Juglone Inactivates Cysteine-rich Proteins Required for Progression through Mitosis*

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The parvulin peptidyl-prolyl isomerase Pin1 catalyzes cis-trans isomerization of p(S/T)–P bonds and might alter conformation and function of client proteins. Since the trans conformation of p(S/T)–P bonds is preferred by protein phosphatase 2A (PP2A), Pin1 may facilitate PP2A-mediated dephosphorylation. Juglone irreversibly inhibits parvulins and is often used to study the function of Pin1 in vivo. The drug prevents dephosphorylation of mitotic phosphoproteins, perhaps because they bind Pin1 and are dephosphorylated by PP2A. We show here however that juglone inhibited post-mitotic dephosphorylation and the exit of mitosis, independent of Pin1. This effect involved covalent modification of sulfhydryl groups in proteins essential for metaphase/anaphase transition. Particularly cytoplasmic proteins with a high cysteine content were vulnerable to the drug. Alkylation of sulfhydryl groups altered the conformation of such proteins, as evidenced by the disappearance of antibody epitopes on tubulin and the mitotic checkpoint component BubR1. The latter activates the anaphase-promoting complex/cyclosome, which degrades regulatory proteins, such as cyclin B1 and securins, and is required for mitotic exit. Indeed, juglone-treated cells failed to assemble a mitotic spindle, which correlated with perturbed microtubule dynamics, loss of immunodetectable tubulin, and formation of tubulin aggregates. Juglone also prevented degradation of cyclin B1, independently of the Mps1-controlled mitotic spindle checkpoint. Since juglone affected cell cycle progression at several levels, more specific drugs need to be developed for studies of Pin1 function in vivo.

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Juglone (5-hydroxy-1,4-naphthalenedione) is a benzoquinone that covalently modifies thiol groups of cysteine residues in parvulin, one of which is essential for PPIase activity (4). It is thought that the inhibition of isomerase activity by juglone is caused by partial unfolding of the PPIase active site (4). Although juglone can inhibit other proteins (9–11), it is frequently used to explore the relevance of Pin1 function in vivo (12–14), especially since it often phenocopies effects of Pin1 dominant negative mutants or Pin1 knockdown. For instance, PP2A-mediated dephosphorylation of the Pin1-interacting proteins, Raf-1, Cdc25c, Pim-1, Myc, and Tau, critically relies on Pin1 (15–18). In line with this, juglone prevents the dephosphorylation of MPM2 antigens (19), which constitute a subset of Pin1-interacting mitotic phosphoproteins, as well as of NHERF-1 (20) and Disabled-2 (21). For that matter, it is thought that cis-trans isomerization of p(S/T)–P bonds by Pin1 regulates dephosphorylation of PP2A targets by facilitating the accessibility of this phosphatase to its substrates (see Ref. 8).

Several Rab GTPases, including endosomal Rab4a, are phosphorylated by Cdk1 on S/T-P sites within their hypervariable region (22, 23). Rabs are key regulators of membrane traffic (see Ref. 24), and their phosphorylation at the onset of mitosis might
be important in the concomitant down-regulation of intracellular transport (22, 23). We previously found that phosphorylated Rab4a binds Pin1 in mitotic cells (25) and that PP2A dephosphorylates Rab4a when cells exit prometaphase.3 During the analysis of Pin1 function in dephosphorylation of Rab4a, we also employed juglone and made a number of unanticipated observations on the target of the drug. Here we show that treatment of mitotic cells with juglone prevented postmitotic dephosphorylation via pathways that did not involve Pin1. Juglone appears to cause this effect by alkylating sulfhydryl groups in proteins critical for metaphase-anaphase transition, which precludes mitotic exit.

EXPERIMENTAL PROCEDURES

Reagents and Materials—An antibody against phospho-Ser204–Rab4a (Rab4apS204)) was raised in rabbits with the keyhole limpet hemocyanin-coupled CRQLRpsPRRTQAPN peptide (where pS represents phosphoserine). Rabbit polyclonal antibodies against human Rab4a and human Pin1 have been described (23, 25). Other antibodies were from the indicated sources: mouse monoclonal MP2 and BubR1 and rabbit antibodies against the catalytic subunit of PP2A (PP2Ac) and methylated PP2Ac (Upstate Cell Signaling Solutions), mouse monoclonal anti-β-catenin (BD Transduction Laboratories), mouse monoclonal anti-α- and anti-β-tubulin (Sigma), and mouse monoclonal cyclin B1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). HRP-labeled and fluoroescently labeled secondary antibodies were purchased from Pierce, Jackson Laboratories, and Molecular Probes. Purified protein phosphatase PP2A1 was from Upstate Cell Signaling Solutions, SP600125 was from Sigma, and Alexa488-Annexin V and propidium iodide were from Molecular Probes.

Cell Lines and Synchronization—The CHO-Rab4a cell line (23) and mouse embryonic fibroblasts (MEFs) from Pin1−/− mice (26) were described in the references indicated. Spontaneously immortalized Pin1−/− MEFs were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin and transfected with pFRSV–Rab4a (23) using calcium phosphate. Clones were suspended at a density of 106 cells/ml in 10 mM Hepes, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2. Alexa488–Annexin V and propidium iodide were added for 15 min at room temperature according to the manufacturer’s instructions. Samples were immediately analyzed on a FACS Vantage SE cell sorter (Becton Dickinson) using the 488-nm laser. Results were quantitated in Cellquest. Pin1−/− MEFs were treated for 15 h with increasing concentrations of juglone with 1 μM juglone. Cells were fixed in ice-cold methanol and subsequently processed for indirect immunofluorescence microscopy. Cells were labeled for α-tubulin and stained with Alexa488-conjugated anti-mouse IgG. DNA was visualized with 4’,6-diamidino-2-phenylindole. Coverslips were mounted and dried, and cells were viewed with a Zeiss LSM5 confocal microscope.

Flow Cytometry—Interphase CHO cells were treated with increasing concentrations of juglone for 180 min. Cells were harvested, washed in phosphate-buffered saline, and resuspended at a density of ~1 × 107 cells/ml in 10 mM Hepes, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2. Alexa488–Annexin V and propidium iodide were added for 15 min at room temperature according to the manufacturer’s instructions. Samples were immediately analyzed on a FACSVantage SE cell sorter (Becton Dickinson) using the 488-nm laser. Results were quantitated in Cellquest. Pin1−/− MEFs were treated with increasing concentrations of juglone. During the last hour, Hoechst 33342 (10 μg/ml) was added to stain DNA. Cells were trypsinized, and cell cycle profiles were obtained by cytometric analysis on a FACSVantage SE cell sorter using the UV laser. The number of cells in different cell cycle phases (M1–M4) was evaluated in Cellquest.

RESULTS

Rab4a is phosphorylated during mitosis by Cdk1 on Ser204 (23) and dephosphorylated by PP2A when cells exit prometaphase.4 We here employed the parvinulin PPlase inhibitor

3 C. Fila and P. van der Sluijs, unpublished results.

4 C. Fila and P. van der Sluijs, manuscript in preparation.
juglone initially, to analyze a possible role of Pin1 in the dephosphorylation of Ser\textsuperscript{204}.

**Juglone Titration on CHO Cells—** Because high concentrations of juglone affect cell viability (27, 28), we performed a titration study to establish an optimal concentration of the inhibitor. CHO cells were incubated with increasing concentrations of juglone and stained with Alexa\textsuperscript{488}-Annexin V to assess the extent of apoptosis and with propidium iodide to distinguish between living and dead cells by fluorescence-activated cell sorting analysis. As shown in the bar diagram in Fig. 1A, less than 10 µM juglone did not cause apoptosis (R2 and R3) or necrosis (R1 and R2), compared with controls. Concentrations above 10 µM increased the number of both apoptotic and necrotic cells. Most of the Annexin V-labeled cells were also positive for propidium iodide, showing that the majority had already entered late stages of apoptosis. In the subsequent *in vivo* experiments in CHO cells, we used 7.5 µM juglone, since this concentration did not induce apoptosis or cell death and is close to the minimal concentration that inactivates parvulins *in vitro* (4).

**Juglone Prevents Postmitotic Dephosphorylation of Rab4a—** Mitotic CHO-Rab4a cells were released from prometaphase arrest and incubated in the presence of 7.5 µM juglone, since this concentration did not induce apoptosis or cell death and is close to the minimal concentration that inactivates parvulins *in vitro* (4).

**FIGURE 1. Juglone inhibits postmitotic dephosphorylation of Rab4a.** A, interphase CHO cells were treated for 3 h with increasing concentrations of juglone. Binding of Alexa\textsuperscript{488}-annexin V (A488-annexin V) and permeability to propidium iodide was analyzed by fluorescence-activated cell sorting. Density dot plots were used to quantitate the ratio apoptotic/Annexin V-positive (R2 and R3) and dead/propidium iodide-positive (R1 and R2) as presented in the bar diagram. B, Interphase (I) and mitotic (M) CHO-Rab4a transfectants were incubated with 7.5 µM juglone (+) or solvent (–). Lysates were prepared at the indicated periods of time after nocodazole (noc) washout and were analyzed by Western blot with antibodies against Rab4a(pS204) and Rab4a. α-Tubulin served as loading control. C, MPM2 antigens were detected in detergent lysates of juglone-treated interphase as well as mitotic cells taken 0 and 180 min after nocodazole washout.
The entire set of MPM2 antigens was dephosphorylated after 180 min of nocodazole washout in control cells (Fig. 1). This indicates an initial level. We therefore concluded that juglone prevented dephosphorylation of mitotic phosphoproteins, including Rab4a. Similar results were reported for Disabled-2, NHERF-1, and MPM2 antigens and proposed to be caused by inhibition of PP2A-mediated dephosphorylation due to loss of Pin1 function (19–21).

**Juglone Does Not Inhibit PP2A**—PP2A is a heterotrimer consisting of a scaffolding (A), a regulatory (B), and a catalytic (C) subunit (30). Since juglone is known to inhibit proteins other than just parvulins (9–11), and since naphtoquinones inactivate protein-tyrosine phosphatases (31), we first investigated whether juglone inhibited PP2A directly and if postmitotic dephosphorylation of Rab4a is unlikely to be due to inhibition of the phosphatase.

**Juglone Blocks Postmitotic Dephosphorylation in Pin1−/− Cells**—Having shown that inhibited dephosphorylation of mitotic phosphoproteins (Fig. 1, B and C) is not caused by a direct effect of juglone on PP2A (Fig. 2, A and B), we next investigated whether the block was due to inhibition of Pin1. For that matter, we studied dephosphorylation of mitotically phosphorylated Rab4a in MEFs derived from Pin1−/− mice (26). The cells lacked immunologically detectable Pin1, as evidenced by a Western blot with a polyclonal antibody against Pin1 (Fig. S1). As a consequence of Pin1 deficiency, the cells also contained ~40% less of the Pin1 client protein β-catenin (Fig. S1), whose stability correlates directly with Pin1 expression levels (32). The reduced β-catenin levels in Pin1−/− MEFs also suggested that the cells do not have salvage mechanisms to counteract the loss of Pin1 activity. Pin1−/− MEFs were next released from the mitotic block and incubated in the presence of 7.5 μM juglone for up to 60 min. The cells were then harvested at different periods of time after nocodazole washout, and detergent lysates were analyzed for the amounts of Rab4a(pS204) and total Rab4a (Fig. 3). Even in a genetic model for the loss of Pin1 function, juglone inhibited the dephosphorylation of Rab4a in the Pin1−/− MEFs (Fig. 3). These results showed that the effect of juglone is not caused by a mechanism involving Pin1.

**Juglone Decreased Tubulin Content in Lysates**—Careful analysis of the experiments with the Pin1−/− cells revealed an additional effect of juglone treatment. We found that the amount of α- and β-tubulin decreased during the release of the mitotic block in the presence of juglone (Fig. 3). This observation was also made in interphase cells that were treated for 60 min with juglone, documenting that the cell cycle stage was immaterial to this effect of the drug. The disappearance of α- and β-tubulin was not caused by leakage of cytosolic proteins, because actin levels remained the same throughout the experiment. To account for the loss of α- and β-tubulin from the cigarette
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**FIGURE 4. Juglone causes tubulin aggregation.** CHO cells (A) or Pin1−/− MEFs (B) were released from mitotic arrest by washout of nocodazole (noc) and incubated for 60 min with increasing amounts of juglone. Lysates were analyzed for Rab4a(pS204), α-tubulin, actin, and MPM2 antigens.

Although 7.5 μM juglone caused aggregation of tubulin in Pin1−/− MEFs, we did not observe this in CHO cells (Fig. 1B). To investigate whether or not the effect on the Triton X-100 solubility of tubulin (from now on called solubility) was restricted to Pin1−/− MEFs, we performed juglone titrations on CHO cells and Pin1−/− MEFs. Nocodazole-arrested mitotic cells were harvested and incubated for 60 min in medium containing increasing juglone concentrations. Cell lysates were then analyzed for Rab4a(pS204), MPM2 antigens, α-tubulin, and actin. As shown in Fig. 4A, juglone concentrations above 2 μM inhibited dephosphorylation of Rab4a and MPM2 antigens in CHO cells. The amount of soluble α-tubulin only started to decrease at juglone concentrations above 10 μM. The initial experiments with CHO cells in which we found inhibited post-mitotic dephosphorylation (Fig. 1B) were done with 7.5 μM, which did not affect tubulin solubility (Fig. 4A). In Pin1−/− MEFs, the dephosphorylation of phospho-Rab4a was already inhibited by as little as 0.1 μM juglone (Fig. 4B), whereas tubulin became insoluble at juglone concentrations above 2 μM (Fig. 4B). Thus, the dose-dependent effects of the Pin1 inhibitor on dephosphorylation of mitotic phosphoproteins and tubulins were not limited to Pin1−/− MEFs but were also recapitulated in another cell line. The experiments in Figs. 2 and 4 also showed that dephosphorylation of mitotic phosphoproteins is a more sensitive read-out than the generation of insoluble tubulin.

Juglone Affects Tubulin Function via Alkylation of SH Groups—Juglone can covalently modify free SH groups of cysteine residues, which is the basis for the inactivation of Pin1 (4) and probably of other proteins (33). To examine if the effects of juglone on tubulin and dephosphorylation of mitotic phosphoproteins are caused by such reactivity, we incubated mitotic Pin1−/− MEFs with increasing concentrations of juglone in the absence or presence of an excess of l-cysteine. In Pin1−/− MEFs, the dephosphorylation of phospho-Rab4a was already inhibited by as little as 0.1 μM juglone (Fig. 4B), whereas tubulin became insoluble at juglone concentrations above 2 μM (Fig. 4B). Thus, the dose-dependent effects of the Pin1 inhibitor on dephosphorylation of mitotic phosphoproteins and tubulins were not limited to Pin1−/− MEFs but were also recapitulated in another cell line. The experiments in Figs. 2 and 4 also showed that dephosphorylation of mitotic phosphoproteins is a more sensitive read-out than the generation of insoluble tubulin.

**FIGURE 5. Role of SH groups in tubulin aggregation.** A, Pin1−/− MEFs were released from mitotic block in nocodazole (noc)-free medium and were incubated with increasing amounts of juglone in the presence of l-cysteine. Lysates were analyzed for Rab4a(pS204), Rab4a, and α-tubulin by Western blotting. B, polymerization of phosphocellulose-purified brain tubulin in the presence of the indicated reagents was assayed by measuring dynamic light scattering at 340 nm.
The modification and aggregation of tubulin will probably affect its incorporation into microtubules. We therefore performed in vitro polymerization reactions with phosphocellulose-purified brain tubulin to investigate whether or not juglone directly interfered with the assembly of tubulin into microtubules. Highly purified bovine brain tubulin, devoid of microtubule-associated proteins, was resuspended in PIPES buffer in the presence of different concentrations of juglone. Reactions with taxol and colchicine served as controls, since they either stimulate or inhibit tubulin polymerization, respectively (35). As shown in Fig. 5B, 5 μM juglone reduced tubulin polymerization to about 60%, and 10 μM blocked it to nearly the same extent as colchicine. The addition of 5 mM DTT to the reaction containing 10 μM juglone rescued tubulin polymerization and restored it to ~80%. Taken together, these data showed that juglone acted directly on tubulin and inhibited tubulin polymerization through modification of its SH groups.

Given the effects of juglone on tubulin solubility in vivo and the assembly of tubulin polymers, we determined whether microtubule organization is affected by the drug. Pin1−/− MEFs and CHO cells were incubated for 120 min with different concentrations of juglone. Cells were then fixed and labeled for α-tubulin. Low concentrations of juglone that prevented post-mitotic dephosphorylation of Rab4a but did not induce overt tubulin aggregation (1 μM for MEFs and 5 μM for CHO cells) also did not affect the morphology of the microtubule networks in both cell lines (Fig. S2A). Juglone concentrations that cause clear immunodetectable aggregation of tubulin (5 μM for MEFs and 15 μM for CHO) essentially resulted in the complete disassembly of microtubule networks, and a shift toward cytosolic tubulin staining similar to what is seen when cells were treated with the microtubule-depolymerizing drug colchicine (Fig. S2A). Since low concentrations of juglone did not affect morphological integrity of microtubule networks in interphase CHO cells and Pin1−/− MEFs, we extended the incubation period with low juglone concentrations to 12 h and assessed the effect on tubulin solubility in a sedimentation assay. Colchicine was included as control, since it depolymerizes microtubules. Cells were lysed, and α-tubulin was then analyzed by Western blotting of the Triton X-100-soluble/insoluble fractions. In order to detect aggregated α-tubulin in the detergent-insoluble fraction, we loaded 5 times more of this fraction than of the soluble pool. The amount of pelleted α-tubulin increased 2.5-fold in the presence of 1 μM juglone compared with nontreated control cells (Fig. S2B). In the presence of 10 μM juglone, the entire pool of soluble α-tubulin was aggregated (not shown). Although there was clearly a dose-dependent increase in the amount of pelleted α-tubulin, this represented a relatively modest fraction compared with the total pool. In colchicine-treated cells, α-tubulin was only detectable in the supernatant, as might be expected given its microtubule-depolymerizing effect. Polymerized αβ-tubulin in microtubules is in dynamic equilibrium with a soluble pool. Alterations in the concentration of soluble tubulin will therefore affect the equilibrium and indirectly the integrity of microtubules. In addition, it is likely that the small molecule inhibitor will also affect microtubules directly by modifying surface-exposed cysteines in microtubule-tubulin.

Juglone Does Not Arrest Cells in Metaphase—Because juglone treatment disrupted microtubule networks, we also evaluated whether juglone can arrest asynchronously growing cells in metaphase. To this aim, Pin1−/− MEFs were treated for 12 h with different concentrations of the drug, and the appearance of the mitotic marker protein phospho-Rab4a was determined. As controls, we included taxol, nocodazole, and colchicine, which arrest cells in mitosis (35). Unlike the three spindle poisons, juglone treatment of interphase cells failed to generate the Rab4a(pS204) epitope in Pin1−/− MEFs (Fig. 6A) and CHO cells (not shown). The cells were next investigated by flow cytometry to evaluate potential juglone-mediated effects on DNA content. Histograms of cell populations treated with increasing juglone concentrations are shown in Fig. 6B. Up to 5 μM juglone caused a gradual decrease of cells in G1/M phase (M1) and a concomitant increase in the number of cells residing in S phase (M2) (Fig. 6B, table). Higher concentrations also reduced the number of cells in S phase with an accompanying increase of cells with sub-G1 DNA content (M4) (Fig. 6B, table). Thus, low juglone concentrations arrested cells in particular stages of their life cycle, whereas high doses of the drug or long term treatment induce cells to undergo apoptosis. Similar results were obtained with CHO cells that were first synchronized at the G1/S boundary by thymidine and then released into S phase for 2.5 h and finally treated with 7.5 μM juglone for 6 h. Whereas the same treatment with nocodazole allowed for the generation of robust signals of Rab4a(pS204) and MPM2 antibodies (Fig. S3A) as well as a significant increase of cells with 4 N DNA content (Fig. S3B, table), juglone failed to arrest cells efficiently in prometaphase of mitosis. Thus, although juglone and colchicine both disrupted microtubules (Fig. S2), the different sedimentation characteristics of tubulin (Fig. S2B) and the inability of juglone to block cells in metaphase (Fig. 6 and Fig. S3) showed that the two drugs interfere with microtubule function in a distinct manner.

Juglone Inhibits Mitotic Spindle Assembly—Short term treatment with low doses of juglone did not disrupt interphase microtubule networks (Fig. 4C). Such concentrations might however disturb microtubule polymerization under conditions that require higher microtubule dynamics and increased tubulin turnover. For instance, at the onset of mitosis, the catastrophe rate of microtubules changes, because assembly of the mitotic spindle requires shorter and more dynamic microtubules (36). Highly dynamic microtubules might be more susceptible toward reagents that cause subtle alterations in structure and function of tubulin. To pursue this idea, we investigated microtubule rearrangements in Pin1−/− MEFs during exit from mitosis. Pin1−/− MEFs were arrested in prometaphase, released from the mitotic block, and seeded on poly-L-lysine-coated coverslips in the presence of 1 μM juglone. Cells were fixed after different periods of time and labeled for α-tubulin, and DNA was stained with 4′,6-diamidino-2-phenylindole. Control cells that were released from the prometaphase block passed through mitosis, as visualized by the ordered appearance of characteristic mitotic profiles (Fig. 7A and Fig. S4, left column). Initially, the cells contained clearly condensed chromatin and predominantly nonpolymerized tubulin. After 15 min, most of the cells established a spindle.
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apparatus, and chromosomes were aligned in the metaphase plate. After 30 min, cells were in the process of being pulled toward opposite spindle poles and thus had passed the metaphase/anaphase transition. After 60 min, almost all cells displayed early or late stages of cytokinesis; some had already flattened and reestablished a typical interphase microtubule network. In contrast, cells that were treated with juglone (Fig. 7A and Fig. S4, right column) retained a tubulin staining pattern that is typical for prometaphase (cf. time 0) and failed to assemble a mitotic spindle or align chromosomes in the metaphase plate. In agreement with these results, the mitotic index, as quantitated using 4',6-diamidino-2-phenylindole staining of condensed chromatin (Fig. S4) or with phosphohistone 3 labeling (not shown), remained constant at ~90% during juglone treatment. Identical results were found in CHO cells treated with juglone than interphase microtubules.

Juglone Prevents Postmitotic Protein Dephosphorylation in the Absence of Active Spindle Checkpoint Factors—To investigate a direct role of the mitotic spindle checkpoint in the abolished degradation of cyclin B1 and the inhibited dephosphorylation of mitotic phosphoproteins, we examined the activity of spindle checkpoint factors in juglone-treated cells. These represent regulators of the Mad and Bub families (see Ref. 37). In the case of spindle damage or a lack of tension on spindle microtubules, they are activated and recruited to the kinetochore region of the chromatids. The serine/threonine kinase Mps1 (monopolar spindle 1) is an essential upstream regulator whose activity is essential for the initiation of the checkpoint signaling cascade (38) through phosphorylation of other checkpoint proteins, such as Mad2 and Bub1 (see Ref. 37). We treated mitotic Pin1<sup>−/−</sup> MEFs and U2OS cells with either nocodazole or juglone and 10 μM SP600125, a known Mps1 inhibitor (39). The inhibition of Mps1 kinase would allow cells to override the spindle checkpoint and to dephosphorylate mitotic proteins.
also in the presence of a spindle poison (i.e. in the absence of a functional mitotic spindle) (39). As shown in Fig. 8, A and B, nocodazole maintained Pin1−/− MEFs and U2OS cells in prometaphase, as evidenced by the Rab4a(pSer204) and MPM2 signals, respectively. When we added SP600125 in combination with the spindle poison, both Rab4a(pS204) (Fig. 8A) and MPM2 antigens (Fig. 8B) were dephosphorylated. When we repeated the experiment with juglone instead of nocodazole, SP600125, surprisingly, did not affect the phosphorylation status of mitotic phosphoproteins, since neither Rab4a nor MPM2 antigens (Fig. 8B) became dephosphorylated.

We also analyzed U2OS cell lysates for the essential spindle checkpoint kinase BubR1. It is extensively phosphorylated by Cdk1 and Polo-like kinase 1 during mitosis (40), which is easily visualized by a mobility shift on SDS-polyacrylamide gels, as shown in Fig. 8B. The addition of SP600125 clearly diminished the mobility shift of BubR1 in cells that were treated with nocodazole. The effect of SP600125 is due to inhibition of Mps1 and the inactivation of the checkpoint and reflects the rapid dephosphorylation of BubR1. We then analyzed the expression of BubR1 in U2OS cells that were treated with juglone. As shown in the Western blot of Fig. 8B, BubR1 was not detectable anymore, irrespective of whether SP600125 was present or not. BubR1 is a cytoplasmic protein containing 21 cysteine residues, of which the SH groups are expected to be in the free form. This represented a similar situation as tubulin with its high cysteine content, suggesting that juglone also covalently modified the free thiol groups of BubR1 and affected its folding in a normal functional state. Since the extent of MPM2 staining is the same free thiol groups of BubR1 and affected its folding in a normal functional state. Since the extent of MPM2 staining is the same...
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DISCUSSION

We investigated a possible role of Pin1 in the dephosphorylation of mitotic phosphoproteins by PP2A at the end of mitosis. We initially focused on Rab4a, since it is targeted by Cdk1, in complex with Pin1 during mitosis (25), and dephosphorylated when cells exit M phase. In experiments with the Pin1 inhibitor juglone, we found that dephosphorylation of Rab4a and of MPM2 antigens was strongly reduced. Previously, it was shown that juglone inhibits dephosphorylation of Pin1-interacting mitotic phosphoproteins, such as NHERF-1 and Disabled-2, a phenotype that was thought to represent a specific effect of Pin1 inhibition (18–21). This paradigm was largely built on studies in which Pin1 client proteins required the isomerase for dephosphorylation by PP2A during interphase (15–17).

Juglone is a naphthoquinone, a class of organic compounds whose biological effects are largely caused by the formation of reactive oxygen species through redox activation and the covalent modification of free thiols to form thioethers (see Refs. 41 and 42)). To avoid cell death through apoptosis or necrosis during our experiments, we first conducted juglone titration studies with CHO cells to establish an optimal concentration. Therefore, the juglone concentrations used in our study were orders of magnitude below those often used in earlier studies aimed at inhibiting Pin1 in vivo (12–14, 27). Moreover, we treated the cells only for relatively short periods of time with the drug.

Our results clearly showed that juglone-mediated inhibition of mitotic phosphoprotein dephosphorylation (MPM2 antigens) at the end of mitosis is not caused by inhibition of Pin1. Likewise, the drug did not inactivate PP2A or factors involved in the methylation of the catalytic subunit of PP2A. Instead, juglone inhibited the dynamics of microtubules and prohibited the assembly of a mitotic spindle at concentrations that did not cause apoptosis or necrosis. These in vivo findings are consistent with the reported covalent binding of \( \text{p}-\text{benzoquinone derivatives to tubulin in vitro} \) (43, 44).

The lowest juglone concentrations that prevented mitotic exit, however, left the microtubule networks in interphase cells untouched. This strongly suggested that mitotic microtubules are more sensitive toward juglone. Indeed, at the onset of mitosis, the array of long and stable microtubules is replaced by a set of short and fragile microtubules with a faster tubulin turnover (45). This architectural editing is necessary for spindle formation and chromosome segregation (36). In addition to the complete inhibition of mitotic spindle formation or the alignment of chromosomes in the metaphase plate, juglone also inhibited destruction of cyclin B1, the essential cofactor of Cdk1. Both observations suggested that the compromised mitotic exit might be a result of the activation or maintenance of the mitotic spindle checkpoint. However, this idea is not tenable given the data obtained with the Mps1 inhibitor SP600125. If juglone were to maintain the mitotic spindle checkpoint, then inhibition of Mps1 kinase activity should override this and cause dephosphorylation of mitotically phosphorylated proteins. Instead, Rab4a phosphorylation and the amount of MPM2 antigens were the same with or without SP600125, revealing that the effect of juglone on mitotic protein dephosphorylation is not due to the activation/maintenance of the mitotic spindle checkpoint.

During the experiments with SP600125, we uncovered an additional and previously unknown target of juglone that has a function in the orderly progression of the cell cycle. As with tubulin, juglone also caused the disappearance of BubR1 immunoreactivity. BubR1 is an essential kinase of the mitotic checkpoint response and is heavily phosphorylated by Cdk1 and plk1 during mitosis (40). Through interaction with Cdc20, it can negatively regulate the APC/C (46). Tubulin and BubR1 are both cytoplasmic proteins with a high content of cysteine residues whose free SH groups are amenable to alkylation by \( \text{p}-\text{benzoquinones} \). The binding domain of Cdc20 on BubR1 contains 8 Cys residues. It is quite possible that the alkylation of one or more of these SH groups by juglone will also interfere with the normal function of the APC/C degradation machinery.

The abrogated spindle assembly correlated with the modification of SH groups in tubulin that are critical for polymerization into microtubules. Our in vivo findings are in agreement with the covalent binding of \( \text{p}-\text{benzoquinone derivatives to tubulin in vitro} \) (43, 44). Low concentrations of juglone already altered critically cysteines, consistent with the observation that mutation of even a single cysteine in \( \alpha \)-tubulin inhibits its polymerization (47). This modification changes the local charge distribution in peptide side chains and can cause conformational alterations that perturb folding and may induce aggregation. A plausible consequence of these alterations is the disappearance of epitopes that are recognized by specific antibodies, as we found for \( \alpha \)-tubulin and BubR1. Misfolded BubR1 will be unlikely to assemble properly with Mad2 and Bub3 in the mitotic checkpoint complex, since reduced levels of BubR1 dramatically affect mitotic progression (48, 49). Because BubR1 is a pseudosubstrate inhibitor of Cdc20 (50), it would be expected that juglone interferes with the function of APC/C and pleiotropically perturbs progress into anaphase. Another possibility is that juglone directly inhibits the APC/C. This could mimic the activation of the spindle checkpoint and would prevent the destruction of cyclin B1 even in the absence of an active checkpoint, as we found with the Mps1 inhibitor.

Although juglone exhibited some characteristics of a spindle poison, it did not arrest unsynchronized or presynchronized cells in prometaphase of mitosis. In fact, we found that long term treatment with low juglone concentrations caused the accumulation of cells in S phase and a concomitant decrease of cells with a \( \text{N} \) DNA content. Given the effect of juglone on proteins with such distinct functions as Pin1, \( \alpha \)-tubulin, and BubR1, it may also target cell cycle regulators necessary for the \( \text{S/G}2 \) and \( \text{G}2/\text{M} \) transitions. Indeed, it has been reported that juglone (at much higher concentrations than we described in vivo) “freezes” cells in S phase, which was thought to reflect the inhibition of Pin1 (51). The involvement of Pin1 should, however, not be inferred from inhibitor studies with juglone (51, 52), because the effects can be recapitulated in Pin1 \(^{-/} \) cells, as we showed. Moreover, the higher concentrations of juglone that were used to inhibit the \( \text{S/G}2 \) transition can also induce oxidative stress, DNA damage, and subsequent apoptosis (42, 44). Importantly, we only observed an increased number of cells with sub-\( \text{G}1 \) DNA content (Fig. 6B) with
juglone concentrations that were significantly higher than required for the inhibition of protein dephosphorylation and spindle formation.

Juglone-induced tubulin modification might evoke additional downstream effects that could correlate with phenotypes in response to alternative strategies for interfering with Pin1 function. Especially, changes in properties like subcellular localization, cell shape, and intracellular transport can be an indirect consequence of disturbed microtubule networks. In summary, we showed that juglone exerts biological effects that might not only be useful in studying the functional relevance of Pin1 in vivo; they could also provide the therapeutic basis to treat pathological conditions in which Pin1 is aberrantly upregulated, such as cancer (8).

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