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Independent Mechanistic Inhibition of Cdc25 Phosphatases by a Natural Product Caulibugulone

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ABSTRACT

Caulibugulones are novel but poorly characterized cytotoxic isoquinoline quinones and iminoquinones identified in extracts from the marine bryozoan *Caulibugula intermis*. We now report that the caulibugulones are selective in vitro inhibitors of the Cdc25 family of cell cycle-controlling protein phosphatases compared with either human vaccinia H1-related phosphatase (VHR) or tyrosine phosphatase 1B (PTP1B). The in vitro inhibition of Cdc25B by caulibugulone A was irreversible and attenuated by reducing agents or catalase, consistent with direct oxidation of the enzyme by reactive oxygen species. Mechanistically, caulibugulone A directly inhibited cellular Cdc25B activity, generated intracellular reactive oxygen species and arrested cells in both G_1 and G_2/M phases of the cell cycle. Caulibugulone A also caused the selective degradation of Cdc25A protein by a process that was independent of reactive oxygen species production, proteasome activity, and the Chk1 signaling pathway. Instead, caulibugulone A stimulated the phosphorylation and subsequent activation of p38 stress kinase, leading to Cdc25A degradation. Thus, caulibugulone inhibition of cellular Cdc25A and B phosphatases occurred through at least two different mechanisms, leading to pronounced cell cycle arrest.

Caulibugulones A to F are novel secondary metabolites originally extracted from the marine bryozoan *Caulibugula intermis* (Milanowski et al., 2004). The isoquinoline quinones caulibugulones A, B, and C and the isoquinoline iminoquinones caulibugulones E and F caused growth arrest of murine IC-2^{WT} cells with IC_{50} values ranging from 0.03 to 1.67 μg/ml (Milanowski et al., 2004). We previously reported the total syntheses for these naturally occurring compounds and their ability to inhibit Cdc25B in vitro (Alagille et al., 2004; Wipf et al., 2004). Because the mechanisms responsible for caulibugulone-mediated growth arrest were not defined, the goal of this study was to further characterize the mode of action of these natural products.

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Much of our work has been focused on identifying small molecule inhibitors of Cdc25 phosphatases because of their critical role in regulating the cell cycle controlling cyclin dependent kinases (Cdk) (Lyon et al., 2002). All three Cdc25 phosphatases permit G_2/M transition by dephosphorylating and activating the Cdk1/cyclin B complex. Cdc25A also induces transition from G_1 to S phase by dephosphorylation of Cdk2/cyclin E and Cdk2/cyclin A (Hoffmann et al., 1994; Blomberg and Hoffmann, 1999), and both Cdc25B and Cdc25C have been implicated in initiating entry into S phase (Garner-Hamrick and Fisher, 1998; Turowski et al., 2003). Cdc25A and Cdc25B but not Cdc25C are overexpressed in human cancer cells and seem to contribute to oncogenesis (Kristjansdottir and Rudolph, 2004).

Endogenous regulation of Cdc25 phosphatases is also a central feature of mammalian cell cycle checkpoints. Clinically used anticancer agents, such as topoisomerase I inhibitors, topoisomerase II inhibitors, and DNA-damaging agents

ABBREVIATIONS: Cdk, cyclin-dependent kinase; ATM, ataxia telangiectasia mutated; ATR, ATM and Rad-3 related; VHR, vaccinia H1-related phosphatase; PTP1B, tyrosine phosphatase 1B; DA3003-1, 6-chloro-7-(2-morpholin-4-yl-ethylamino)-quinoline-5,8-dione; JUN1111, 7-(2-morpholin-4-yl-ethylamino)-quinoline-5,8-dione; 5169131, 3-benzoyl-naphtho[1,2-*b*]furan-4,5-dione; OMFP, *O*-methyl fluorescein phosphate; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's minimal essential medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; DCF, 2′,7′-dichlorodihydrofluorescein; HA, hemagglutinin; tet, tetracycline; NAC, *N*-acetyl-L-cysteine; SB 203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; ROS, reactive oxygen species; Rb, retinoblastoma protein; siRNA, small interfering RNA; NSC 95397, 2,3-bis-(2-hydroxy-ethylsulfonyl)-[1,4] naphthoquinone.

(UV, ionizing radiation), decrease Cdc25A protein levels leading to cell cycle arrest (Agner et al., 2005). The resulting proteosomal degradation of Cdc25A is accomplished by activation of the ATM/ATR-Chk1/Chk2 cascade and phosphorylation of Cdc25A on key serine residues followed by ubiquitination and degradation of Cdc25A. Phosphorylation of all three of the Cdc25 phosphatases is also important for creating 14-3-3 binding sites to sequester the phosphatases away from their substrates and induce cell cycle arrest (Boutros et al., 2006).

We and others have observed that the most potent small molecule inhibitors of the Cdc25 phosphatases are frequently quinone-derived compounds (Lazo et al., 2001, 2002; Lyon et al., 2002; Brisson et al., 2004; Kristjansdottir and Rudolph, 2004; Brisson et al., 2005; Boutros et al., 2006). We previously hypothesized (Brisson et al., 2005) that quinoid inhibitors could disrupt Cdc25B phosphatase activity by oxidation of the catalytically essential cysteine residue in the enzyme's active site through production of reactive oxygen species. Therefore, in the current study we analyzed this mode of action for the newly synthesized caulibugulone quinones. Caulibugulones A to E were previously shown to be in vitro inhibitors of Cdc25B with IC_{50} values ranging from 2.7 to 32.5 μM, demonstrating specificity for Cdc25B over two other known phosphatases: VHR and PTP1B (Wipf et al., 2004). We now report that caulibugulone A inhibited all human Cdc25 isoforms, generated a modest level of reactive oxygen species in cells, irreversibly inhibited Cdc25B, and caused G_1 and G_2/M phase cell cycle arrest. Surprisingly, caulibugulone A inhibited Cdc25A by a completely separate mechanism, namely, Cdc25A degradation, which relied on the p38 stress kinase but was independent of reactive oxygen species, proteosome activity, and the Chk1 signaling pathway. Depletion of Cdc25A was observed with other quinone Cdc25 inhibitors, suggesting it was a general and possibly important pharmacological phenomenon.

Materials and Methods

Chemicals. The syntheses of caulibugulones A to E, DA3003-1, and JUN1111 were reported previously (Wipf et al., 2004; Brisson et al., 2005). 5169131 was purchased from Chembridge Research Laboratories, Inc. (San Diego, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted.

In Vitro Enzyme Assays. Epitope-tagged $His_6Cdc25A_1$, $His_6Cdc25B_2$, and glutathione transferase-$Cdc25C_1$ were expressed in *Escherichia coli* and purified by nickel-nitrilotriacetic acid (His_6) or glutathione-Sepharose resin as described previously (Lazo et al., 2001). Human recombinant VHR and PTP1B were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Enzyme activities in the absence and presence of inhibitors were measured using the artificial substrate *O*-methyl fluorescein phosphate (OMFP) at concentrations equal to the K_m of each enzyme and at the optimal pH for individual enzyme activity in a 96-well microtiter plate assay based on previously described methods (Lazo et al., 2001). Fluorescence emission from the product was measured after a 20-min (VHR and PTP1B) or 60-min (Cdc25) incubation period at ambient temperature with a multiwell plate reader (Cytofluor II; Applied Biosystems, Foster City, CA; excitation filter, 485-nm/20-nm bandwidth; emission filter, 530-nm/30-nm bandwidth). IC_{50} concentrations were determined using Prism 3.0 (GraphPad Software Inc., San Diego, CA). For reversibility studies with inhibitors, we used a protocol similar to a dilution method described previously (Sohn et al., 2003). $Cdc25B_2$ full-length enzyme (60 mM Tris, 2 mM EDTA, and 150 mM NaCl, pH 8.0) was preincubated with ~3 times the IC_{50} (20 μM caulibugulone A) for 0, 5, or 20 min at room temperature. Separately, the enzyme was also incubated with the DMSO vehicle. After preincubation, the reaction was diluted >10-fold to determine remaining enzyme activity by the above-mentioned phosphatase assay using OMFP, and results were compared with enzyme incubated with the DMSO vehicle.

Cell Cycle Analysis and Measurement of Cellular ROS by Flow Cytometry. tsFT210 cell synchronization and flow cytometry assays were performed as described previously (Osada et al., 1997) using a FACSCalibur flow cytometer (BD Biosciences PharMingen, San Diego, CA). HeLa cells (1×10^6), cultured in Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS), were trypsinized, resuspended in phosphate buffer saline (PBS), and preloaded with 2′,7′-dichlorodihydrofluorescein (DCF) diacetate dye (Invitrogen, Carlsbad, CA). Cells were washed in PBS and resuspended in PBS buffer containing 3 μM propidium iodide. Cells were then treated for 10 min with DMSO, 1 mM H_2O_2, caulibugulone A, or JUN1111. DCF and propidium iodide fluorescence were measured by flow cytometry, and data were analyzed using ModFit LT cell cycle analysis software (Verity Software House, Topsham, ME).

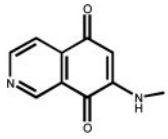
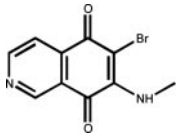
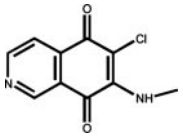
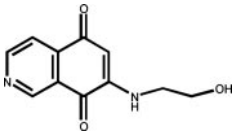
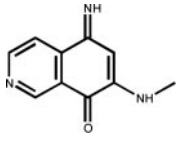
Direct Inhibition of Cdc25B in U2OS Cells. Previously described (Theis-Febvre et al., 2003; Bugler et al., 2006) U2OS cells overexpressing HA-$Cdc25B_3$ under the tetracycline (tet)-repressible promoter (a generous gift from Prof. Bernard Ducommun, Centre National de la Recherche Scientifique, Université Paul Sabatier, Toulouse, France) were maintained in DMEM constituted with 10% FBS, 100 μg/ml G418 (Geneticin; Invitrogen), 1% penicillin-streptomycin, and 2 μg/ml tet. Cells were plated for 23 h with tet to suppress ectopic Cdc25B expression and without tet to stimulate ectopic Cdc25B expression. Cells were then treated with 40 μM etoposide for 1 h followed by addition of 200 ng/ml nocodazole alone or in the presence of 1 to 30 μM caulibugulone A for 23 h. Cells were harvested in ice-cold lysis buffer (50 mM Tris HCl, pH 7.5, containing 250 mM NaCl, 5 mM EDTA, and 0.1% Triton X-100) supplemented with various protease and phosphatase inhibitors. Phosphorylated Histone H3 (Ser10), a well established marker of mitotic arrest (Hendzel et al., 1997), was detected by Western blot using a rabbit polyclonal antibody from Millipore Corporation (Billerica, MA).

Treatment of HeLa Cells and Western Blotting. HeLa cells were cultured in DMEM containing 10% FBS. Cells ($0.5–1 \times 10^6$) were plated in 10-cm dishes and treated with DMSO (vehicle) or with the following conditions: 1 to 1000 μM H_2O_2 for 2 h; 1 to 30 μM quinone (caulibugulone A or E, JUN1111, DA3003-1, or 5169131) for 2 h; pretreatment with 20 mM *N*-acetyl-L-cysteine (NAC) for 2 h followed by addition of caulibugulone A for 2 h; pretreatment with 1, 10, or 20 μM SB 203580, a p38 inhibitor (Calbiochem, San Diego, CA) for 1 h followed by addition of caulibugulone A for 2 h; or pretreatment with 5 μM MG132 for 15 h followed by 30 μM caulibugulone A for 2 h. In some studies, cells were exposed to 60-J/m^2 UV irradiation with or without MG132 or SB 203580 pretreatment. Cycloheximide (25 μg/ml) was added to cells in the presence of DMSO vehicle or 30 μM caulibugulone A for 0 to 30 min for Cdc25A half-life determination. After treatments, cells were lysed in ice-cold lysis buffer as stated above. Cdc25 phosphatase protein levels were detected by Western blotting with the following antibodies: mouse monoclonal anti-Cdc25A (F6; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse monoclonal anti-Cdc25B (BD Biosciences, San Jose, CA), and mouse monoclonal anti-Cdc25C (H-6; Santa Cruz Biotechnology, Inc.). Phospho-Chk1 (Ser345) and phospho-Chk2 (Thr68) antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA). Phospho-p38 (Thr180/Tyr182) and total p38 antibodies were purchased from Cell Signaling Technology Inc.

Results

Crystallographic data (Reynolds et al., 1999) and molecular modeling studies (Lavecchia et al., 2006) suggest that the active sites in the Cdc25 family members are sufficiently different, suggesting that selectivity among catalytic inhibitors of Cdc25 phosphatases may be possible. Thus, we expanded our previous investigation of caulibugulone inhibition of Cdc25B (Wipf et al., 2004) to examine the selectivity of these compounds against all three human Cdc25 phosphatases. Using identical reaction conditions, we found caulibugulone A to E inhibited recombinant human Cdc25 A, B, and C phosphatase activity in vitro with IC_{50} values ranging from 1.51 to 32.5 μM, with caulibugulone E being the least potent (Table 1). Consistent with molecular modeling predictions, all of the caulibugulones, like some of the *para*-quinones studied previously (Brisson et al., 2005), showed a modest (2- to 3-fold) preference for Cdc25A versus Cdc25B or Cdc25C (Table 1). Caulibugulone A, D, and E were specific for the Cdc25 phosphatase family, compared with VHR and PTP1B where the IC_{50} values exceeded 1 mM, whereas caulibugulones B and C were less specific. We focused our attention on caulibugulone A because of its potency, its higher specificity compared with caulibugulones B and C, and the relative simplicity of its chemical structure.

TABLE 1
IC_{50} values of caulibugulones for inhibition of recombinant human protein phosphatases
All values are micromolar concentrations and are the mean ± S.E.M. of three or more independent determinations

Caulibugulone	Structure	Cdc25A	Cdc25B[a]	Cdc25C	VHR[a]	PTP1B[a]
A		3.38 ± 0.62	6.74 ± 1.28	5.43 ± 1.22	>1000	>1000
B		1.51 ± 0.23	2.72 ± 0.51	2.68 ± 0.23	130 ± 23	183 ± 24
C		2.57 ± 0.60	5.40 ± 0.73	3.31 ± 0.28	175 ± 2	322 ± 32
D		4.89 ± 0.82	19.1 ± 0.25	10.8 ± 0.46	>1000	>1000
E		18.2 ± 1.13	32.5 ± 3.57	16.6 ± 0.98	>1000	>1000

[a] Previously reported in Wipf et al. (2004).

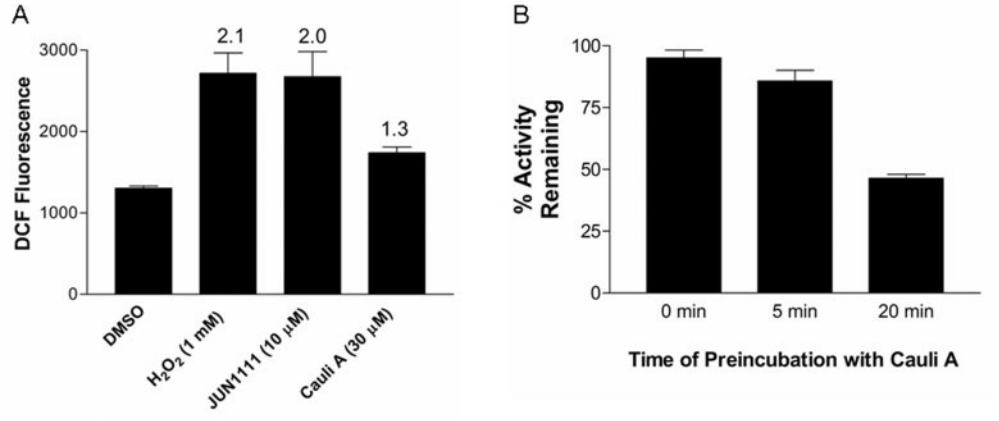


Fig. 1. Caulibugulone A is an irreversible inhibitor that produces ROS. A, HeLa cells were preloaded with DCF dye and incubated for 10 min with DMSO vehicle, 1 mM H_2O_2, 10 μM JUN1111, or 30 μM caulibugulone A. ROS were detected by flow cytometry. Results are mean ± S.E.M. (n = 3). B, Cdc25B was preincubated with 20 μM caulibugulone A (3 × IC_{50}) for 0, 5, and 20 min and diluted >10-fold before an in vitro assay for phosphatase activity with the substrate OMFP. Results are mean ± S.E.M. (n = 4).

Our previous studies (Brisson et al., 2005) revealed that quinone inhibitors of Cdc25 phosphatases induce irreversible oxidation of the catalytic cysteine of Cdc25B through the production of ROS. Consistent with this hypothesis, the degree of inhibition by caulibugulone A was reduced with increasing concentrations of dithiothreitol, respectively. Addition of 80 U/ml catalase also increased the IC_{50} of caulibugulone A against Cdc25B by 16-fold, suggesting that H_2O_2 may be involved in the oxidation of Cdc25B (data not shown). Because these data suggested that caulibugulone A might oxidize Cdc25B, we investigated the ability of caulibugulone A to produce ROS in HeLa cells. As seen in Fig. 1A, 30 μM caulibugulone A caused a small increase in DCF fluorescence in HeLa cells that was less than that seen with 1 mM H_2O_2 or with 10 μM JUN1111, a quinone inhibitor of Cdc25B previously shown to produce ROS (Brisson et al., 2005). Irreversible inhibition of Cdc25B in vitro was also seen when the enzyme was pretreated with caulibugulone A for up to 20 min using a previously described dilution method (Sohn et al., 2003) (Fig. 1B). These data suggest that caulibugulone A emulated other quinone inhibitors by irreversibly inhibiting Cdc25B through the production of ROS.

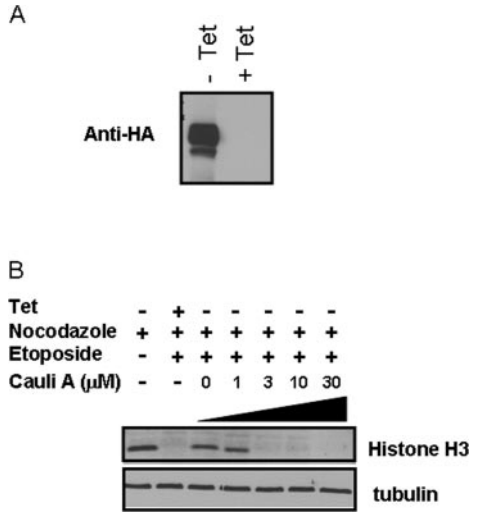


Fig. 2. Caulibugulone A directly inhibits Cdc25B in cells. A, HA-$Cdc25B_3$ expression was induced in U2OS cells by tetracycline removal from the medium for 23 h. Ectopic $Cdc25B_3$ expression was detected by Western blotting using an anti-HA tag antibody. B, cells either expressing or not expressing ectopic $Cdc25B_3$ were treated for 1 h with etoposide followed by 23 h with 1 μM nocodazole alone or nocodazole plus caulibugulone A at 1, 3, 10, or 30 μM. As a positive control, a subset of cells was treated with nocodazole alone. Cdc25B inhibition was assessed by examining phosphorylated Histone H3 (Ser10) levels in cell lysates using a phospho-specific antibody and Western blotting.

To determine whether caulibugulone A inhibited cellular Cdc25B, we exploited a U2OS cell line that conditionally overexpresses hemagglutinin (HA) and His_6-tagged $Cdc25B_3$ under the control of the tet-promoter compared with the nearly undetectable endogenous Cdc25B levels seen in non-transfected U2OS cells as described previously (Bugler et al., 2006) (Fig. 2A). Removal of tet resulted in robust expression of Cdc25B as revealed by the epitope tagged protein (Fig. 2A). Treatment of cycling cells with nocodazole, a potent mitotic blocker, resulted in prominent phosphorylation of Ser10 on Histone H3, a well established marker for mitotic cells (Hendzel et al., 1997) (Fig. 2B). The topoisomerase II inhibitor etoposide activates a G_2 checkpoint, preventing cells from progressing into mitosis in the absence or presence of nocodazole. When overexpression of Cdc25B was blocked (+ tet) and cells were treated with etoposide and nocodazole, the etoposide prevented continuation into mitosis, causing arrest at the G_2 phase of the cell cycle and absence of the phospho-Histone H3 signal (Fig. 2B). In contrast, when tet-free U2OS cells overexpressing Cdc25B were treated with both etoposide and nocodazole, they bypassed the etoposide-mediated G_2 checkpoint and exhibited a high phospho-Histone H3 signal, indicative of mitotic trapping by nocodazole. When tet-free U2OS cells overexpressing Cdc25B were

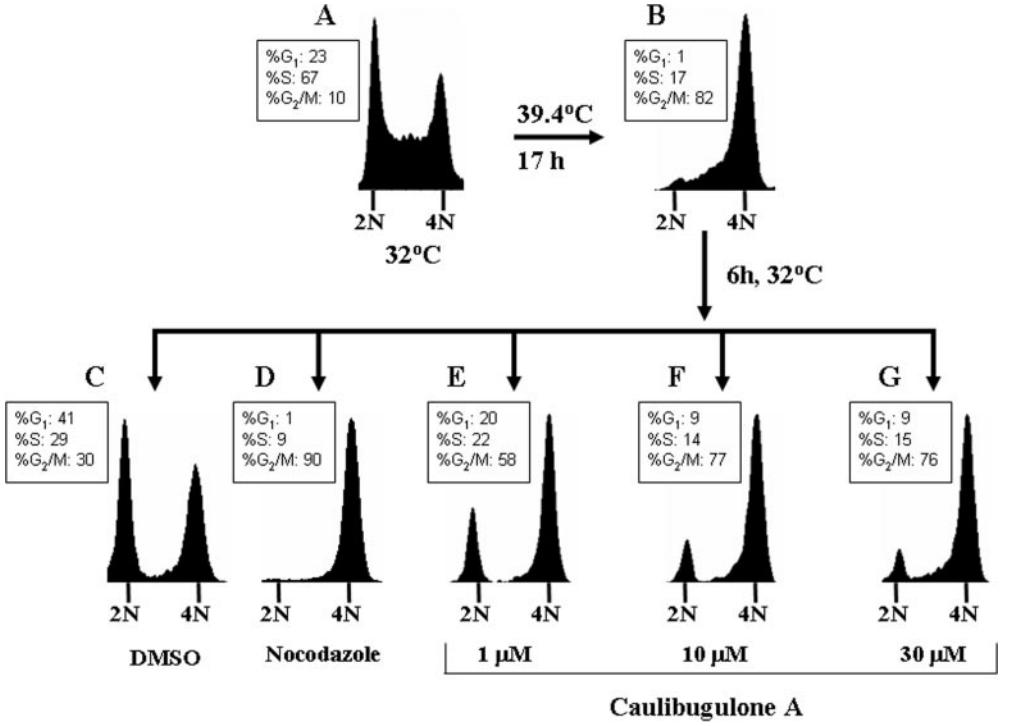


Fig. 3. Caulibugulone A induces G_2/M cell cycle arrest. Murine tsFT210 cells were cultured at the permissive temperature of 32°C and then incubated for 17 h at 39.4°C to arrest cells in G_2/M. DMSO or drug was added to cells for an additional 6 h at 32°C. A, asynchronous control. B, G_2/M-arrested cells after temperature shift for 17 h at 39.4°C. C, DMSO treated cells. D, cells treated with 1 μM nocodazole (positive control). E to G, cells treated with 1 to 30 μM caulibugulone A. This was representative of two independent experiments.

treated with etoposide, nocodazole, and caulibugulone A, we observed a concentration-dependent caulibugulone A elimination of Histone H3 phosphorylation, consistent with Cdc25B inhibition, thus allowing for etoposide-mediated G_2 arrest (Fig. 2B). Furthermore, exposure of synchronized tsFT210 cells to 1, 10, and 30 μM caulibugulone A induced a profound G_2/M cell cycle arrest (Fig. 3) in agreement with the direct inhibition of Cdc25 phosphatases. Exposure to as little as 1 μM caulibugulone A reduced progression through mitosis (Fig. 3E). Caulibugulone A treatment of tsFT210 cells that were released from mitotic arrest resulted in a G_1 cell cycle arrest (Fig. 4), also consistent with Cdc25 phosphatase inhibition.

We next investigated the fate of Cdc25B protein levels after caulibugulone A treatment because growth factors and some DNA-damaging agents have been shown to increase Cdc25B (Oguri et al., 2003, 2004). After treatment of HeLa cells with caulibugulone A, Cdc25B protein levels remained largely unaltered (Fig. 5A). The slight decrease in Cdc25B levels seen with 30 μM caulibugulone A was not reproducible in subsequent experiments. In contrast, there was a reproducible complete loss of Cdc25A with 30 μM caulibugulone A as well as a mild decrease in Cdc25C protein levels. It is noteworthy that addition of the iminoquinone caulibugulone E had no effect on Cdc25 phosphatase protein levels. The loss of Cdc25A was also observed in MDA-MB-231 and MCF7 human breast adenocarcinoma cells (Fig. 5B). Caulibugulone A at 30 and 10 μM decreased the levels of Cdc25A in MDA-MB-231 cells below the DMSO vehicle control, whereas the same concentrations eliminated Cdc25A levels in MCF7 cells. These results were reminiscent of decreased Cdc25A levels that we observed with the naphthoquinone NSC 95397 in PC-3 human prostate cancer cells (Nemoto et al., 2004). Therefore, we tested three other previously described quinone Cdc25 phosphatase inhibitors, DA30003-1, JUN1111, and 5169131, at concentrations known to induce cell cycle arrest (Brisson et al., 2004, 2005), for their ability to decrease endogenous Cdc25A protein levels. All three of these inhibitors depleted Cdc25A protein levels (Fig. 5C), suggesting that the depletion of Cdc25A was a somewhat general characteristic of other known quinone inhibitors of the Cdc25 phosphatases.

Recent evidence suggests that H_2O_2 could decrease Cdc25A transcription through formation of a signal transducer and activator of transcription 3 and Rb repressor complex at the Cdc25A promoter, leading to a reduction in Cdc25A (Barre et al., 2005). Because caulibugulone A produced a modest amount of cellular ROS and catalase blocked caulibugulone inhibition of Cdc25B in vitro, we tested a possible role for H_2O_2 in caulibugulone-mediated reduction of Cdc25A protein levels. Although addition of H_2O_2 to Rb-positive HCT116 colon carcinoma cells caused a concentration-dependent loss in Cdc25A protein consistent in previous studies (Barre et al., 2005), this loss of Cdc25A protein was also observed in HeLa cells where Rb protein levels are decreased and the Rb pathway is inactivated because of overexpression of the human papillomavirus E7 (Goodwin and DiMaio, 2000) (Fig. 6A). Pretreatment of HeLa cells with the antioxidant NAC prevented the H_2O_2-mediated loss of Cdc25A (Fig. 6B). In contrast, pretreatment with the same concentration of NAC did not alter the caulibugulone-mediated reduction in Cdc25A (Fig. 6C). Thus, the loss of Cdc25A after caulibugulone A treatment seemed to be independent of H_2O_2 production.

Constitutive Cdc25A protein degradation is thought to occur through a ubiquitin-proteasome-mediated pathway that is accelerated by DNA damage via the ATM/Chk1 or ATR/Chk2 signaling cascade (for review, see Busino et al., 2004). To determine whether the loss of endogenous Cdc25A was due to protein degradation rather than transcriptional or translational regulation, we treated cells with cycloheximide from 0 to 30 min to halt protein synthesis in the presence or absence of caulibugulone A (Fig. 7A). The half-life of Cdc25A

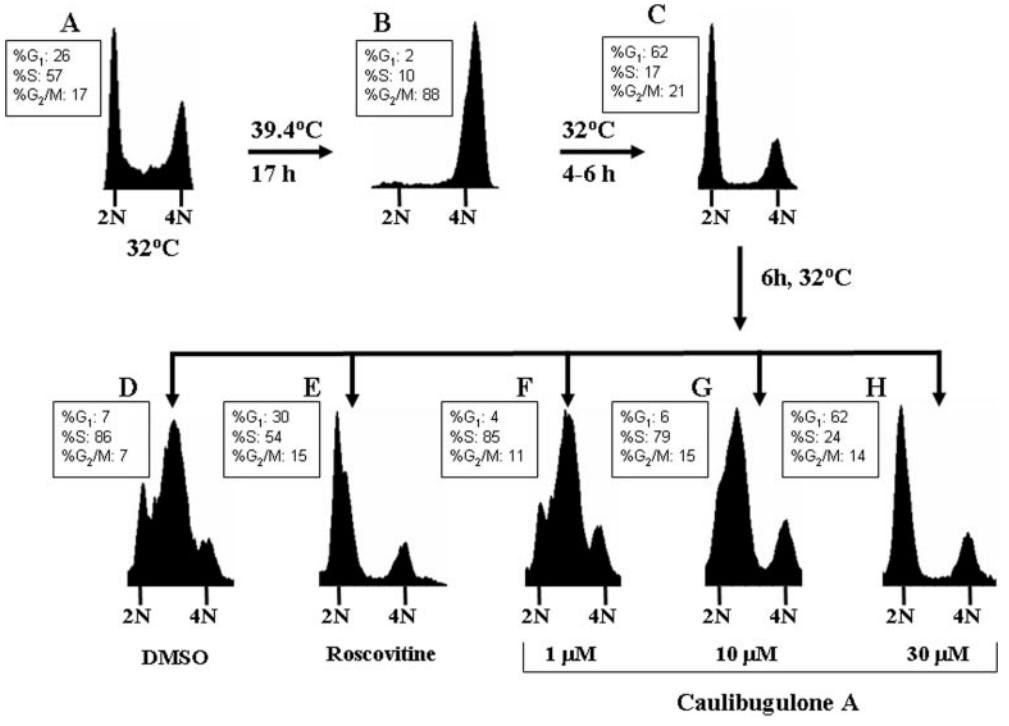


Fig. 4. Caulibugulone A induces G_1 cell cycle arrest. tsFT210 cells were cultured at the permissive temperature of 32°C and then incubated for 17 h at 39.4°C to arrest cells in G_2/M. Cells were then released for 4 to 6 h at 32°C to reinitiate cell cycle progression into G_1. DMSO or drug was added for an additional 6 h at 32°C. A, asynchronous control. B, G_2/M-arrested cells after temperature shift for 17 h at 39.4°C. C, G_1-arrested cells after temperature shift back to 32°C. D, DMSO-treated cells. E, cells treated with 50 μM roscovitine (positive control). F to H, cells treated with 1 to 30 μM caulibugulone A. This was representative of two independent experiments.

in the presence of DMSO was approximately 20 min, whereas the degradation of Cdc25A was greatly accelerated in the presence of caulibugulone A with a half-life of approximately 5 min, indicating that caulibugulone A enhanced Cdc25A protein degradation. However, Cdc25A degradation by caulibugulone A was not mediated by Chk1 or Chk2 pathways. Exposure of HeLa cells to UV resulted in Chk1 activation as revealed by Ser345 phosphorylation (Fig. 7B). In contrast, neither caulibugulone A nor caulibugulone E caused phosphorylation of Chk1. We did observe phosphorylation of Chk2 (Thr68) after addition of caulibugulone A (10 or 30 μM) or caulibugulone E (30 μM) (Fig. 7B), but neither 10 μM caulibugulone A nor 30 μM caulibugulone E reduced Cdc25A levels (Fig. 5A). Therefore, we think the activation of phospho-Chk2 by caulibugulones A and E did not correlate with the changes observed in Cdc25A protein levels.

We previously observed that the proteasome had an important role in degradation of Cdc25A after treatment of cells with the naphthoquinone NSC 95397 (Nemoto et al., 2004). Therefore, we investigated the functional importance of proteasome activity for the degradation seen with caulibugulone A. When HeLa cells were pretreated with the proteasome inhibitor 5 μM MG132 for 15 h, constitutive Cdc25A turnover was blocked, as evidenced by an increase in the Cdc25A protein levels over the DMSO vehicle control (Fig. 7C). The Cdc25A degradation induced by UV irradiation was also blocked by pretreatment with MG132. When we pretreated cells under the same conditions with MG132 and then added caulibugulone A for 2 h, caulibugulone A was still capable of degrading Cdc25A to levels well below that seen with MG132 alone (Fig. 7C). These data indicated that the decrease in Cdc25A protein levels seen with caulibugulone A treatment was unlikely to be mediated by a proteasome-dependent mechanism.

Activation of the p38 signaling pathway by UV irradiation and other stressors has been implicated in the regulation of Cdc25 phosphatase activity (Boutros et al., 2006). Therefore, we examined cells treated with caulibugulone A for p38 activation using a phospho-specific antibody detecting dual phosphorylation at Thr180/Tyr182 on p38 (Fig. 8A). Significant p38 activation was detected in cells treated with caulibugulone A as well as with other quinone inhibitors of Cdc25 phosphatases, namely, DA3003-1 and JUN1111. No changes in total p38 protein levels were noted. Upon preincubation with the selective p38 kinase inhibitor SB 203580, the caulibugulone-mediated degradation of Cdc25A was blocked in a concentration-dependent manner (Fig. 8B). In contrast, SB 203580 did not prevent the UV-induced decrease in Cdc25A caused by DNA damage signaling pathways (Fig. 8B). These results suggest that Cdc25A degradation induced by caulibugulone A was regulated through an alternative p38 kinase pathway, which is distinct from pathways involved in UV irradiation.

Discussion

The caulibugulone natural products represent a unique class of cytotoxic marine-derived compounds with an unknown mechanism of action (Milanowski et al., 2004). The specificity of the caulibugulones for inhibition of Cdc25 phos-

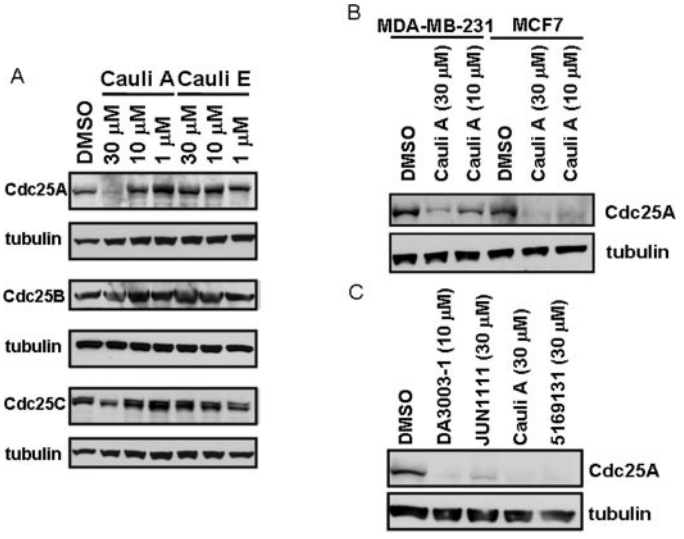


Fig. 5. Endogenous Cdc25A protein levels decrease after treatment of HeLa cells with quinone inhibitors. A, HeLa cells were treated with DMSO vehicle or 1, 10, or 30 μM caulibugulone A or E for 2 h before cell lysis and detection of Cdc25A, -B, or -C by Western blot. B, MDA-MB-231 and MCF7 cells were treated with DMSO vehicle or 10 or 30 μM caulibugulone A for 2 h before cell lysis and detection of Cdc25A by Western blot. C, HeLa cells were treated with DMSO vehicle, 10 μM DA3003-1, or 30 μM JUN1111, 5169131, or caulibugulone A before cell lysis and detection of Cdc25A by Western blot. Blots are representative of at least two independent experiments.

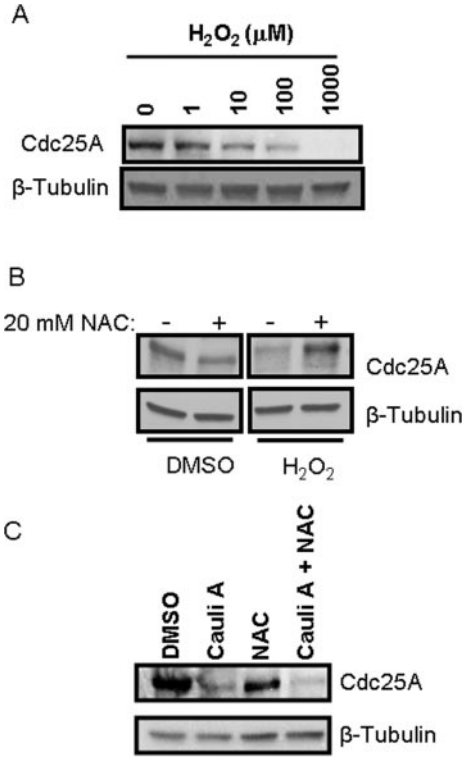


Fig. 6. H_2O_2 production by caulibugulone A does not contribute to Cdc25A degradation. A, HeLa cells were treated with 1 to 1000 μM H_2O_2 for 2 h before detection of Cdc25A levels by Western blot. B, Cdc25A levels after HeLa cells were pretreated with or without 20 mM NAC for 2 h followed by addition of DMSO vehicle or 1 mM H_2O_2 for 2 h. C, HeLa cells were pretreated with or without 20 mM NAC for 2 h followed by a 2-h incubation with DMSO vehicle, 30 μM caulibugulone A alone, or caulibugulone A with NAC pretreatment before detection of Cdc25A by Western blot. Blots are representative of at least two independent experiments.

phatases (Table 1) stimulated interest in examining the mechanism responsible for mammalian growth inhibition. Recent evidence suggested that at least some *para*-quinones act as inhibitors of the Cdc25B phosphatase in part by oxidizing and inactivating the catalytic cysteine of the enzyme through redox cycling and production of ROS (Brisson et al., 2005). Similar to several other quinone-based Cdc25 inhibitors, caulibugulone A produced ROS in cells (Fig. 1) and induced irreversible Cdc25B inhibition that was sensitive to catalase. These data support the hypothesis that redox cycling could be one mechanism of caulibugulone A inhibition of Cdc25B.

A common response to some forms of cellular stress is a marked decrease in Cdc25A levels, resulting in cell cycle arrest. Caulibugulone A addition to HeLa cells at 30 μM caused a complete loss of Cdc25A through protein degradation (Figs. 5 and 7A) that could mediate cell cycle arrest (Figs. 3 and 4). Three other quinone inhibitors were also capable of degrading Cdc25A, consistent with our previous findings with NSC 95397 (Nemoto et al., 2004), suggesting that this is a fairly common phenomenon among known quinone inhibitors of Cdc25 phosphatases. In contrast to the Cdc25A decrease caused by H_2O_2 in HeLa cells, the Cdc25A loss by caulibugulone A was not prevented with NAC pretreatment (Fig. 6), indicating that H_2O_2 was an unlikely mediator of the Cdc25A degradation induced by caulibugulone A.

The DNA damage checkpoint response induced by UV or ionizing irradiation is known to activate the ATM/ATR kinase signaling pathways, which in turn phosphorylate Chk1 and Chk2 protein kinases, respectively (Boutros et al., 2006). Chk1 and Chk2 are responsible for regulating Cdc25 phosphatase activity to maintain cell cycle checkpoint integrity. Chk1 and Chk2 phosphorylate Cdc25A at Ser124/178/293, targeting the enzyme for ubiquitination, and ultimately, degradation by the proteasome, leading to cell cycle arrest (Busino et al., 2004). Caulibugulone A, however, did not induce Chk1 phosphorylation and activation (Fig. 7B). Although modest Chk2 phosphorylation was seen with 10 and 30 μM caulibugulone A (Fig. 7B), the phosphorylation of Chk2 after 10 μM caulibugulone A did not correlate with Cdc25A down-regulation, because this concentration of caulibugulone A did not change Cdc25A levels (Fig. 5A). In addition, 30 μM caulibugulone E caused prominent phosphorylation of Chk2 but had no effect on Cdc25A protein levels (Figs. 5A and 7B). Further support that Cdc25A degradation was not mediated by Chk1 or Chk2 activation was evident by the inability of the proteasome inhibitor MG132 to prevent the degradation of Cdc25A in the presence of caulibugulone A (Fig. 7C). This is contradictory to our previously published data that suggested a proteosome-dependent decrease in Cdc25A levels with NSC 95397 (Nemoto et al., 2004). In the previous experiment, PC-3 cells were pretreated for only 1 h with 10 μM MG132, resulting in incomplete proteosome inhibition and a mild increase in endogenous Cdc25A levels. We think that our interpretation of the previous data, suggesting a proteosome-dependent mechanism, would have been different under the more stringent conditions of the current study. Together, these data excluded the ATM/ATR pathways as the primary participants responsible for the caulibugulone A-mediated degradation of Cdc25A, and they suggested an unknown alternative proteosome-independent pathway of regulation.

The stress-activated protein kinase p38 has a recognized role in delaying entry into mitosis by controlling Cdc25 phosphatase activity when cells are stressed (Boutros et al., 2006). Under conditions of osmotic stress, p38 phosphorylates Cdc25A at Ser75, leading to degradation of Cdc25A (Goloudina et al., 2003). Khaled et al. (2005) also observed p38 phosphorylation of Cdc25A at Ser75 and Ser123 and subsequent degradation upon interleukin-3 and interleukin-7 cytokine withdrawal from lymphocytes, leading to G_1/S arrest. The p38 signaling pathway has also been implicated in instigating G_2/M arrest after UV irradiation by phosphorylation of Cdc25B (Ser309/361) and Cdc25C (Ser216) (Bulavin et al., 2001). It is now thought that a downstream target of p38, mitogen-activated protein kinase-activated protein kinase-2, is responsible for the phosphorylation of

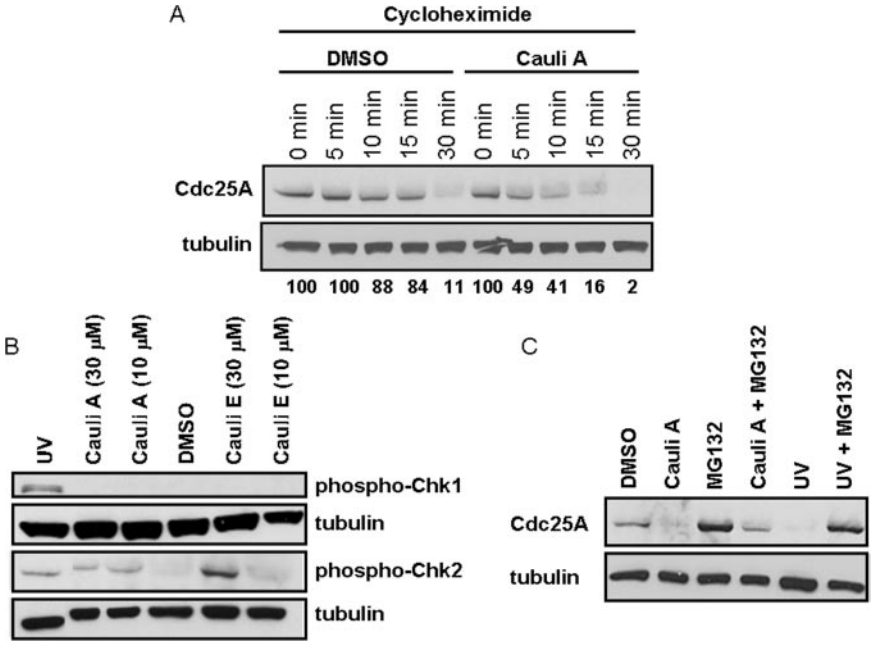


Fig. 7. Caulibugulone A instigates Cdc25A protein degradation via a proteasome-independent mechanism. A, HeLa cells were exposed to 25 μg/ml cycloheximide from 0 to 30 min in the presence of DMSO or 30 μM caulibugulone A followed by cell lysis and detection of Cdc25A levels by Western blot. Quantification of the Western blot was performed by densitometry and band intensity for Cdc25A bands, as indicated below the panel, were normalized to tubulin and expressed as percentage of the corresponding 0-min control. B, phosphorylated Chk1 and Chk2 were detected in HeLa cells after treatment with 60 J/m^2 UV (positive control), 10 or 30 μM caulibugulone A or E for 2 h. C, HeLa cells were pretreated for 15 h with DMSO vehicle control (lanes 1, 2, and 5) or 5 μM MG132 (lanes 3, 4, and 6). Cells were then treated for 2 h with DMSO vehicle (lane 1), 5 μM MG132 (lanes 3, 4, and 6), 30 μM caulibugulone A (lanes 2 and 4), or exposed to 60 J/m^2 UV (lanes 5 and 6) and incubated for 1 h. Cdc25A levels were assessed by Western blot. Blots are representative of at least two independent experiments.

Cdc25B and C, leading to association with 14-3-3 and sequestration in the cytoplasm away from Cdk1/cyclin B (Manke et al., 2005). It is interesting, therefore, that caulibugulone A, which activated p38 (Fig. 8A), caused a loss of Cdc25A with little or no loss of either Cdc25B or C (Fig. 5A). The p38-specific inhibitor SB 203580 partially restored Cdc25A levels in a concentration-dependent manner (Fig. 8B), further implicating p38 kinase. This degradation of Cdc25A by p38 activation seems to be proteosome-independent (Fig. 7C), indicating a new, previously unrecognized mechanism by which Cdc25A is degraded. Initial studies with p38 siRNA targeted to the α isoform were capable of decreasing p38 α levels by ~50% in cells treated with caulibugulone A; however, phosphorylated p38 levels were still present in α p38 siRNA transfected cells compared with control siRNA upon caulibugulone A treatment, resulting in Cdc25A degradation (data not shown). Further studies are underway to determine whether decreasing the levels of the α isoform of p38 to >50% will be sufficient in significantly decreasing phosphorylated p38 or whether other isoforms (β, γ, or δ) of p38 need to be targeted. Due to the role of p38 in Cdc25B sequestration, it is also possible that this is yet another mechanism by which Cdc25B is regulated by caulibugulone A and other quinone inhibitors. These data provide added support for further studies of the potential antineoplastic actions of caulibugulone A as the p38 pathway has been shown to be activated by other clinically used chemotherapeutic agents (Hirose et al., 2003, 2004; Mikhailov et al., 2004).

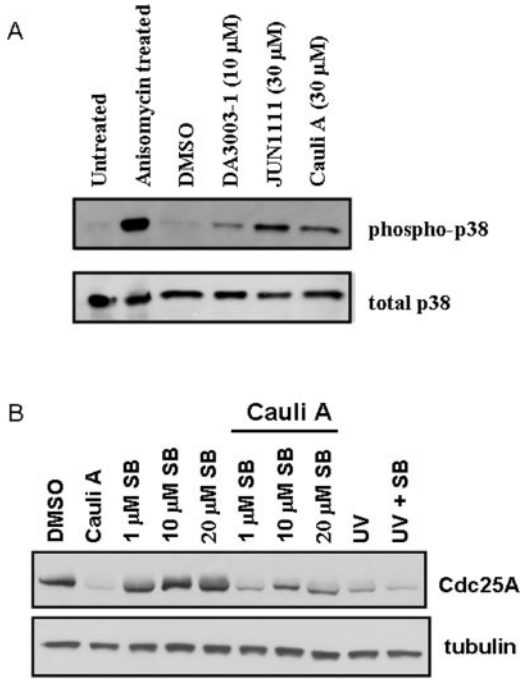


Fig. 8. Caulibugulone A degrades Cdc25A by activation of p38 kinase. A, Phospho-p38 and total p38 protein levels in HeLa cells were detected after treatment with DMSO vehicle anisomycin (positive control from Cell Signaling Technology Inc.), 10 μM DA3003-1, 30 μM JUN1111, or 30 μM caulibugulone A for 2 h. B, cells were pretreated with and without 1 to 20 μM SB 203580 for 1 h followed by exposure to DMSO or 30 μM caulibugulone A before cell lysis and detection of Cdc25A levels by Western blot. UV treated cells at 60 J/m^2 alone or pretreated with the SB 203580 inhibitor were used as controls. Blots are representative of at least two independent experiments.

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