Pharmacologic inhibition of CDC25 phosphatases impairs interphase microtubule dynamics and mitotic spindle assembly

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Abstract

The CDC25 cell cycle regulators are promising targets for new pharmacologic approaches in cancer therapy. Inhibitory compounds such as BN82685 have proven to be effective in specifically targeting CDC25 in cultured cells and in inhibiting tumor cell growth. Here, we report that BN82685 impairs microtubule dynamic instability and alters microtubule organization and assembly at the centrosome in interphase cells. Treatment of mitotic cells with BN82685 delays mitotic spindle assembly, chromosome capture, and metaphase plate formation. Furthermore, we show that combining low concentrations of both BN82685 and paclitaxel inhibits the proliferation of HT29 human colon cancer cells. Our results show a role for CDC25 phosphatases in regulating microtubule dynamics throughout the cell cycle and suggest that combinations of CDC25 inhibitors with microtubule-targeting agents may be of therapeutic value. [Mol Cancer Ther 2007;6(1):318–25]

Introduction

CDC25 phosphatases are essential cell cycle regulators that are responsible for the dephosphorylation and subsequent activation of cyclin-dependent kinases (CDK) associated with their cyclin regulatory subunits. It was initially proposed that the three human CDC25 phosphatases (A, B and C) were involved in the control of G_1-S transition, the progression through late G_2, and into mitosis, respectively. However, it is now recognized that all three isoforms probably contribute to the mitotic process (for review, see ref. 1). Although CDC25B and CDC25C have been reported to be dispensable for mouse development, whereas CDC25A is essential, it is clear from small interfering RNA–based studies that all three isoforms participate in the control of the activity of CDK1 at various stages of mitosis (2). CDC25A and CDC25B are proposed to play a role in the entry into mitosis, with CDC25A being involved in the control of chromosome condensation and CDC25B in regulating the centrosomal activation of CDK/cyclin complexes (2, 3). CDC25C is thought to be less crucial for the control of entry into mitosis, but is largely involved in the amplification and maintenance of the CDK/cyclin activity during mitosis (1).

Changes in CDC25A and CDC25B expression and their association with high aggression and poor prognosis have been largely documented for a number of tumor types (see ref. 4 for review). It is still not totally clear how these changes contribute to the oncogenic process, or whether this is related to their cell cycle regulatory functions or to their specific roles in the checkpoint response to DNA damage (5, 6). Indeed, CDC25B expression level has been shown to be critical for the control of entry into mitosis after DNA damage (7, 8).

CDC25 phosphatases are considered as promising targets in cancer therapy. To this aim, a number of academic laboratories and pharmaceutical companies have identified and developed CDC25 inhibitory compounds over the last few years (for reviews, see refs. 4, 9). These inhibitors are active against all three CDC25 phosphatases *in vitro* and have been shown to efficiently target CDC25 activities in cultured cells (10–15). Specifically, these compounds have been shown to inhibit cancer cell proliferation *in vitro*, and we recently reported that some of them, such as BN82002 and BN82685, are active *in vivo* in mouse xenografted human tumor models (14, 15). BN82685 inhibits the growth of human tumor cell lines with an IC_{50} in the submicromolar range and its specificity towards CDC25 phosphatases was shown *in vitro* and *in vivo* (15).

It is well established that during the cell cycle, microtubule dynamics are regulated by CDK activities (16, 17) through the phosphorylation of microtubule-associated proteins and modulation of their activity (18). It has also recently been shown that CDK1 can directly phosphorylate β-tubulin and modulate microtubule assembly at mitosis (19). As CDKs require dephosphorylation of specific residues to become active, their effects on microtubule dynamics are therefore probably also indirectly controlled by the CDC25 phosphatases. The activating effects on mitotic spindle assembly observed upon CDC25B overexpression also supports this model (20).

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In the work reported here, we have investigated the effect of the previously described CDC25 inhibitor, BN82685, on the assembly of microtubules in interphase and during progression into mitosis. We have found that inhibiting CDC25 phosphatase activities alters microtubule dynamic instability, impairs assembly at the centrosomes in interphase cells, and perturbs the mitotic process by impairing mitotic spindle assembly. In addition, we report the additive effect achieved by combining a CDC25 inhibitor with paclitaxel, and propose that the combination of a microtubule-targeting agent and a CDC25 inhibitory compound may represent a promising therapeutic approach.

Materials and Methods

Cell Culture

HeLa cells were grown as previously described (21). HT29 cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L of L-glutamine, 100 units/mL of penicillin, and 0.1 mg/mL of streptomycin. All cell lines were maintained at 37°C in a 5% CO_2 atmosphere.

In vivo Microtubule Nucleation Assay

HeLa cells were treated or not with 250 nmol/L of BN82685 for 2 h, and then placed on ice for 80 min to completely depolymerize microtubules. Cells were then transferred to fresh media prewarmed to 30°C for 3, 10, 30, or 60 min. Cells were permeabilized briefly in 80 mmol/L of PIPES (pH 6.8), 1 mmol/L of EGTA, 1 mmol/L of $MgCl_2$, 0.5% Triton X-100, washed once with PBS and then fixed in ice-cold methanol for 6 min at −20°C. Microtubules were visualized by staining for α-tubulin and γ-tubulin.

In vitro Tubulin Polymerization Assay

Tubulin polymerization assays were carried out using the tubulin polymerization assay kit from Cytoskeleton, Inc. (Vancouver, British Columbia, Canada), following the manufacturer's instructions. All small molecules were

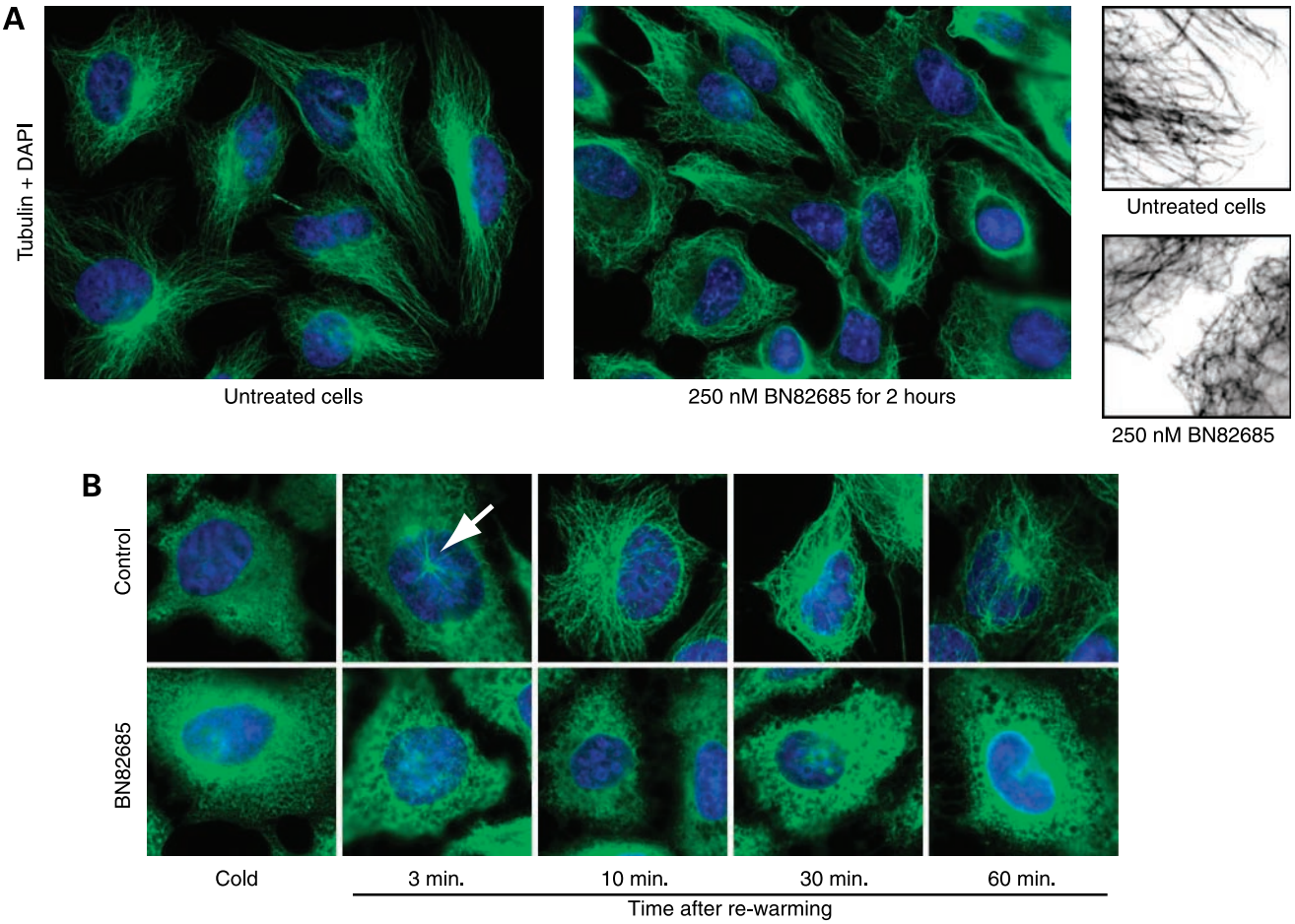
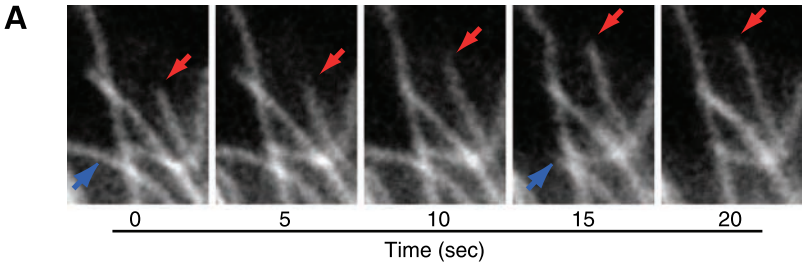
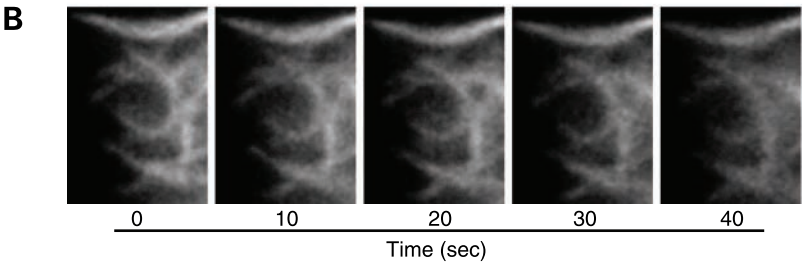


Figure 1. Inhibition of CDC25 phosphatases impairs microtubule repolymerization. **A,** HeLa cells were treated or not for 2 h with BN82685 (250 nmol/L) and fixed and stained for tubulin (*green*) and DNA (*blue*). *Right,* enlargements of cells from the left with inverted contrast to visualize details of the microtubule network. **B,** control and BN82685-treated HeLa cells were cold-treated and rewarmed for 3, 10, 30, or 60 min at 30°C before immunofluorescence detection of tubulin (*green*) and DNA (*blue*). *Arrowhead,* microtubule aster nucleated in control cells. In BN82685-treated cells, microtubule assembly is impaired and no sign of nucleation is visible.





C

	Control	BN82685
Frequency catastrophe (s^{-1})	0.065 ± 0.024	-
Frequency rescue (s^{-1})	0.053 ± 0.012	-
Growth rate (μm/s)	0.220 ± 0.083	0.02
shrink rate (μm/s)	0.340 ± 0.122	0.02

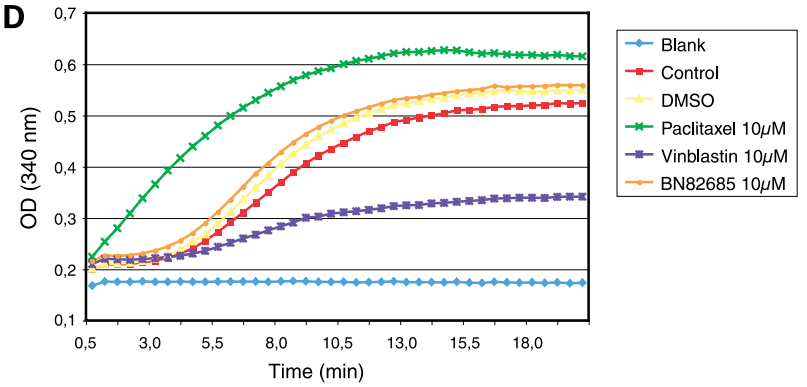


Figure 2. BN82685 impairs the microtubule dynamic but not *in vitro* microtubule assembly. **A** to **C,** HeLa cells expressing α-tubulin-GFP were treated (**B**) or not (**A**) for 2 h with BN82685 (250 nmol/L) and imaged by time-lapse videomicroscopy (see movie 1, Supplementary Material). **A** and **B,** selected images extracted from this movie. **A,** growing microtubules (*red arrows*); shrinking microtubules (*blue arrows*). **C,** quantification of the data acquired from microtubules in individual cells. Values shown are means ± SD. In treated cells, the extremely low number of growing or shrinking microtubules are insufficient for calculation of SD and measurements of catastrophe and rescue frequencies. **D,** *in vitro* tubulin polymerization assay. The extent of tubulin polymerization at 37°C was monitored over time (in minutes) using a polymer-specific dye (arbitrary fluorescence units) in the presence of control buffer, DMSO buffer, paclitaxel (10 μmol/L), vinblastin (10 μmol/L), or BN82685 (10 μmol/L). *Points,* means of three independent experiments.

stored as 100% DMSO (v/v) stock solutions. Vinblastine was purchased from Sigma (St. Louis, MO).

Cell Treatment and Proliferation Assay

Paclitaxel was obtained from Sigma and BN82685 from IPSEN. Both were dissolved in DMSO at 10^{-2} mol/L. To test the effect of BN82685 in combination with paclitaxel on cell proliferation, cells were seeded into 96-well plates at 2,000 cells per well and incubated overnight. Paclitaxel and BN82685 were added at various concentrations, either alone or simultaneously. Plates were then incubated under the same conditions for a further 5 days. This experiment was done thrice with four repetitions per concentration tested. Cell proliferation was measured using the colorimetric assay, WST1, based on the cleavage of the tetrazolium salt WST1 by mitochondrial dehydrogenases in viable cells, leading to formazan formation (Roche Diagnostic, Basel, Switzerland).

Dose-Effect Analyses

The percentages of metabolically active (viable) cells in compound-treated populations were compared with untreated control cells following 5 days of incubation. The synergy between BN82685 and paclitaxel was analyzed using the multiplicative model. The expected effect (22) was calculated as [(OD of BN82685-treated cells × OD of paclitaxel-treated cells) / OD of control cells] × 100. When ''expected effect'' and ''observed effect'' were equal, the effects were considered as additive (22).

Antibodies

The human polyclonal CREST (calcinosis–Raynaud phenomenon–esophageal dismobility–sclerodactyly–telangiectasia) antibody from a patient with scleroderma was a generous gift from Dr. J. Goetz (Strasbourg University, Strasbourg, France) and used at a dilution of

1:800. Mouse anti–α- and γ-tubulins (Sigma) were used at 1:2,000 and 1:1,000 dilutions, respectively. Secondary antibodies labeled with Alexa 488 or Alexa 594 (Molecular probes) were used at 1:800 dilutions.

Immunofluorescence Microscopy

Cells were seeded onto glass coverslips and fixed 24 h later in cold methanol for 6 min at −20°C. Incubation with primary and secondary antibodies were done in PBS, 0.1% Tween and 3% FCS. DNA was visualized using 4′,6-diamidino-2-phenylindole staining. Images were acquired using DMIRE2 and DM6000 microscopes (Leica Microsystems, Wetzlar, Germany) fitted with a Roper COOLsnap ES CCD camera, and subsequently processed using the MetaMorph and Photoshop software packages.

Time-lapse Videomicroscopy and Analysis of Dynamic Instability

HeLa cells expressing α-tubulin green fluorescent protein were plated on glass chamber slides and grown in DMEM containing 10% fetal bovine serum at 37°C with 5% CO_2. Cells were imaged using a Leica DMIRB (Leica Microsystem) with a 100 W mercury lamp. Images were acquired with a CoolSnapEs cooled CCD camera (Roper Scientific, Inc., Tucson, AZ). Metamorph software (Universal Image, Downingtown, PA) was used to control the CCD camera. All variables, including the growth and shrinking rates of microtubules, were monitored using the track point function of the metamorph software.

Results

Inhibition of CDC25 Phosphatases by BN82685 Impairs Interphase Microtubule Assembly

We first investigated the effects of pharmacologic inhibition of CDC25 on the microtubule network organization in HeLa cells. We used the CDC25 inhibitor, BN82685, a previously described compound that efficiently inhibits CDC25 activity *in vitro* (IC_{50}, 171 nmol/L) and HeLa cell proliferation (IC_{50}, 350 nmol/L; ref. 14). As shown in Fig. 1A, after 2 h of treatment with BN82685, microtubules were not correctly organized, were shorter and more undulated, and did not always seem to nucleate from the centrosomes, but nucleated rather diffusely and often formed a ring around the nucleus. This observation prompted us to examine the effect of CDC25 inhibition on the assembly of microtubules at the centrosomes in more detail. To this aim, we used an *in vivo* microtubule nucleation assay in cultured HeLa cells pretreated or not with BN82685. Cells were cold-shocked to fully disassemble microtubules and then the cells were incubated with warm medium to allow reassembly, before fixation and immunofluorescence detection of tubulin. As shown in Fig. 1B, microtubules were completely disassembled after 80 min of cold treatment. In control cells, microtubules rapidly reassembled from the centrosomes upon rewarming, and within ~10 min, an interphase microtubule network was reconstituted. In contrast, cells treated with 250 nmol/L of BN82685 did not display any obvious signs of microtubule reassembly, even after 60 min of rewarming. In the experiment shown in Fig. 1B, a temperature of 30°C was used to slow down the microtubule reassembly process, but comparable results were obtained at 37°C for

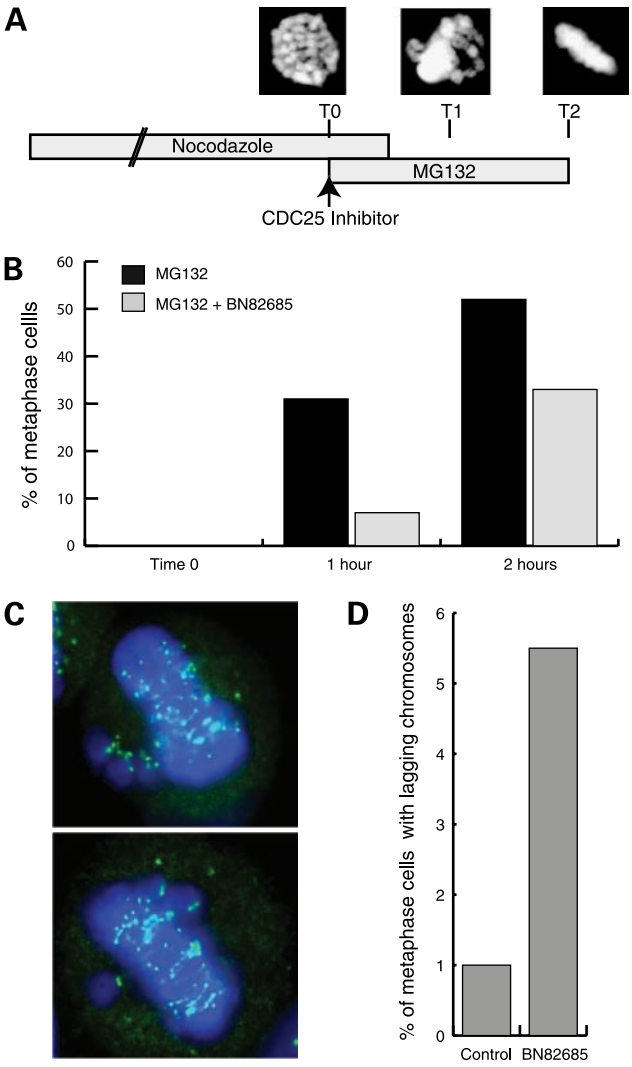


Figure 3. Inhibition of CDC25 phosphatases by BN82685 delays progression from prometaphase to metaphase and increases the rate of metaphase cells with lagging chromosomes. **A,** experimental scheme. HeLa cells were treated with 80 nmol/L of nocodazole for 4 h, then together with 25 μmol/L of MG132 for a further 30 min. The culture medium was removed and replaced with medium containing only MG132. BN82685 (500 nmol/L) was added at time 0 (T_0), T_1 (1 h), and T_2 (2 h). *Inset,* HeLa cells treated as indicated were fixed and stained with 4′,6-diamidino-2-phenylindole. **B,** the percentages of true metaphase cells were determined from among the mitotic cell population. The cells that had not been blocked in prometaphase by the 4-h treatment with nocodazole were not considered. Metaphase cells were counted at time 0, then after 1 and 2 h in the presence of MG132, with or without BN82685. **C** and **D,** HeLa cells treated as indicated in (**A**) for 2 h were fixed and stained with CREST human polyclonal antibody (*green*) and 4′,6-diamidino-2-phenylindole (*blue*). **C,** two typical cells displaying lagging chromosomes. Progressive alignment of the chromosomes along the metaphase plate is delayed, along with an increase in the percentage of metaphase cells with lagging chromosomes, in comparison to control cells (**D**).

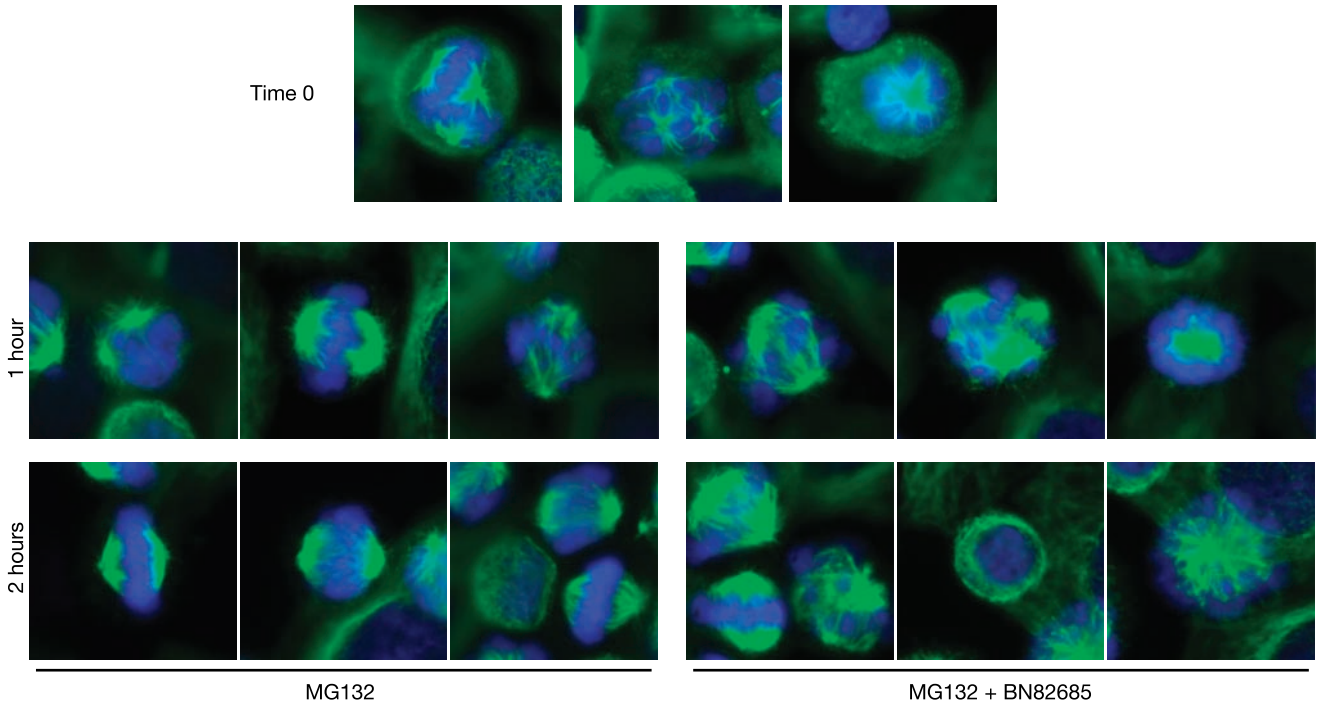


Figure 4. CDC25 inhibition results in altered mitotic spindle organization. HeLa cells were treated as described in Fig. 2. At the indicated times, cells were fixed and stained with a monoclonal anti–α-tubulin antibody (*green*) and 4′,6-diamidino-2-phenylindole (*blue*); selected representative cells. *Left,* cells treated with 25 μmol/L of MG132 only. *Right,* cells treated with MG132 and 500 nmol/L of BN82685.

BN82685-treated cells, whereas a microtubule network formed within 1 to 2 min of rewarming in control cells (data not shown).

BN82685 Impairs the Microtubule Dynamic but not *In vitro* Microtubule Assembly

These observations led us next to examine microtubules dynamic instability by time-lapse videomicroscopy in cells expressing an α-tubulin-GFP construct. As shown in Fig. 2A and B and in the accompanying movie (Supplementary Data)[3] microtubule dynamics were considerably impaired in cells treated with BN82685 as compared with control untreated cells. We monitored several variables of microtubule dynamics including growth rate, shrink rate, catastrophe, and rescue frequencies (Fig. 2C), and found that microtubule dynamic instability was totally abolished in the treated cells.

In order to confirm that the effect observed *in vivo* was dependent on the inhibition of CDC25 and did not result from an interaction between BN82685 and tubulin, we also examined the effect of this compound in an *in vitro* polymerization assay (Fig. 2D). The assembly of tubulin into microtubules was monitored as shown with control and DMSO samples. As expected, paclitaxel (10 μmol/L) caused a rapid increase of *in vitro* microtubule assembly, whereas vinblastine (10 μmol/L) caused a reduced microtubule formation. In similar conditions, BN82685 at concentrations up to 40 μmol/L had no detectable effect (Fig. 2D; data not shown), strongly suggesting that this compound does not interfere directly with tubulin polymerization.

BN82685 Delays Progression from Prometaphase to Metaphase

We next investigated the consequence of using CDC25 phosphatase inhibitors on the assembly of the metaphase mitotic spindle using a new assay based on the following experimental conditions (adapted from ref. 23). As depicted in Fig. 3A, HeLa cells were treated with nocodazole in order to arrest a fraction of these cells at the spindle assembly checkpoint (24) in a prometaphase-like stage (Fig. 3A, *top left, inset*). At time 0, the cells were treated for an additional 30 min with MG132 together with nocodazole, then with a MG132 alone for the rest of the experiment. Upon removal of the nocodazole, the cells that were arrested in prometaphase by activation of the spindle assembly checkpoint (25) were able to progress through mitosis. However, due to the presence of the proteasome inhibitor MG132 (which impairs sister chromatid separation by inhibiting the degradation of securine; ref. 26), the cells stopped progressing through mitosis once the metaphase spindle was fully assembled and the chromosomes were correctly aligned (Fig. 3A, *top right, inset*). In a parallel experiment, the CDC25 inhibitor, BN82685, was added to the assay at T_0, at the same time as MG132. As shown in Fig. 3B, control cells treated only with MG132 progressively accumulated in metaphase, as expected. In contrast, the

[3] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

cells that were treated with BN82685 together with MG132 showed delayed metaphase plate formation by ~75% and 40% after 1 and 2 h of treatment, respectively. Additional experiments done with several other chemically unrelated CDC25 inhibitory compounds resulted in a similar phenotype (data not shown). Taken together, these results indicate that CDC25 inhibition with specific chemical compounds retards the mitotic spindle assembly process.

CDC25 Inhibition Increases the Percentage of Metaphase Cells with Lagging Chromosomes

As CDC25 inhibition delayed metaphase plate formation, we hypothesized that this may correlate with an increased frequency of lagging chromosomes. This issue was examined using an experimental scheme similar to that presented in Fig. 3A. After treatment, the cells were fixed and stained with 4′,6-diamidino-2-phenylindole to visualize DNA, together with CREST human autoantibodies, which recognize kinetochores (27). This allowed the identification of cells with lagging chromosomes (two typical examples of which are shown in Fig. 3C), following 2 h of treatment with BN82685. In control experiments with MG132 alone, the percentage of cells displaying similar lagging chromosomes always progressively decreased with time and was ~1% in the experiment presented in Fig. 3D. The percentage of lagging chromosomes was significantly increased after 2 h of treatment with the CDC25 inhibitor and was ~5.5% in the specific example shown in Fig. 3D.

CDC25 Inhibition Results in Altered Mitotic Spindle Organization

We next examined in more detail the morphologic aspect of the effect observed upon treatment of mitotic cells with a CDC25 inhibitor. Cells released from a nocodazole arrest in the presence of BN82685 and MG132 were subjected to immunofluorescence staining of the mitotic spindle using antitubulin monoclonal antibodies. At time 0, in the presence of nocodazole, the cells were arrested at the spindle assembly checkpoint and displayed abnormal mitotic phenotypes with multipolar or monopolar spindles (Fig. 4). Upon release from nocodazole block, most of the control cells treated with MG132 alone progressively reassembled a mitotic spindle and accumulated in a true metaphase state within the 2 h of the experiment. In contrast, cells treated with BN82685 presented abnormal mitotic figures such as monopolar aster-like spindles, multipolar spindles, or ''donut''-like spindles, 2 h after release from the nocodazole treatment.

Additive Effects of CDC25 Inhibitors and Microtubule-Targeting Drugs

The data presented in Figs. 1–4 strongly suggested that CDC25 inhibition was indirectly responsible for altering microtubule dynamics and events that are required for microtubule assembly in interphase, and to build a bipolar mitotic spindle required for correct segregation of the sister chromatids during mitosis. We therefore hypothesized that treatment with CDC25 phosphatase inhibitors, in combination with drugs targeting microtubules, could be of interest in cancer therapy.

This issue was investigated by treating the human colon cancer cell line HT29 with a combination of low concentrations of both the microtubule disrupting agent paclitaxel (5 nmol/L) and the CDC25 inhibitor BN82685 (125–500 nmol/L) for 5 days and evaluating their effects on cell proliferation. The combination of BN82685 and paclitaxel exhibited greater concentration-dependent inhibition of cell proliferation compared with each agent alone (Fig. 5A). However, the variation between ''observed data'' and ''calculated values'' using the model of Momparler (22) for *in vitro* evaluation of combination chemotherapy was not significantly different (Fig. 5B). In consequence, the combination was considered to have an additive effect on the HT29 proliferation at the presented concentrations. Similar conclusions were reached with higher concentrations of each agent (data not shown).

Discussion

In the search for new therapeutic avenues in cancer therapy, cell cycle regulatory proteins are now considered as potential and relevant targets. Major efforts have been made to identify compounds that inhibit CDKs or

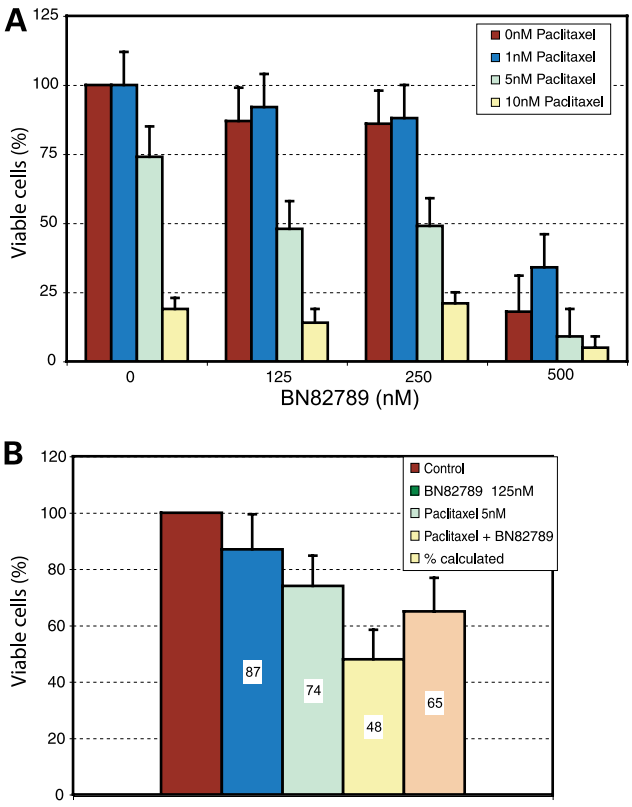


Figure 5. Additive effects of CDC25 inhibitors and microtubule-targeting drugs. **A,** HT29 human colon carcinoma cells were treated with increasing concentrations of BN82685 and paclitaxel for 5 d. Cell proliferation was measured using a WST1 assay, and the percentages of viable cells were compared with the controls. **B,** the ''observed data'' of the combination were compared with the ''calculated values'' according to the formula of Momparler (22).

their regulators to consequently cause cell cycle arrest and inhibition of proliferation (28). As major activators of CDKs, the CDC25 phosphatases have been recognized as promising targets and a number of inhibitory agents have already been identified (for review, see refs. 4, 9). These compounds are still under development, but there are already strong indications, such as their *in vivo* activity on xenografted tumor growth (14, 15), that they may be available for clinical studies in the near future. It is therefore of timely interest to consider the possibility of using these inhibitory compounds in association with other pharmacologic strategies.

Microtubule-targeting compounds are widely used in cancer chemotherapy and major benefits could be obtained with compounds such as paclitaxel. The main target of these inhibitors is the mitotic spindle, the assembly of which is considerably altered both by microtubule depolymerizing agents, such as *Vinca* alkaloids and their derivatives, and by microtubule bundling agents, such as paclitaxel (29).

In this study, we have investigated whether the use of a CDC25 inhibitor could have an additive effect to that obtained with microtubule-targeting compounds and have found this to be the case, along with several other key findings. First of all, we found that interphase cells treated with BN82685 displayed an abnormal organization of the microtubule network and that repolymerization of microtubules from the centrosomes after cold shock–induced disassembly, was significantly impaired. Furthermore, we show by time-lapse videomicroscopy that interphase microtubule dynamic instability is abolished in CDC25 inhibitor–treated cells. Taking into account these observations, we argued that a compound such as BN82685 might also affect the organization of the mitotic spindle and the progression into mitosis. We made use of an original assay adapted from ref. (23), that allowed us to focus on the behavior of cells progressing from a nocodazole-induced prometaphase arrest to the formation of a metaphase plate. We found that inhibiting CDC25 activities in cells that were already engaged in mitosis delayed the assembly of the mitotic spindle and the capture and positioning of the chromosomes along the metaphase plate. This result would be surprising if one were to consider a simplistic model in which after CDC25-dependent activation of CDK-cyclin complexes at the G_2-M transition, mitosis could freely proceed. However, recent data suggesting that CDC25A, CDC25B, and CDC25C play more complex roles during mitosis, rules out this model and indicates that CDC25 activities are necessary for the duration of mitosis, and are probably required for local and discrete activation of a subpopulation of CDKs. The data we report here therefore suggest that CDC25 phosphatase activities are probably required to activate CDKs and modulate microtubule dynamics, thus, contributing to the correct assembly of the mitotic spindle and the even segregation of chromosomes.

Taking these data into account, we have investigated the effects of the association of CDC25 inhibitors with microtubule-targeting agents. We report that treatment with BN82685, together with paclitaxel, has an additive effect on the proliferation of human HT29 colon cancer cells. We therefore propose that the combination between a microtubule-targeting agent, such as paclitaxel, and a CDC25 phosphatase-targeting compound might represent a promising combination in cancer therapy. Further investigation will be required to strengthen these observations and validate the interest of using such drug combinations *in vivo* on animal models.

Acknowledgments

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