Inhibition of human tumor cell growth *in vivo* by an orally bioavailable inhibitor of CDC25 phosphatases

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Abstract

Cell cycle regulators, such as the CDC25 phosphatases, are potential targets for the development of new anticancer drugs. Here we report the identification and the characterization of BN82685, a quinone-based CDC25 inhibitor that is active *in vitro* and *in vivo*. BN82685 inhibits recombinant CDC25A, B, and C phosphatases *in vitro*. It inhibits the growth of human tumor cell lines with an IC_{50} in the submicromolar range, independently of their resistance to chemotherapeutic agents. This inhibitory effect is irreversible on both the purified CDC25 enzyme *in vitro* and on tumor cell proliferation. The specificity of BN82685 towards the CDC25 phosphatases is shown by an increase in cyclin-dependent kinase 1 tyrosine 15 phosphorylation, by the reversion of the mitosis-inducing effect of CDC25B overexpression in HeLa cells, and by the lack of a growth inhibitory effect in an assay based on the use of a CDC25-independent fission yeast model. Finally, when administered p.o., BN82685 is shown to inhibit the growth of the human pancreatic tumor Mia PaCa-2 xenografted in athymic nude mice. BN82685 is therefore a promising new compound targeting CDC25, which confirms the interest of the inhibition of these enzymes as an anticancer therapeutic strategy. [Mol Cancer Ther 2005;4(9):1378–87]

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Introduction

The proposal of targeting the cell cycle machinery for the treatment of human disease is receiving increasing interest, and a number of small molecules designed to modulate cell cycle control are already under development as therapeutic agents (for review, see ref. 1). These novel compounds promise to open a new era in cancer therapy using drugs that will target the cell cycle deregulation, which is the hallmark of tumor cells.

Among the main regulators of cellular proliferation that have been the focus of a great deal of interest are the CDC25 phosphatases. These enzymes are involved in the activation of the cyclin-dependent kinases (CDK), the central regulators of the cell division cycle, at the major cell cycle phase transitions (for review, see refs. 2, 3). For instance, at the onset of mitosis, tyrosine 15 of CDK1 is dephosphorylated, thereby leading to the activation of the CDK1-cyclin B1 complex, which then phosphorylates a number of mitotic substrates.

There are three members of the human CDC25 family. CDC25A seems to control the activity of the CDKs both at G_1-S and at mitosis (4). CDC25B and CDC25C are involved in the control of the transition from G_2 to mitosis (2); however, there are also some reports of their involvement in S phase (5, 6). The complexity of the analysis of the substrate specificity of the CDC25 phosphatases is probably related to the existence of a very large number of CDC25A, B, and C isoforms. These multiple phosphatases are generated both at the transcriptional level, with numerous splice variants existing (7–10), and at the posttranslational level by phosphorylation events (see for instance refs. 4, 11, 12) that are likely to modulate their activity or/and substrate specificity. The specific role of these variants remains to be elucidated and is the focus of current research.

Overexpression of members of the CDC25 family has been found in many different human tumors such as breast cancer, pancreatic ductal adenocarcinoma, prostate cancer, non-Hodgkin's lymphoma, esophageal squamous cell carcinoma, and non–small-cell lung cancer (for further details, see ref. 2 for an excellent review). In a significant number of these cases, a correlation between the overexpression of CDC25A or CDC25B and the prognosis was observed. Recently, it has been reported that CDC25 inhibitors were able to reduce the growth of pancreatic cell lines that expressed high levels of CDC25B (13). Although these studies remain to be completed with a detailed analysis of the involvement of CDC25 expression in tumorigenesis, their findings support the idea that the inhibition of the CDC25 phosphatases may represent a potent novel therapeutic approach for the treatment of cancer.

Over the last few years, efforts in that direction have led to the identification of novel classes of CDC25 inhibitory molecules that are efficient inhibitors of cell cycle progression *in vitro* (2, 14). More recently, several interesting new inhibitor families have been reported, including the indolyl-dihydroxy-quinones (15) and naphtofurandione (16). Both series of compounds have been shown to bind within the pocket adjacent to the active site. We have also reported the characterization of BN82002, a new compound that is not only active against the CDC25 phosphatases *in vitro* but is also inhibitory for tumor growth *in vivo* (17). The most recent inhibitors reported in the literature, the caulibugulones A to E, are novel isoquinoline quinones and iminoquinones that were isolated from an extract of the marine bryozoan *Caulibugula intermis* at the National Cancer Institute and shown to be potent and selective CDC25 inhibitors (18).

In this publication, we report the identification of BN82685, a novel CDC25 phosphatase inhibitor. This compound is shown to specifically inhibit CDC25 *in vitro* and in cultured cells within the nanomolar range. Furthermore, when administered p.o., BN82685 inhibits tumor cell growth *in vivo* in xenografted athymic nude mice.

Materials and Methods

Preparation of BN82685

N,N-Dimethylethylenediamine (2.5 mL, 23 mmol) was added to a mixture of 5-methoxy-2-methyl-4,7-dioxobenzothiazole (3.2 g, 15.3 mmol; ref. 19) in anhydrous methanol (10 mL). The resulting mixture was stirred for 2 hours at 60°C and concentrated to give a red viscous oil. Purification by chromatography over silica gel using 3% methanol in dichloromethane gave 2.6 g (65%) of a red powder: mp 142°C to 143°C; ^{1}H nuclear magnetic resonance (DMSO) δ 7.35 (t, J = 5.2, 1H), 5.48 (s, 1H), 3.26 to 3.20 (m, 2H), 2.75 (s, 3H), 2.49 to 2.47 (m, 2H), 2.18 (s, 6H). To obtain reproducible crystalline forms, various salified forms of BN82685 were prepared. The hydrochloride and benzoate salts showed good physicochemical properties and their preparation is reported below.

For the preparation of the hydrochloride salt, 1.2 equivalents of a 1 N solution of HCl in diethylether were added to an acetone solution BN82685. The hydrochloride salt immediately precipitated as a red powder: mp 176°C to 178°C; ^{1}H nuclear magnetic resonance (DMSO) δ 10.00 (s, 1H), 7.79 (t, J = 6.0, 1H), 5.68 (s, 1H), 3.59 to 3.55 (m, 2H), 3.32 to 3.27 (m, 2H), 2.80 (s, 6H), 2.76 (s, 3H).

For the preparation of the benzoate salt, 1.1 equivalents of benzoic acid in acetone were added to an acetone solution of BN82685. The benzoate salt immediately precipitated as a red powder: mp 178°C to 180°C; ^{1}H nuclear magnetic resonance (DMSO) δ 7.94 to 7.93 (m, 2H), 7.62 to 7.58 (m, 1H), 7.50 to 7.46 (m, 2H), 7.36 (t, J = 5.3, 1H), 5.49 (s, 1H), 3.25 to 3.21 (m, 2H), 2.75 (s, 3H), 2.53 to 2.50 (m, 2H), 2.20 (s, 6H).

Cell Culture Conditions

Human cell lines were purchased from the American Type Culture Collection (Rockville, MA). They were cultivated in DMEM or RPMI 1640 with the addition of 10% FCS, glutamine, and penicillin/streptomycin at 37°C in a 5% CO_2 atmosphere. HeLa cells were transfected using Exgen-500 (Euromedex, Souffelweyersheim, France) following the instructions of the manufacturer. Cell cycle synchronization was achieved as previously described (20).

In vitro CDC25 Phosphatase Assays

The activity of a maltose binding protein-CDC25C recombinant enzyme produced in bacteria (GTP Technology, Labège, France) was monitored using 3-*O*-methylfluorescein phosphate. The assay was done in 384-well plates in a final volume of 50 μL. The maltose binding protein-CDC25 proteins were stored in elution buffer [20 mmol/L Tris-HCl (pH 7.4), 250 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, 10 mmol/L maltose]. They were diluted in assay buffer [50 mmol/L Tris-HCl (pH 8.2), 50 mmol/L NaCl, 1 mmol/L DTT, 20% glycerol]. The final concentrations were 158 to 172 ng/well for CDC25A, 84 ng/well for CDC25B2, 168 ng/well for CDC25B3, 350 ng/well for CDC25C, and 70 to 105 ng/well for the catalytic domain of CDC25C (CDC25C-cat). The concentrations of the enzyme were adjusted to obtain similar fluorescence values. Products were studied in a concentration range of up to 40 μmol/L. The reaction was initiated by the addition of 500 μmol/L of 3-*O*-methylfluorescein phosphate. After 4 hours at 30°C, 3-*O*-methylfluorescein fluorescence emission was measured with a Victor 2 plate reader (excitation filter 475 nm and emission filter 510 nm; Perkin-Elmer, Fremont, CA). The IC_{50} values were calculated from at least two independent experiments with one determination per tested concentration.

Reversibility assays were conducted by preincubating large amounts of CDC25C enzyme (3,500 ng/well) with increasing concentrations (37.5–1,000 nmol/L) of BN82685 hydrochloride salt for various durations (2–30 minutes). The mixture was then diluted 10-fold to decrease both the final CDC25C enzyme concentration to the usual level of 350 ng/well and the BN82685 concentrations to 3.75 to 100 nmol/L amounts before addition of 3-*O*-methylfluorescein phosphate. Incubations were then continued for a further 30 minutes before the fluorescence emission was measured. The results are representative of two independent experiments.

Cell Proliferation Assays

The inhibition of cellular proliferation was determined using a colorimetric assay (WST1) based on the cleavage of the tetrazolium salt WST1 by mitochondrial dehydrogenases in viable cells, leading to the formation of formazan (Roche Diagnostic, Meylan, France). On day 0, human tumor cell lines were seeded into 96-well plates in DMEM or RPMI 1640 supplemented with 10% FCS, 50,000 units/L penicillin, and 50 mg/L streptomycin. On day 1, the cells were treated with increasing concentrations of the drug for 96 hours. The number of cells to be seeded for each cell line had been previously determined to allow the untreated control cells to be in log phase growth at the end of the experiment. These experiments were done at least twice, with either four or eight determinations

per concentration of each compound tested. For each compound, values within the linear part of the sigmoid growth curve were included in a linear regression analysis and were used to estimate the IC_{50}.

Cells Extracts and Immunoblotting

Cells were harvested and lysed for 30 minutes with agitation at 4°C in lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 2.5 mmol/L sodium orthovanadate, 10 mmol/L NaF, 2 mmol/L phenylmethylsulfonyl fluoride, and a cocktail of protease inhibitors (Complete, Roche)]. After centrifugation for 10 minutes at 14,000 rpm, 50 mg of lysate were denatured at 95°C for 5 minutes, electrophoresed on a 7.5% SDS-PAGE gel, and analyzed by Western blotting using antibodies directed against cyclin B1 (GNS1, Santa Cruz Biotechnology, Santa Cruz, CA) and CDK1Y15-p (9111S, Cell Signaling, Beverly, MA).

Immunofluorescence Microscopy

Cells were seeded onto glass coverslips, then fixed and permeabilized 24 hours later as described (11). The antibodies against cyclin B1 (GNS1, Santa Cruz Biotechnology) were used. Images were acquired using the DMIRE2 and DM5000 microscopes (Leica, Rueil-Malmaison, France) fitted with a Roper COOLsnap ES CCD camera, and subsequently processed using the MetaMorph and Photoshop software packages.

Fission Yeast Growth Inhibition Assay

The strains used in this study were wild-type (*leu1-32 ura4 D18*), *cdc25* disruptant (*cdc2-3W cdc25::ura4 leu1-32 ura4 D18*), CDC25A humanized (*cdc25::hs*CDC25A *leu1-32 ura4 D18*), CDC25B1 humanized (*cdc25::hs*CDC25B1 *leu1-32 ura4 D18*), and CDC25B2 humanized (*cdc25::hs*CDC25B2 *leu1-32 ura4 D18*). Details of the construction of these strains can be found in refs. 21–23.

A volume of 200 μL of exponentially growing cells (10^6 cells/mL) was grown at 30°C in 96-well plates with increasing concentrations of BN82685. The inhibition of proliferation was measured by counting the cells after 4 hours of treatment.

Human Tumor Xenografting into Nude Mice

Cells of the human pancreatic carcinoma cell line Mia PaCa-2 were injected s.c. into the flanks of 4- to 6-week-old female athymic NCr-*nu/nu* mice. Tumors were allowed to reach a volume of 75 mm^3. Once the tumors were established, treatment was started by the p.o. administration. Six animals per group were treated with the BN82685 benzoate salt dissolved in water, at doses of either 10 or 15 mg/kg twice a day for 2 days, followed by 5 days without treatment, with three cycles of treatment. Tumor measurements and animal weights were monitored and recorded twice a week. Animal care was in accordance with IPSEN Biomeasure institutional guidelines.

Results

BN82685 Inhibits the Activity of Recombinant CDC25 Phosphatase

The hydrochloride and benzoate salt forms of BN82685 (Fig. 1A) inhibited the phosphatase activity of the purified recombinant fusion protein maltose binding protein-CDC25C in a concentration-dependent manner, with an IC_{50} in the nanomolar range (Table 1). Comparable inhibitory effect was observed when the CDC25C catalytic domain alone was challenged with BN82685 salt forms or when recombinant CDC25A, CDC25B2, and CDC25B3 were tested (Table 1). These results indicated that both salts, hydrochloride and benzoate, were equally active against all members of the CDC25 family.

BN82685 Inhibits CDK1 Dephosphorylation and Delays Entry into Mitosis

The CDC25 phosphatases are known to play an essential role in the control of CDK activity at mitosis. We therefore examined the immediate effect of the BN82685 hydrochloride salt on this key cell cycle step. First, histone H3 phosphorylation, a convenient marker for the detection of mitotic cells, was monitored by flow cytometry. As shown in Fig. 1B, a dose-dependent inhibition of mitosis by BN82685 was rapidly observed. During the same experiment, to directly assess the effect of the inhibition of CDC25 activity by 500 nmol/L BN82685, the level of phosphorylation of CDK1 on tyrosine 15 was examined by Western blot analysis. As shown in Fig. 1C, tyrosine 15 phosphorylation of CDK1 increased in parallel with the inhibition of entry into mitosis. A similar inhibition of CDK1 tyrosine 15 phosphorylation was observed in the human pancreatic tumor cell line Mia PaCa-2 treated with 500 nmol/L BN82685 (data not shown).

The expression level of cyclin B1 was also examined in the same experiment both by Western blot (Fig. 1C) and by immunofluorescence staining (Fig. 1D). Cyclin B1 accumulated with time, indicating that cells were prevented from entering mitosis by inhibition of CDC25. Whereas control cultures displayed the classic staining patterns of G_2 phase cells with cytoplasmic cyclin B1 staining, and mitotic cells with high cyclin B1 levels, cells treated for 4 hours with 500 nmol/L BN82685 accumulated in G_2 phase, with cyclin B1 located in the cytoplasm, indicative of a G_2 phase arrest.

Altogether, these observations suggested that BN82685 acted as an inhibitor of the CDC25 phosphatases in cultured cells.

BN82685 Inhibits Cell Cycle Progression

HeLa cells were used to investigate the effect of the BN82685 on cell cycle progression. Flow cytometry after propidium iodide staining was used to monitor the DNA content of the cells after treatment with BN82685, reflecting their cell cycle status. As shown in Fig. 2A, increasing concentrations of the drug affected the cell cycle distribution with a progressive decrease in the numbers of cells in S phase and an accumulation of cells with either G_1 or G_2 phase DNA contents, suggesting that BN82685 was impairing cell cycle progression at various stages of the cell cycle.

To analyze the effect of BN82685 on cell cycle progression in more detail, we made use of HeLa cells that were synchronized in late G_1 phase by double thymidine block and release. As shown in Fig. 2B, after the double thymidine block, these cells were fully synchronized with

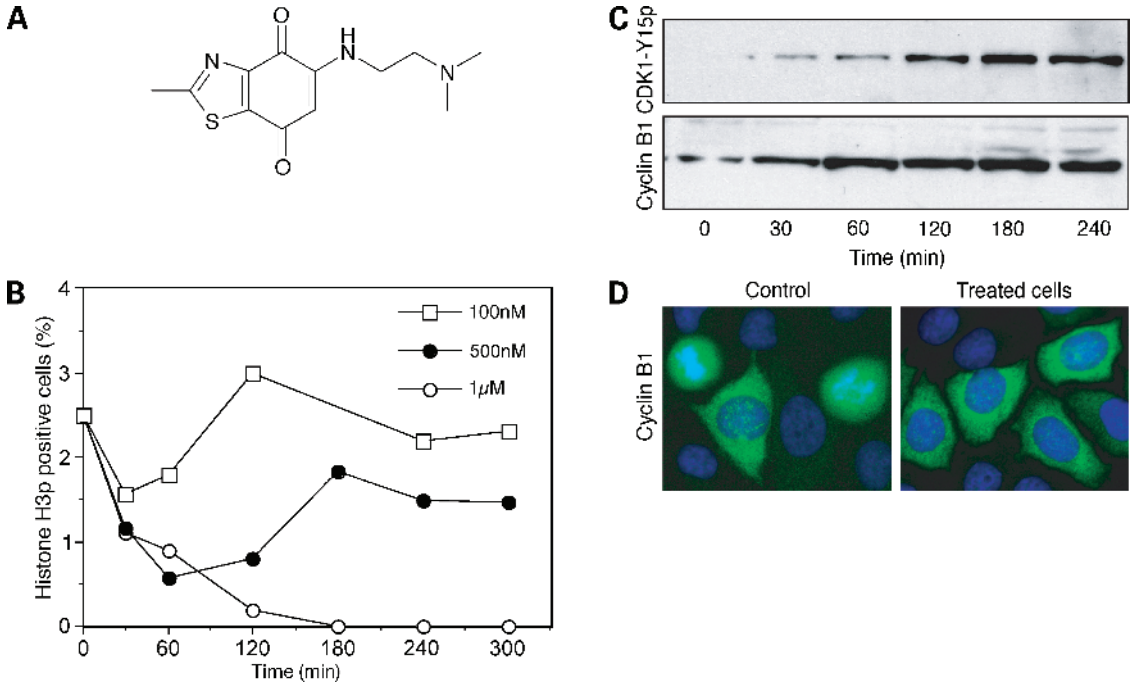

Figure 1. BN82685 inhibits CDK1 tyrosine 15 dephosphorylation and delays entry into mitosis. **A,** chemical structure of BN82685. **B – D,** exponentially growing HeLa cells were treated with 100 nmol/L, 500 nmol/L, or 1 μmol/L of BN82685 for the indicated times. **B,** cells were harvested and subjected to flow cytometry analysis after staining with antibodies raised against phosphorylated histone H3. Data were collected on a BD FACSCalibur. **C,** samples from cells treated with 500 nmol/L BN82685 were collected and processed for Western blot analysis with antibodies raised against tyrosine 15 – phosphorylated CDK1 and cyclin B1. **D,** either untreated HeLa cells or cells that had been treated 4 h with 500 nmol/L BN82685 were fixed, permeabilized, and stained with monoclonal antibodies raised against cyclin B1.

about 80% of the population displaying a G_1 phase DNA content. Ten hours after release, most of the control cells treated with DMSO alone were in G_2 phase (80%). Cells that were treated with increasing concentrations of BN82685 in G_1 phase were delayed in their progression into S phase and remained blocked in G_1 when high concentrations of BN82685 were used. When HeLa cells were treated with BN82685 in G_2 phase (i.e., 10 hours after synchronization; Fig. 2C), those that had been treated with the highest concentrations of BN82685 were unable to progress through the cell cycle and remained blocked in G_2 phase, whereas the control DMSO-treated cells were able to complete mitosis within 10 hours and to undergo the following cell cycle. These experiments indicate that BN82685 is able to delay progression at various stages of the cell cycle including G_1-S and G_2-M transition.

Table 1. BN82685 inhibits purified recombinant members of CDC25 family

	BN82685 hydrochloride salt		BN82685 benzoate salt	
	IC_{50} (nmol/L)	SE	IC_{50} (nmol/L)	SE
CDC25C	171	2	201	37
CDC25C-cat	179	70	117	11
CDC25A	251	80	109	5
CDC25B2	250	8	160	21
CDC25B3	260	41	249	56

NOTE: Enzyme inhibition was assayed on recombinant maltose binding protein-CDC25C, maltose binding protein-CDC25C catalytic domain (CDC25C-cat), maltose binding protein-CDC25B2, and maltose binding protein-CDC25B3 using 3-*O*-methylfluorescein phosphate as a substrate. The IC_{50} (in nanomolar) values were calculated from at least three independent experiments with one determination per concentration tested.

BN82685 Reverts the Mitosis-Inducing Effect of CDC25B

We have previously reported that the specificity of the inhibition of CDC25B phosphatase activity can be determined in cultured cells by testing the ability of a compound to revert the entry into an abnormal mitotic phenotype, described as premature chromosome condensation, which is observed on massive CDC25B overexpression (17, 24).

HeLa cells were transiently transfected with an YFP-tagged CDC25B-S353E mutant expression plasmid, then treated 12 hours later for an additional 12 hours with BN82685. The CDC25B-S353E mutant was used as we have recently shown that its mitosis-inducing activity is greater than that of wild-type CDC25B (11). The percentage of abnormal mitotic figures was determined in the transfected cells expressing CDC25B (YFP-CDC25B positive cells). Thirty-nine percent of the CDC25B transfected cells displayed abnormally condensed chromatin 24 hours after transfection (Fig. 3A). DMSO had no influence on this

result (data not shown). In cells treated with BN82685, the percentage of cells showing premature chromosome condensation decreased in a dose-dependent manner, with only 12% displaying premature chromosome condensation at a concentration of 1 μmol/L, which represents a 70% inhibition of the mitosis-inducing effect of CDC25B overexpression (Fig. 3A). This result indicates that treatment with BN82685 (either the salt or base form) is able to counteract the mitosis-inducing activity of CDC25B phosphatase, and suggests that CDC25 is the major or the only target of this compound as far as mitosis is concerned.

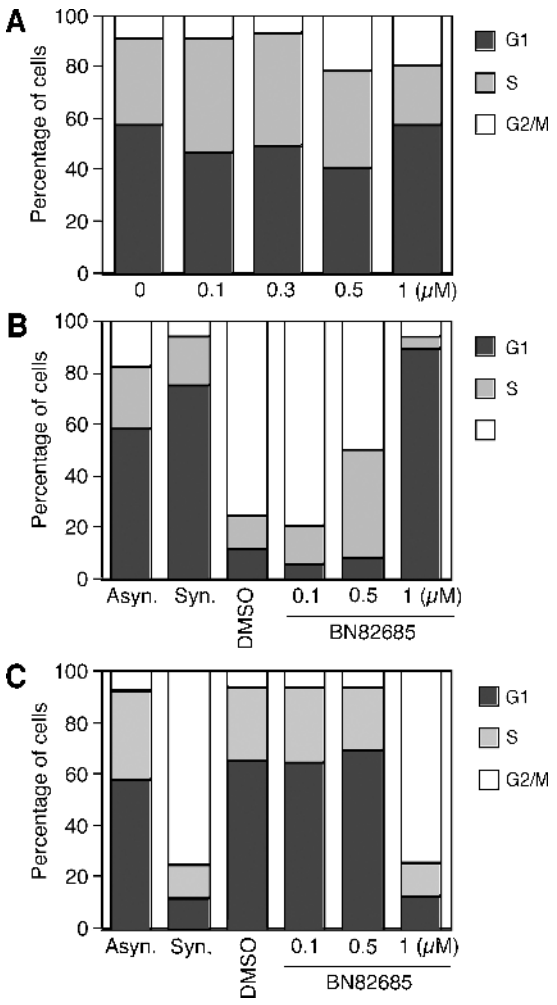

Figure 2. BN82685 inhibits cell cycle progression. **A,** exponentially growing HeLa cells were treated with BN82685 for 24 h. Cells were then harvested and subjected to flow cytometry analysis of their DNA content after propidium iodide staining. Data were collected on a BD FACSCalibur and analyzed using the Modfit software (Verity Software). **B** and **C,** HeLa cells were synchronized in G_1 phase by a double thymidine block protocol then either treated immediately (**B**) or released for 8 h (**C**) to progress in G_2 phase before the addition of BN82685. Cells synchronized (*Syn.*) in G_1 (**B**) or G_2 (**C**) were treated for 10 h with the indicated concentration of BN82685 or DMSO. Cells were then harvested and subjected to flow cytometry analysis of their DNA content after propidium iodide staining. Data were collected and analyzed as in **A**. *Asyn.,* control asynchronous untreated cells.

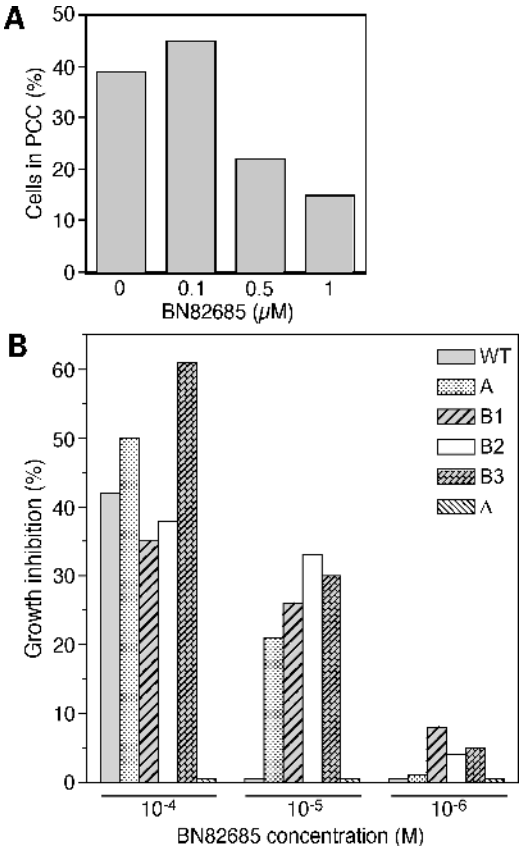

Figure 3. Target specificity of BN82685. **A,** BN82685 reverts CDC25B mitotic inducing activity in HeLa cells. HeLa cells were transiently transfected (see Materials and Methods) with a plasmid allowing the expression of a YFP-tagged version CDC25B-S353E and treated 12 h later for an additional 12 h with the indicated concentration of the BN82685 hydrochloride salt. The cells were fixed and the percentage of transfected cells (YFP-positive cells) in mitosis among at least 300 cells was determined by fluorescence microscopy after 4′,6-diamidino-2-phenylindole staining. **B,** BN82685 inhibition of yeast proliferation depends on CDC25. Exponentially growing *S. pombe* wild type (*WT*), CDC25A humanized (*A*), CDC25B1 humanized (*B1*), CDC25B2 humanized (*B2*), CDC25B3 humanized (*B3*), and *cdc25* disruptant (Δ) cells were used in this experiment. Cells were treated for 4 h with the indicated concentrations of BN82685. Cell growth was monitored by cell counting and the percentage of inhibition was indicated. Representative of 3 to 4 independent experiments.

Use of a Yeast Model to Validate the Target Specificity of BN82685

A yeast-based assay was used to validate the specificity of BN82685 for the CDC25 phosphatases (22). This assay is based on the use of fission yeast strains expressing human CDC25 (A, B1, B2, and B3; refs. 21–23) or genetically engineered to grow in a CDC25-independent manner. This strain does not express an endogenous CDC25 phosphatase but is able to proliferate due to a mutation in cdc2 (*cdc2-3w*) that renders it insensitive to regulation by phosphorylation/dephosphorylation. As shown in Fig. 3B, the inhibition of yeast proliferation by the hydrochloride salt of BN82685 was completely dependent on the expression of the CDC25 target because the growth of the parental *cdc25*

Table 2. BN82685 inhibits the growth of human cell lines

Human cell lines		BN82685 IC_{50} (nmol/L)	
		Hydrochloride salt	Benzoate salt
Human diploid fibroblasts	IMR-90	1,000	ND
Cervix carcinoma	HeLa	350	ND
Pancreas Cancer	Mia PaCa-2	118	185
Hormone-resistant prostate cancer	DU-145	90	115
Hormone-sensitive prostate cancer	LNCaP	ND	358
Hormone-sensitive breast cancer	MCF-7	245	ND
Hormone-resistant breast cancer	MDA-MB231	380	ND
Hormone-resistant breast cancer	BT20	265	ND
Hormone-sensitive breast cancer	T47D	565	ND
Osteocarcinoma	U2OS	250	ND
Glioblastoma	U-87 MG	ND	263
Small cell lung carcinoma	NCI-H69	ND	161
Lung carcinoma	A-427	ND	258
Colon adenocarcinoma	HT-29	ND	397
Colon adenocarcinoma	Caco-2	ND	196
Primary colon tumor	SW480	ND	138
Metastasis from SW480	SW620	ND	150
Melanoma metastasis	A2058	ND	134

NOTE: Growth inhibition of the indicated cell lines for the hydrochloride and benzoate salts was assayed using the WST1 cell proliferation assay. The IC_{50} (in nanomolar) values reported were calculated from at least two independent experiments with eight determinations per concentration tested. ND, not determined.

disruptant strain was not impaired. In contrast, CDC25A and CDC25B humanized strains were found to be slightly more sensitive to BN82685. Because of the low permeability of the yeast cell wall, the concentrations used to inhibit fission yeast growth were higher than those used to inhibit CDC25 phosphatases *in vitro* or in human tumor cells in culture (see below). Although done in a simple unicellular eukaryotic organism, this experiment provides additional experimental evidence for the specific targeting of the CDC25 phosphatases by BN82685.

BN82685 Inhibits Cellular Proliferation *In vitro*

The effect of BN82685, either in its hydrochloride or benzoate salt form, on cell proliferation was evaluated *in vitro* on several human normal and tumor cell lines (Table 2). Whenever both salts were tested in parallel, the hydrochloride and benzoate salts showed equivalent activity on cellular proliferation. All the cell lines examined were sensitive to BN82685, in the range of 250 nmol/L to 1 μmol/L. The most sensitive lines were DU-145, Mia PaCa-2, and the highly aggressive metastatic melanoma cell line A2058 with IC_{50} of 90, 118, and 134 nmol/L, respectively, and the least sensitive cells were the IMR-90 normal fibroblasts with an IC_{50} of 1 μmol/L. BN82685 was as active on the primary colon tumor line SW480 as on its metastatic cell line SW620. There was no evidence of detectable correlation between the *in vitro* IC_{50} and the hormone sensitivity status of any of the cell lines we tested, including MCF-7, MDA-MB231, BT20, and T47D. All of these lines displayed similar sensitivities to BN82685 (Table 2).

We also found that BN82685 was equally active on the HL60 acute promyelocytic leukemia cell line and its cytotoxic agent-resistant derivative lines expressing multidrug resistance protein or P-glycoprotein that renders them resistant to daunorubucin and Adriamycin, respectively. Similarly, BN82685 was active on HL60 cells resistant to mitoxantrone and on CCRF lymphoblastic leukemia cells, independently of their resistance status to etoposide (Table 3).

BN82685 Irreversibly Inhibits CDC25 *In vitro* and in Cultured Cells

We first investigated whether the effect of BN82685 was reversible. The basis of this experiment was to preincubate

Table 3. BN82685 inhibits the growth of human cell lines resistant to cytotoxic drugs

Human cell lines		IC_{50} (nmol/L)				
		BN82685	DNR	ADR	MTZ	VP-16
Acute promyelocytic leukemia*	HL60	607	32	88	ND	ND
Acute promyelocytic leukemia resistant to daunorubicin	HL60-DNR(pgp)	610	1,100	3,100	ND	ND
Acute promyelocytic leukemia resistant to Adriamycin	HL60-ADR(MRP)	643	694	2,430	ND	ND
Acute promyelocytic leukemia*	HL60	480	ND	ND	<100	ND
Acute promyelocytic leukemia resistant to mitoxantrone	HL60/MX2	480	ND	ND	600	ND
Acute lymphoblastic leukemia	CCRF-CEM	300	ND	ND	ND	160
Acute lymphoblastic leukemia resistant to VP-16	CCRF-CEM/C2	290	ND	ND	ND	1,810

NOTE: Growth inhibition of the indicated cell lines was assayed using the WST1 cell proliferation assay. The IC_{50} (in millimolar) values reported were calculated from two independent experiments with four or eight determinations per concentration tested (see Materials and Methods for details).
Abbreviations: DNR, daunorubicin; ADR, Adriamycin; MTZ, mitoxantrone; VP-16, etoposide; MRP, multidrug resistance protein; pgp, P-glycoprotein; ND, not determined.
*The results presented for the acute promyelocytic leukemia cell line HL60 were obtained from two sets of independent experiments.

the phosphatase with the inhibitor at an active concentration, then to dilute the reaction mixture to a concentration at which the inhibitor is inactive. The rationale was that if the inhibitory effect was irreversible, it should persist after the dilution procedure. Large amounts of CDC25C (3,500 ng/well) were incubated with increasing concentrations (37.5–1,000 nmol/L) of BN82685 for increasing durations (2–30 minutes). The reaction mix was subsequently diluted 10-fold to decrease the final concentrations of CDC25C and of the inhibitor to 350 ng/well and 3.75 to 100 nmol/L, respectively, before determination of the phosphatase activity (Fig. 4A). At concentrations ranging from 37.5 nmol/L to 1 μmol/L, BN82685 hydrochloride salt inhibited CDC25C up to 46% at 1 μmol/L. BN82685 was inactive when used at the 10-fold diluted concentration without the preincubation step. When a 2- to 5-minute preincubation was done before dilution, BN82685 was more active. Maximal inhibitory activity (100%) was observed after 20 minutes of preincubation with 1 μmol/L BN82685 (100 nmol/L after dilution). The inhibition of CDC25C by BN82685 at these dilute concentrations at which BN82685 is inactive without the preincubation step indicates that the effect of BN82685 is achieved through the interaction of the inhibitor with CDC25 during the preincubation step. Because this effect is measured at least 30 minutes after the end of the incubation under dilute conditions, it is reasonable to postulate that BN82685 is an irreversible inhibitor.

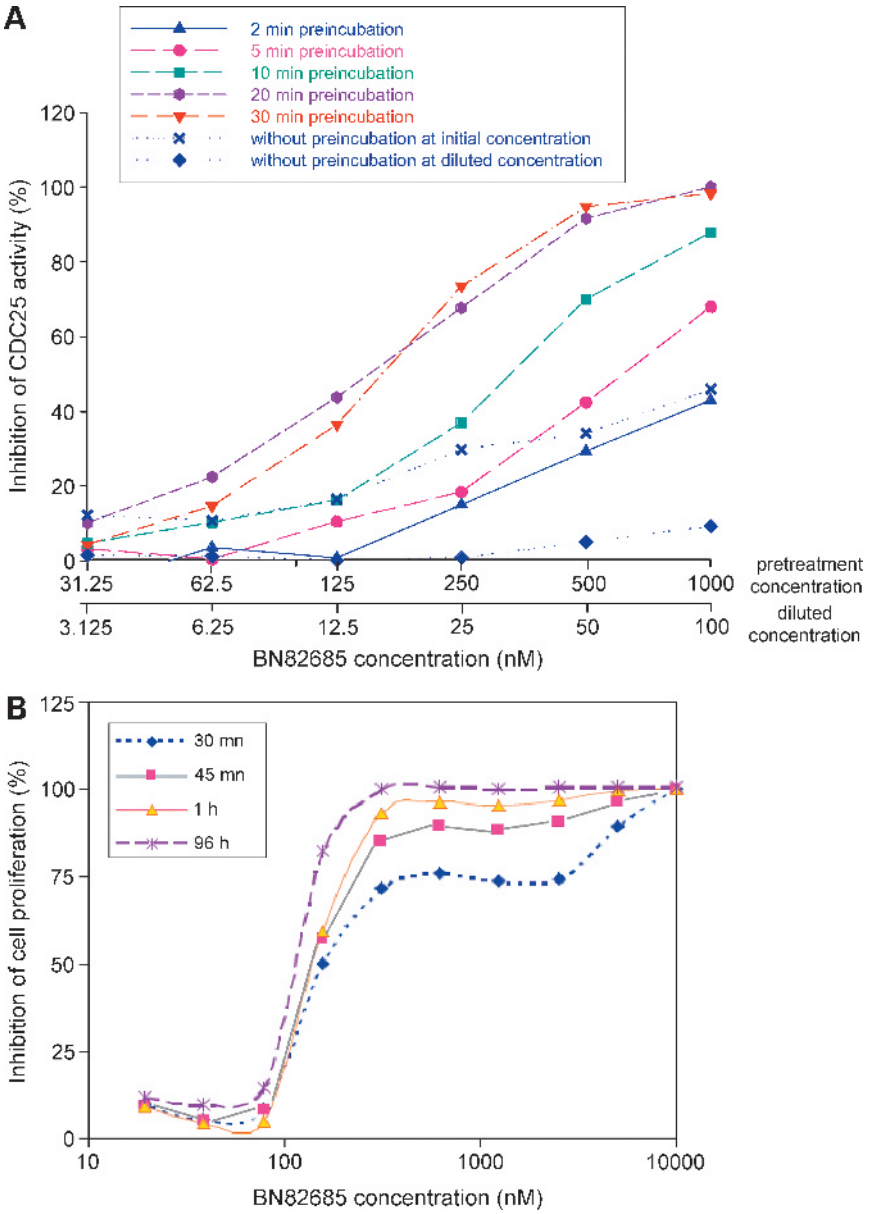

Figure 4. BN82685 hydrochloride irreversibly inhibits CDC25 *in vitro* and in cultured cells. **A,** reversibility assays were conducted by preincubating large amounts of CDC25C enzyme (3,500 ng/wells) with increasing concentrations (37.5 – 1,000 nmol/L) of the BN82685 hydrochloride salt for various lengths of time (2 – 30 min). The reaction mix was then diluted 10-fold to decrease both the final CDC25C enzyme concentration to 350 ng/well and the BN82685 concentrations to between 3.75 and 100 nmol/L before adding the substrate 3-*O*-methylfluorescein phosphate. The reactions were incubated a further 30 min before determination of the phosphatase activity. Representative of two independent experiments. **B,** Mia PaCa-2 cells were treated for 30 min, 45 min, 1 h, or 96 h with increasing concentrations of the drug (up to 10 μmol/L). After the drug treatment, the medium was removed, the cells were washed with PBS, and the fresh medium was added. The inhibition of cellular proliferation was determined using the WST1 colorimetric assay 96 h after the beginning of the treatment. The IC_{50} values were calculated from two independent experiments with eight determinations per concentration tested. The calculated IC_{50} values at 30 min, 45 min, 1 h, and 96 h were 154, 141, 150, and 112 nmol/L respectively.

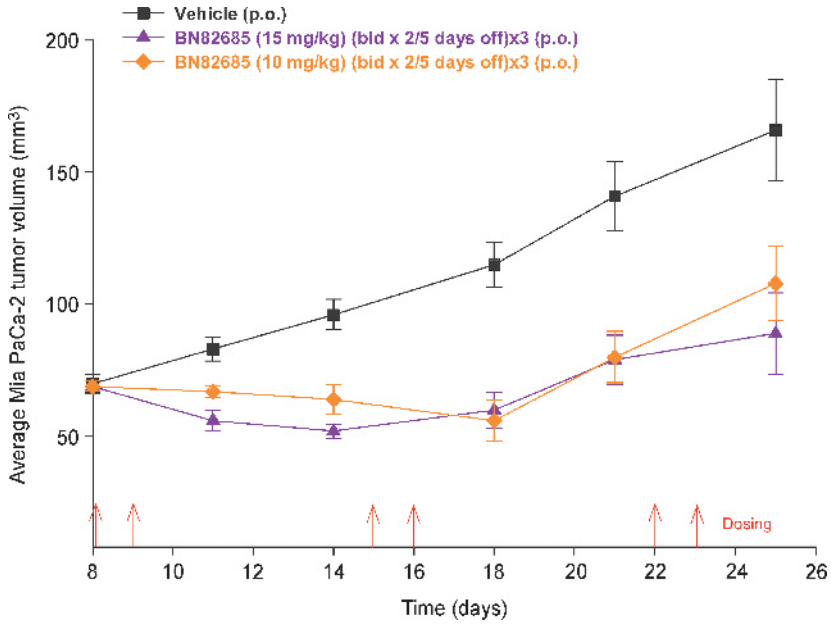

Figure 5. Growth inhibition of human tumor in nude mice treated with BN82685 by p.o. administration. Cells of the human pancreatic carcinoma cell line Mia PaCa-2 were injected s.c. into the flank of female athymic mice. The resulting tumors were allowed to reach a volume about 75 mm^3. Once the tumors were established, treatment of Mia PaCa-2 xenografted mice was started by the p.o. administration at 10 or 15 mg/kg (twice a day for 2 d followed by 5 d without treatment, *arrows*) with three cycles of treatment (*bid*, twice a day).

We next examined whether a transient treatment was sufficient to observe an inhibitory effect on cell growth. For this, we determined the minimal incubation time leading to growth inhibition of cultured cells. DU-145 cells were treated for 30 minutes, 45 minutes, 1 hour, and 96 hours with BN82685 and the effect on their growth was examined after 96 hours. The IC_{50} values at 30 minutes, 45 minutes, 1 hour, and 96 hours were 150, 140, 150, and 110 nmol/L, respectively. The effect of BN82685 was therefore similar after 1 or 96 hours of treatment (Fig. 4B), which indicates that BN82685 has a rapid and irreversible effect on cell proliferation.

Transient *In vivo* Growth Inhibition of Human Tumor Cells Xenografted in Nude Mice

The human pancreatic tumor cell line Mia PaCa-2 was implanted s.c. into nude mice. When the resulting tumors reached a volume of about 75 mm^3, treatment with BN82685 was initiated by the p.o. administration at a dose of either 10 and 15 mg/kg, twice a day for 2 days, followed by 5 days without treatment, with three cycles of treatment. A very limited body weight loss was observed during the treatment with a full and rapid recovery on arrest. This treatment led to stabilization of the growth of the Mia PaCa-2 tumors during the first 10 days of treatment, after which the tumors started to grow again (Fig. 5).

Discussion

The CDC25 phosphatases are currently being considered as attractive targets for the development of new chemotherapeutic agents. A number of recent publications witness the collective drug discovery effort being directed towards the identification of novel compounds that not only inhibit CDC25 activity *in vitro* and in cultured cells but are also active against human tumors *in vivo*.

The new compound we describe here, BN82685, fulfills these objectives. We have been able to show that BN82685, whatever the salt form used, inhibits the activity of purified recombinant CDC25A, B, or C enzymes *in vitro*. The *in vitro* IC_{50} for BN82685 is within the range of 100 to 300 nmol/L, which qualifies this inhibitor as a member of the group of the most potent compounds published to date. BN82685 is also active against cultured tumor cells, with an IC_{50} in the same range. The most sensitive cell lines were DU-145, Mia PaCa-2, and A2058 metastatic, whereas the least sensitive were the human primary fibroblasts (IC_{50}: 1 μmol/L). The inhibition of purified CDC25C by BN82685 was found to be irreversible, as dilution to subactive concentrations after preincubation at a higher concentration resulted in the inactivation of the enzyme. This result was reinforced by cell proliferation studies that showed that a relatively short treatment time (1 hour) with BN82685 was sufficient to arrest proliferation for 96 hours. Because CDC25 phosphatases have short half-lives, inhibition by BN82685 long after elimination suggests that the compound might stay long within the cell. Further studies will be required to know more about its intracellular metabolism.

The demonstration of the target specificity of any new CDC25 inhibitor is a major issue. To validate BN82685, we investigated the effect of this compound using three different approaches. First, we were able to show that HeLa cells treated with BN82685 very rapidly stopped entering mitosis and accumulated a high level of tyrosine 15–phosphorylated CDK1. Second, we made use of the deleterious premature entry into mitosis phenotype that is observed on CDC25B overexpression to show that BN82685 treatment could at least partially revert a biological effect that is strictly dependent on CDC25

phosphatase activity. Third, the activity of BN82685 was tested on yeast strains expressing human CDC25 phosphatases or manipulated to become independent of CDC25 for progression through the cell cycle (21–23, 25). We were able to show that the inhibition of yeast proliferation by BN82685 was completely dependent on the expression of the CDC25 target. The concentrations used to inhibit fission yeast growth were higher than those used to inhibit the CDC25 phosphatases *in vitro* or in human tumor cells in culture, probably reflecting the general impermeability of the yeast cell wall to the drug. Taken together, we believe that these three experiments strongly support the idea that the main cellular targets of BN82685 are the CDC25 phosphatases.

As we have reported, in our *in vitro* assays, BN82685 does not specifically inhibit any one particular member of the CDC25 family. This result is consistent with the observations that we have made on HeLa cells in culture. Treatment of these cells with BN82685 led to growth inhibition and cell cycle arrest at each of the major cell cycle phase transitions, thus not obviously modifying the general cell cycle distribution. However, when HeLa cells were synchronized before treatment with BN82685, we observed that they were delayed or arrested at the G_1-S and G_2-M transitions, where it is known that the CDC25 phosphatases play essential roles. BN82685, like most of the CDC25 inhibitors identified thus far, is not specific for any of the CDC25 isoform. The necessity of development of compounds specific for one of the three human CDC25 phosphatases is still a matter of debate. We believe that a broad CDC25 inhibitory effect might be a more efficient way to control cell proliferation through the targeting of multiple cell cycle essential steps.

We have tested a large panel of human cell lines for their sensitivity to BN82685, including several hormone-sensitive and hormone-resistant cell lines originating from the prostate and the breast, to address the issue of the dependency on hormonal status. As we have shown here, there was no difference in sensitivity to BN82685 between the hormone-resistant and hormone-sensitive cell lines, thus indicating that although CDC25B has been reported to interact with both the estrogen and androgen receptors and to act as a transcriptional coactivator (26, 27), the efficiency of these inhibitors does not seem to be affected by hormonal status.

We also examined whether the sensitivity to BN82685 was affected in cell lines that have been selected on the basis of their resistance to chemotherapeutic agents such as daunorubicin, Adriamycin, mitoxanthrone, or etoposide. As reported, growth inhibition by BN82685 was clearly found to be unaffected and, therefore, independent of classic mechanisms of resistance by drug efflux pumps such as P-glycoprotein or multidrug resistance protein.

As BN82685 is a quinone, an important issue is to examine whether DT-diaphorase, an enzyme that reduces quinone to hydroquinone that is often overexpressed in tumors (28, 29), could modulate the antiproliferative activity of BN82685. Preliminary experiments that have to be confirmed suggest that the expression of this reducing enzyme is not limiting for the clinical benefit of compounds derived from BN82685.

Finally, we examined the efficiency of BN82685 *in vivo* on human tumors xenografted in nude mice. We were able to show that BN82685 efficiently delayed the initial growth of Mia PaCa-2 tumors. BN82685 is therefore one of the few inhibitors of CDC25 that, thus far, have been proven to be active *in vivo* by p.o. administration (17).

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