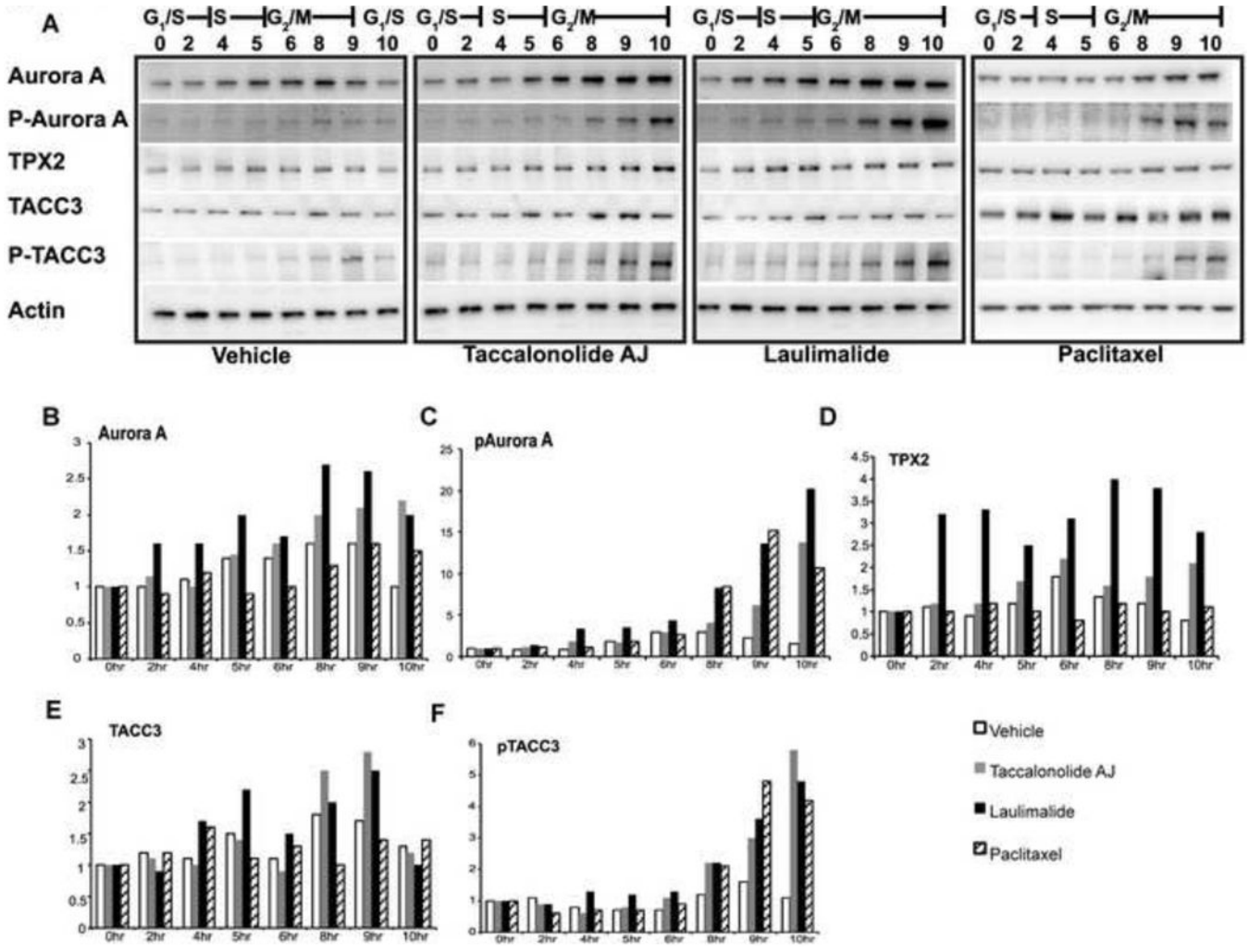

Figure 5. Effects of microtubule stabilizers on Aurora A and its interacting proteins

(A) The effects of taccalonolide AJ, laulimalide and paclitaxel on the expression and activation of Aurora A were evaluated by preparing whole cell lysates of synchronized HeLa cells harvested 2–10 h after release. Cell cycle phase as determined by DNA content using flow cytometry is depicted. (B) Quantitation of western blots in (A). Proteins were quantified by densitometry using GeneTools software. Relative values were obtained by dividing each value by the time 0 time point.

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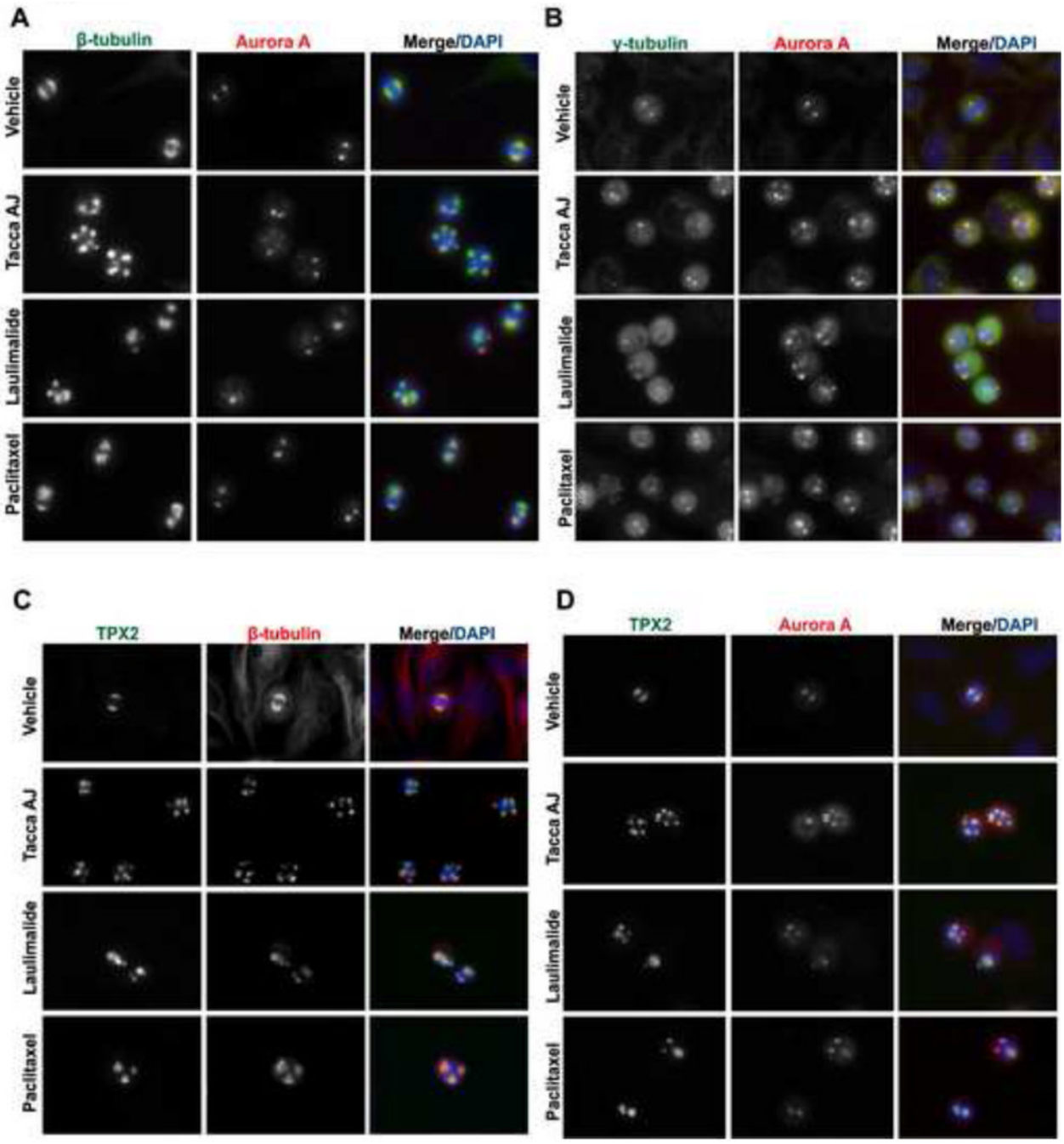

Figure 6. Microtubule stabilizers cause differential localization of TPX2 and Aurora A

HeLa cells were treated with vehicle, taccalonolide AJ, laulimalide or paclitaxel for 18 h. Representative images of cells in mitosis were taken evaluating the localization of β-tubulin, TPX2, γ-tubulin and Aurora A. DNA was visualized by DAPI staining. (A) Co-localization of Aurora A with microtubules. (B) Co-localization of Aurora A with γ-tubulin at the centrosome. (C) Co-localization of TPX2 with microtubules. (D) Co-localization of TPX2 and Aurora A.