A Novel Synthetic Inhibitor of CDC25 Phosphatases: BN82002

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ABSTRACT

CDC25 dual-specificity phosphatases are essential regulators that dephosphorylate and activate cyclin-dependent kinase/cyclin complexes at key transitions of the cell cycle. CDC25 activity is currently considered to be an interesting target for the development of new antiproliferative agents. Here we report the identification of a new CDC25 inhibitor and the characterization of its effects at the molecular and cellular levels, and in animal models.

BN82002 inhibits the phosphatase activity of recombinant human CDC25A, B, and C in vitro. It impairs the proliferation of tumoral cell lines and increases cyclin-dependent kinase 1 inhibitory tyrosine phosphorylation. In synchronized HeLa cells, BN82002 delays cell cycle progression at G1-S, in S phase and at the G2-M transition. In contrast, BN82002 arrests U2OS cell cycle mostly in the G1 phase. Selectivity of this inhibitor is demonstrated: (a) by the reversion of the mitotic-inducing effect observed in HeLa cells upon CDC25B overexpression; and (b) by the partial reversion of cell cycle arrest in U2OS expressing CDC25B. We also show that BN82002 reduces growth rate of human tumor xenografts in athymic nude mice.

BN82002 is a original CDC25 inhibitor that is active both in cell and animal models. This greatly reinforces the interest in CDC25 as an anticancer target.

INTRODUCTION

The cell cycle is controlled by evolutionarily conserved mechanisms that ensure the strict regulation of the division process and the accurate segregation of the genome in two daughter cells. These mechanisms are able to integrate intra- and extracellular signals and, therefore, play a central role in the control of cell proliferation. Cdc25 dual-specificity phosphatase was initially identified in yeast model organisms as an essential cell cycle regulator (1). Its activity is required at mitosis to dephosphorylate and activate Cdc2 (cyclin-dependent kinase; CDK1), a well-known key cell cycle regulator. Three human homologues, CDC25A, B, and C, have subsequently been identified (reviewed in Ref. 2). They share common structural properties, and the ability to dephosphorylate critical tyrosine and threonine residues. Their known substrates are CDKs associated with their cyclin regulatory subunits. Dephosphorylation by CDC25 phosphatases allows CDK activation and the subsequent induction of cell cycle progression. CDC25A is involved in the control of the G1-S transition where it dephosphorylates the CDK2/cyclin A complex (3) and probably also acts in the control of entry into mitosis (4). CDC25B has been proposed to play the role of the starter of mitosis by dephosphorylating and activating CDK2/cyclin A and CDK1/cyclin B (2). CDC25C is recognized as an activator of CDK1/cyclin B at mitosis (2) where it dephosphorylates tyrosine 15 and threonine 14. It also plays a role in the control of the initiation of S phase (5).

The elucidation of the specific role of each isoform at specific stages of the cell cycle is a major issue that is still currently under investigation.

Overexpression of CDC25A and B, but not C, has been observed in a variety of cancers (i.e., breast, ovary, head and neck, and colon) with a striking association with tumor aggressiveness and poor prognosis (6–9). This overexpression of CDC25B has been observed concomitantly with the loss of other regulators such as p27 in high-grade B-cell non-Hodgkin’s lymphomas and may, therefore, possibly contribute to their aggressivity (10). Targeted overexpression of CDC25B in mice is responsible for an increased sensitivity to the carcinogen DMBA (11, 12). CDC25B is also overexpressed in neurons of Alzheimer’s disease patients, and it has been suggested that this might be responsible for an inappropriate activation of mitotic CDK/cyclin complexes resulting in the neurodegenerative process (13).

CDC25 phosphatases are therefore considered to be relevant physiopathological targets supporting the identification and the characterization of chemical inhibitors (14, 15).

However, over the last few years, only a very limited number of CDC25 inhibitors have been described in the literature. Menadione has been reported to be a noncompetitive inhibitor of recombinant CDC25 phosphatase and to induce a cell cycle delay in treated cells (15–17). More potent quinone derivatives such as NSC663284 and NSC95397 have also been identified (18). These molecules display competitive kinetics against CDC25 and inhibit tumor cell proliferation (19, 20). SC-α δelta 9 was identified in the screening of a small molecule library as a potent and selective inhibitor of the CDC25 family of dual-specificity phosphatases (21). However, it has been recently reported that structural resemblance of SC-α δelta 9 to phospholipids allows it to inhibit cellular phospholipase C and is responsible for its antiproliferative effects (22). Indolylidihydroxiquinones have been shown to effectively compete with CDC25 substrate in vitro and to inhibit cell proliferation (23). A few other products such as the steroid derivative dithiocarboxy cholesterol (24) and natural products such as dysidiolide have also been reported to inhibit CDC25 activity in vitro and to cause inhibition of HL60 cell proliferation (25, 26).

Here, we report the identification of a new inhibitor of CDC25 phosphatase and demonstrate that it impairs cell cycle progression and reduces tumor growth in animal models. This compound represents a promising chemical structure active both in cell and animal models, and, therefore, greatly reinforces the interest in the CDC25 cell cycle regulator as an anticancer target.

MATERIALS AND METHODS

Preparation of BN82002. 5-(Dimethylamino)-2-hydroxy-3-methoxybenzaldehyde (Ref. 27; 1 g, 5.12 mmol) was added dropwise to a mixture of N-methyl-2-(4-nitrophenyl)ethylene hydrochloride (1.22 g, 5.63 mmol) and triethylanine (1.1 ml, 7.7 mmol) in anhydrous methanol (30 ml) at room temperature under argon, and the resulting mixture was stirred for 18 h.

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Sodium borohydride (0.213 g, 5.63 mmol) was then added, and stirring was maintained for 4 additional hours. The reaction mixture was then partitioned between cold water (10 ml) and dichloromethane (50 ml), and the aqueous phase was additionally extracted with dichloromethane. The combined organic layers were dried over magnesium sulfate, filtered, and concentrated to give 1.10 g of a brown viscous oil. Purification by chromatography over silica gel using 3% methanol in dichloromethane gave 0.626 g (34%) of a brown solid: mp 92–93°C. 1H NMR (DMSO) δ 8.90 (s, 1H), 8.15–8.12 (d, J = 8.7, 2H), 7.52–7.50 (d, J = 8.7, 2H), 6.28 (s, 1H), 6.04 (s, 1H), 3.70 (s, 2H), 3.57 (s, 2H), 2.94 (t, J = 7.1, 2H), 2.74–2.70 (m, 8H), 2.21 (s, 3H).

Cell Culture Conditions. Human parental cell lines were purchased from American Type Culture Collection (Rockville, MD). They were cultivated in DMEM with 10% FCS, glutamine, and penicillin/streptomycin at 37°C in 5% CO₂ atmosphere. HeLa cells were transfected using Exgen-500 (Euromex) following the manufacturer’s instructions. Cell cycle synchronization was achieved as described previously (28). The U2OS cell line conditionally expressing hemagglutinin (HA)-tagged CDC25B1 (under the control of the tet-promoter) is described previously (29). Expression of CDC25B was achieved by tetracycline removal form the culture media.

In Vitro Enzymatic Assay. The activity of a maltose binding protein-CDC2C recombinant enzyme produced in bacteria (GTP Technology, Labège, France) was monitored using 3-O-methylfluorescein phosphosphate (OMFP) or fluorescein diphosphate. The assay was performed in 384-well plates in a final volume of 50 μl. The maltose binding protein-CDC2C proteins were kept in elution buffer [20 mM Tris-HCl (pH 7.4), 250 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10 mM Maltese]. They were diluted in assay buffer [50 mM Tris-HCl (pH 8.2), 50 mM NaCl, 1 mM EDTA, and 20% glycerol]. The final concentrations were 158 ng/well for CDC25A, 84 ng/well for CDC25B, 168 ng/well for CDC25C, and 70 ng/well for the catalytic domain of CDC25C (CDC25C-cat). Products were studied in concentration response up to 40 μM. The reaction was initiated by addition of 500 μM of O-methylfluorescein phosphate. After 4 h at 30°C, 3-O-methylfluorescein fluorescent emission was measured with Victor 2 (Perkin-Elmer; excitation filter: 475 nm and emission filter: 510 nm).

CD45 enzyme activity was monitored using either the synthetic substrate OMFP or its natural substrate. For testing on OMFP substrate at 500 μM, the CD45 enzyme (SE-135; BIOMOL, Plymouth Meeting, PA) was diluted at 5.7 μg/well. The synthetic substrate pp60c-src (see “Materials and Methods”) than on CDC25C using the peptidic substrate found to be 20-fold less active on CD45 using the peptidic substrate pp60c-src (see “Materials and Methods”) than on CDC25C using the OMFP synthetic substrate. The IC₅₀ and the SD were calculated from a linear regression analysis and were used to estimate the IC₅₀.

Flow Cytometry Analysis of DNA Content. Cells were subjected to flow cytometry analysis of their DNA content after propidium iodide staining as described previously (30). Data were collected on a BD FACSCalibur cytometer and analyzed using the Modfit software (Verity Software).

Human Tumor Xenografts in Nude Mice. Cells of the human pancreatic carcinoma cell line MIA PaCa-2 were injected s.c. into the flank of female athymic NCr-nu/nu mice. 4–6 weeks old. Tumors were allowed to reach a volume of 100 mm³. Once tumors were established, treatment was started by i.p. route as indicated in the figure legend. Six animals/group were treated by BN82002 dissolved in DMSO. Tumor measurements and animal weights were monitored and recorded twice per week. Animal care was in accordance with institutional guidelines.

RESULTS

BN82002 Inhibits the Activity of Recombinant CDC25 Phosphatases. BN82002, a novel CDC25 inhibitor, was identified in the course of a molecular library screening based on the inhibition of the enzymatic activity of a recombinant CDC25C phosphatase. BN82002 inhibits in vitro the activity of CDC25C phosphatase in a concentration dose manner with an IC₅₀ of 5.4 μM. Menadione used as positive control leads to an inhibition of 18.8 μM. The inhibitory effect of BN82002 and menadione was also observed when only the activity of CDC25C catalytic domain is challenged leading to IC₅₀ values also in the micromolar range. In addition, other CDC25 family members including CDC25A and the CDC25B–2–3 variants (31) were inhibited by both compounds within the same range (Table 1). Selectivity was also investigated on the CD45 tyrosine phosphatase. BN82002 was found to be 20-fold less active on CD45 using the peptidic substrate pp60c-src (see “Materials and Methods”) than on CDC25C using the OMFP synthetic substrate. Similarly, Menadione was >20-fold less active on CD45 than on CDC25C. The chemical structure of BN82002 presented in Fig. 1 is a novel tertiary (2-hydroxy-3-methoxy-5-dimethylamino-1-benzylamino) that can be considered original compared with known CDC25 inhibitors (15, 23).

BN82002 Inhibits in Vitro Human Tumor Cell Proliferation. The effect of BN82002 on cell proliferation was evaluated in vitro on several human tumor cell lines (Table 2). Menadione, which has been reported to inhibit cell proliferation, was used as a control. All of the examined cell lines were sensitive to BN82002 and Menadione in a concentration-dependent manner in the low micromolar range. The most sensitive was the pancreatic cancer cell line MIA PaCa-2 with an IC₅₀ of 7.2 μM, and the less sensitive cell line was the colon cancer

<table>
<thead>
<tr>
<th>IC₅₀ (±) SD μM</th>
<th>Menadione</th>
<th>BN82002</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDC25A</td>
<td>7.1 (1.1)</td>
<td>2.4 (1.2)</td>
</tr>
<tr>
<td>CDC25B</td>
<td>14.2 (2.6)</td>
<td>3.9 (0.01)</td>
</tr>
<tr>
<td>CDC25B3</td>
<td>19.6 (5.7)</td>
<td>6.3 (4.1)</td>
</tr>
<tr>
<td>CDC25C</td>
<td>18.8 (2.9)</td>
<td>5.4 (3.4)</td>
</tr>
<tr>
<td>CDC25C-cat</td>
<td>9.1 (1.7)</td>
<td>4.6 (0.5)</td>
</tr>
</tbody>
</table>

A NEW PHOSPHATASE CDC25 INHIBITOR

Enzyme inhibition was assayed on recombinant MBP-CDC25A, MBP-CDC25C, MBP-CDC25C catalytic domain, MBP-CDC25B2, and MBP-CDC25B3 proteins using 3-O-methylfluorescein phosphate as substrate. The IC₅₀ and the SD were calculated from at least two independent experiments with 8 determinations per tested concentration (see “Materials and Methods” for details).
in cells treated with 50 \( \mu M \) BN82002, the activity of CDC25 was inhibited by 50 \( \mu M \), a concentration that fully inhibits cell proliferation, a mechanism that was only modestly affected with a slight decrease in S phase and an increase in cells containing both a G1 and a G2 DNA content, suggesting that the cells treated with BN82002 were arrested at various stages of the cell cycle.

To analyze the effect of BN82002 on cell cycle progression in more detail, we made use of HeLa cells that were synchronized in late G1-phase by double thymidine block and release. As shown in Fig. 4A, left panel, after double thymidine block these cells were fully synchronized with >80\% of the population containing a G1 phase.

### Table 2: BN82002 inhibits in vitro growth of human tumor cell lines

Growth inhibition was assayed on human pancreatic carcinoma cell line (Mia PaCa-2), human prostate carcinoma (DU-145), human glioblastoma (U-87 MG), human prostate carcinoma (LNCaP), human colon carcinoma cell line (HT-29), human osteosarcoma (U2OS), and HeLa cells using the WST1 cell proliferation assay. The IC\(_{50}\) and the SE reported are calculated from at least two independent experiments with 8 determinations per tested concentration (see "Materials and Methods" for details).

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Menadione (IC(_{50}) (SE))</th>
<th>BN82002 (IC(_{50}) (SE))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mia PaCa-2</td>
<td>6.2 (0.15)</td>
<td>7.2 (0.9)</td>
</tr>
<tr>
<td>DU-145</td>
<td>10.6 (0)</td>
<td>13.5 (0.8)</td>
</tr>
<tr>
<td>U-87 MG</td>
<td>5.3 (1.3)</td>
<td>13.9 (3.5)</td>
</tr>
<tr>
<td>LNCaP</td>
<td>15 (2.2)</td>
<td>22.4 (3.1)</td>
</tr>
<tr>
<td>HT-29</td>
<td>9.8 (2.6)</td>
<td>32.6 (8.9)</td>
</tr>
<tr>
<td>U2OS</td>
<td>6.3 (1.9)</td>
<td>11.2 (0.6)</td>
</tr>
<tr>
<td>HeLa</td>
<td>15.5 (0.5)</td>
<td>23 (4.5)</td>
</tr>
</tbody>
</table>

**BN82002 Inhibits Endogenous CDC25 Phosphatase Activity in Whole Cells.** We investigated the molecular bases of the inhibitory activity of BN82002 on cultured cells by monitoring its effect on the tyrosine phosphorylation status of a natural CDC25C substrate, the CDK1 kinase. Indeed, the tyrosine 15 dephosphorylation of CDK1 is fully dependent on the activity of the CDC25C phosphatase. MIA PaCa-2 cells established from a human pancreatic cancer were treated with increasing concentrations of BN82002 for 1 h. Twenty-four h later, the cells were lysed and processed for Western blot analysis using an antibody specific for the tyrosine 15-phosphorylated form of the CDK1 kinase. Actin was used as loading control. As shown in Fig. 2A, BN82002 at 50 \( \mu M \) and 100 \( \mu M \) induced an accumulation of the tyrosine 15-phosphorylated form of CDK1. The inhibition of CDK-1 dephosphorylation suggests that BN82002 efficiently down-regulates the activity of the CDC25C phosphatase toward CDK1 in cultured MIA PaCa-2 cells.

To additionally characterize the effect of BN82002 on the activity of CDC25, we made use of a human osteosarcoma cell line U2OS that has been engineered to express a HA epitope-tagged CDC25B phosphatase under the control of the tetracycline-repressible promoter. As already reported (29), CDC25B phosphatase was expressed at a detectable level starting from 12 h after tetracycline removal from the culture medium. After 16 h of CDC25B expression these cells were treated with 2 h with BN82002 (5 or 50 \( \mu M \)) then harvested. Cell extracts were subjected to immunoprecipitation with anti-HA antibodies, and the phosphatase activity of CDC25B was assayed. As shown in Fig. 2B, in cells treated with 50 \( \mu M \) BN82002, the activity of CDC25B was inhibited by ~60\%.

Taken together, these results indicate that treatment with BN82002 inhibits CDC25 phosphatase activity in cultured cells. The effects of this compound on cell cycle progression were therefore examined.

**BN82002 Inhibits Cell Cycle Progression.** HeLa cells, a convenient model for cell cycle studies, were used to investigate by flow cytometry after propidium iodide staining the effect of BN82002 on cell cycle progression. As shown in Fig. 3, after 24 h of treatment by
DNA content. Four h after release (Fig. 4B, left panel) most of the cells were in S phase (70%), and at 8 h the majority (65%) were in G2 phase (Fig. 4C, left panel). These G1-, S- and G2-synchronized populations (Fig. 4A, B, and C, respectively) were treated for 3 and 10 h with BN82002 or with DMSO as a control. As shown in Fig. 4A, right panels, cells that were treated in G1 phase with BN82002 remained longer in G1 phase and were delayed in their progression to S phase. At 10 h, most of the treated cells were still in S phase, whereas the control population was already in G2 phase. When cells were treated with BN82002 in S phase (Fig. 4B, right panel), they also displayed a significant delay in their progression to G2 phase. At 10 h, most of the cells were still in S phase, whereas the control population had already passed mitosis (G2 to G1 DNA content) and entered the next cell cycle. Finally, HeLa cells that were treated in G2 phase with BN82002 also displayed a major delay in their progression to mitosis (Fig. 4C, right panel). After 10 h, control cells were almost all in G1 phase, whereas half of the BN82002-treated cells were still in the G2 phase. From this set of experiments, we concluded that BN82002 is able to target and to delay cell cycle progression at different stages.

**CDC25 Overexpression Partially Reverts the Effects of BN82002 on Cell Cycle Progression.** The U2OS cell line conditionally expressing CDC25B described above was used to investigate whether the expression of a high level of CDC25B (29) was able to counteract the ability of BN82002 to impair cell cycle progression.

In contrast to what we observed with HeLa cells (Fig. 3), U2OS cells treated for 24 h with 50 μM BN82002 (in the presence of tetracycline to repress CDC25B expression), mostly (88%) arrested their cell cycle with a G1 DNA content (Fig. 5). When treatment with 50 μM BN82002 was applied to cells that were grown previously for 20 h in the absence of tetracycline to allow CDC25B expression, the effect of BN82002 was partially reversed with only 65% of cells in G1 and an ~30% population that was still in S phase. Expression of CDC25B had no significant effect on the control DMSO-treated cells.

**BN82002 Reverts CDC25B Mitotic-Inducing Effect.** It has been demonstrated previously that the overexpression of CDC25B in HeLa cells results in illegitimate and premature entry into an abnormal mitotic process with cells displaying a phenotype described as premature chromosome condensation (32). We made use of this observation to set up a cellular assay allowing us to monitor the activity of the BN82002 inhibitor on a biological feature that is directly dependent on CDC25 activity. HeLa cells were transiently transfected with a HA-epitope tagged CDC25B-expressing plasmid and were treated 12 h later for an additional 12-h duration with 50 μM BN82002. Cells were fixed and examined by immunofluorescence after HA and 4′,6-diamidino-2-phenylindole staining. The percentage of abnormal mitotic figures was determined among the transfected cells expressing CDC25B. Twenty three percent of the CDC25B transfected cells displayed abnormal mitotic figures, whereas only 4% of the DMSO-treated cells exhibited this phenotype.
weekly pancreatic cell MIA PaCa-2. Drug treatment was initiated when the BN82002 was evaluated in athymic mice xenografted with the human Nude Mice. On the basis of the mitotic inducing activity of the CDC25B phosphatase. This observation indicates that treatment with BN82002 is able to counteract the chromosome condensation cells was only 12%, which represents a result. In cells treated with BN82002, the percentage of premature condensation) 24 h after transfection. DMSO had no influence on this displayed abnormal condensed chromatin (premature chromosome condensation) 24 h after transfection. DMSO had no influence on this result. In cells treated with BN82002, the percentage of premature chromosome condensation cells was only 12%, which represents a 45% inhibition of the mitosis-inducing effect of CDC25B. This result indicates that treatment with BN82002 is able to counteract the mitotic inducing activity of the CDC25B phosphatase. This observation indicates that in cultured cells CDC25 is indeed a target for the BN82002 compound.

**In Vivo Growth Inhibition of MIA PaCa-2 Cells in Athymic Nude Mice.** On the basis of the in vitro results, the activity of BN82002 was evaluated in athymic mice xenografted with the human pancreatic cell Mia PaCa-2. Drug treatment was initiated when the tumors reached a volume of 100 mm³ and continued once a day for 10 days at 15 mg/kg or once a week for 3 weeks at 40 mg/kg. BN82002 stabilized tumor growth during the once daily × 10 treatment and induced a delay in the once weekly × 3 schedule. The benefit of this stabilization was still visible after the arrest of the treatment (Fig. 6). No toxicity in terms of weight loss and no animal death were noted during the course of the experiments. In contrast, no activity of menadione was observed up to 100 mg/kg in this model.

**DISCUSSION**

A number of laboratories are currently evaluating the possibility of targeting cell cycle regulators as a new strategy in cancer therapy (33, 34). Indeed, cell-cycle deregulation is a hallmark of tumor cells, and a number of neoplasias show abnormal expression or mutation of key regulators (35). Over the past few years, CDK inhibition has been of considerable interest because of the essential role of these enzymes in the control of cell cycle progression. Several potent CDK inhibitors have been identified and characterized, and few of them are currently undergoing Phase I and II clinical trials (reviewed in Refs. 36, 37). Because of their critical role in the activation of CDKs, CDC25 phosphatases are also considered attractive putative targets for the development of new chemotherapeutic drugs and are currently the object of drug discovery efforts in pharmaceutical companies (15, 38). Most of the inhibitors described thus far lack the efficacy that is required to enter clinical trials. However, they provide a structural basis for the development of new classes of CDC25-directed therapeutic agents (see Introduction).

The work presented here focuses on the characterization of BN82002, a new inhibitor of CDC25 phosphatases discovered in the course of a compound library screen set up to identify small molecules able to inhibit in vitro the catalytic activity of a recombinant CDC25C phosphatase. BN82002 shows activity similar to Menadione, but is less active than the compounds published recently from the Lazo et al. (19). However, based on the recent reviews describing CDC25 inhibitors, BN82002 represents an original structure that is chemically accessible.

The active site of CDC25 contains a catalytic cysteine (39). BN82002, like Menadione and NSC663284 (18), might inactivate CDK5 by inducing disulfide bond linkage through quinone redox reaction. We have found that addition of Glutathion (20 μM) together with BN82002 or Menadione to the CDC25C enzyme assay increased the Menadione IC50 by 3.9-fold and BN82002 IC50 by 4.3-fold. Glutathion by itself had no effect. Glutathion might prevent the link of BN82002 or Menadione on the catalytic cysteine in the active site of the enzyme.

As reported by Rudolph et al. (40), the specificity of CDC25 phosphatases for their CDK substrates resides within the catalytic domain itself and in region(s) outside the phosphate binding site of the enzyme. On the basis of this observation, we made use of a full-length recombinant CDC25C protein to evaluate the inhibitory activity of BN82002. However, using OMFP as substrate, no significant difference could be observed between the inhibition of the full-length CDC25C enzyme and the corresponding catalytic domain. Our initial attempts to set up a robust assay with a natural CDK-cyclin phosphorylated substrate were not successful (data not shown). Consequently, the assays reported here were only performed with artificial substrates. In addition, we found that the ratio of selectivity for one compound on two different phosphatases could vary considerably upon minor changes of the experimental parameters (i.e., pH) or artificial ligand choice.⁴ On the basis of these technical limitations, no additional efforts were made to perform selectivity studies on isolated recombinant phosphatases. As an alternative, we have explored the effects of BN82002 on whole cells where the natural endogenous substrate CDK-cyclin complexes are present in a biological context.

We present several lines of evidence, which strongly suggest that BN82002 targets CDC25 phosphatases in cultured cells. First, in Mia PaCa-2 cells, CDK1 tyrosine phosphorylation is increased by treatment with BN82002, indicating that CDC25 phosphatase activities efficiently revert the aberrant mitotic-inducing effect observed in a specific biological assay based on the phenotype observed upon conditional expression of a CDC25 phosphatase in the U2OS cell line. Finally, we observed that the growth inhibitory effect of BN82002 is partly counteracted by the conditional expression of a CDC25 phosphatase in the U2OS cell line, suggesting that a large excess of CDC25 is able to titrate out the inhibitor.

It is not surprising that the cell cycle of HeLa cells treated with BN82002 is globally affected with, as shown in Fig. 4, a delay in progression at various stages. This observation is in accordance with

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⁴ Unpublished observations.
BN82002 being active against several members of the CDC25 phosphatase family (variants B and C). Thus, cell cycle inhibition is observed at the various stages of the cell cycle where these enzymes are required. Why U2OS cells are only arrested in G1 phase is unclear and remains to be investigated.

We have shown here that BN82002 delays tumor growth in xenografted athymic mice. Very little information is currently available on the study of the antiproliferative activity of CDC25 inhibitors in animal models. Consistent antineoplastic activity of bisperoxovanadium was described recently (41). Here, we report a novel example of CDC25 inhibitor active in vivo. As observed with the above-mentioned vanadate-related compound, the main antitumor effect of BN82002 is a decrease of the tumor growth rate.

In summary, we have identified BN82002 as a new inhibitor of CDC25, a family member of dual-specificity phosphatases involved in the control of cell cycle progression. This compound is active in vitro, in cultured cells, and in vivo. On the basis of its targeted mechanism of action, an inhibitor of CDC25 such as BN82002 should prevent cell proliferation of a broad spectrum of cell types. It will therefore be of major relevance to learn more about the turnover of the CDC25 family members and of their substrates. Additional studies will be necessary to identify in which type of cancers, modifications of these parameters should be considered in the first place, as they may affect the response of cell cycle inhibitors including BN82002.

This work will reinforce the interest in considering inhibition of cell cycle driving enzymes such as CDKs and their activating CDC25 phosphatases as a therapeutic strategy.

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