

# Reversine, a novel Aurora kinases inhibitor, inhibits colony formation of human acute myeloid leukemia cells

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## Abstract

The demonstration that the small synthetic molecule reversine [2-(4-morpholinoanilino)-*N*<sup>6</sup>-cyclohexyladenine] promotes the dedifferentiation of committed cells into multipotent progenitor-type cells has raised hopes on the exploitation of this small chemical tool for the generation of stem cells. Here, we show that reversine causes a failure in cytokinesis and induces polyploidization. These effects of reversine are due to the inhibition of Aurora A and B, two related kinases that are implicated in several aspects of mitosis and that are frequently amplified and overexpressed in human tumors. Reversine inhibits the phosphorylation of histone H3, a direct downstream target of Aurora kinases. Similarly to the Aurora kinase inhibitor VX-680, which has recently entered phase II clinical trials for cancer treatment, reversine inhibited colony formation of leukemic cells from patients with acute myeloid leukemia but was significantly less toxic than VX-680 on cells from healthy donors. The crystal structure of the reversine-Aurora B kinase complex shows that reversine is a novel class of ATP-competitive Aurora kinase inhibitors. Thus, although our studies raise serious doubts on the application of reversine in regenerative medicine, they support the paradigm that reversine might be a useful agent in cancer chemotherapy. [Mol Cancer Ther 2008;7(5):1140–9]

## Introduction

Reversine, a substituted purine analogue, was originally shown to promote the dedifferentiation of myotubes derived from the murine myoblast cell line C2C12 (1).

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**Note:** A.M. D'Alise and G. Amabile have contributed equally to this work.

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More recently, Anastasia et al. showed that reversine can facilitate the differentiation of human fibroblasts into skeletal muscle cells *in vitro* and *in vivo* (2). Thus, reversine has the ability to reprogram somatic cells to a state of increased plasticity that can be manipulated to direct differentiation in different cell types.

The identification of conditions that set the stage for cell differentiation is also relevant to cancer therapy, where the ability to force the differentiation of cancer cells is expected to counteract their proliferation potential. At the beginning of our studies, we asked if pretreatment with reversine could promote the differentiation of cancer cells. As shown below, our results are consistent with the hypothesis that reversine is a facilitator of tumor cell differentiation. For instance, reversine induces the expression of reelin, a neuronal marker selectively expressed in GABAergic neurons (3) in the human embryonal carcinoma cell line N TERA-2 (NT2). Reversine also induces the expression of CD11b, a gene marker of cell differentiation, in the HL60 leukemic cell line.

In an attempt to provide a molecular explanation for the function of reversine, we tested its activity on a broad panel of protein kinases and found that reversine specifically inhibits the function of Aurora kinases both *in vitro* and in cell-based assays. Aurora kinases are serine/threonine kinases that play a prominent role in mitotic regulation (4, 5). The Aurora A kinase, the "polar" kinase, resides at spindle poles during mitosis and is implicated in the control of centrosome duplication, maturation, and separation (6). Aurora A is also important for the formation of a bipolar mitotic spindle. Aurora B, the "equatorial" kinase, is part of a chromosome passenger complex that controls the organization of the centromere-kinetochore region and is directly implicated in the control of microtubule-kinetochore attachment, in which it plays an essential function by correcting faulty microtubule-kinetochore attachments. Later in the cell cycle, Aurora B plays a prominent role in cytokinesis (7). Much less is known about the physiologic role of the Aurora kinase C: it appears that it is a chromosomal passenger protein, like Aurora kinase B, whose function it can complement (8).

Aurora kinases have also emerged as valuable targets in cancer therapy, not least because they appear to be frequently overexpressed in human tumors (7). Several small-molecule inhibitors of Aurora kinases, initially generated in the pharmaceutical industry, have leaked into general laboratory use. In addition to classic studies of genetic inactivation in different cell types and organisms, the small-molecule compounds targeting Aurora kinases in living cells have helped dissecting the specific cellular functions of the Aurora A and B family members. Most notably, the effects on cell division of Hesperadin (Boehringer Ingelheim) and ZM447439 and related compounds

(AstraZeneca) have been described in detail (9–11). Collectively, these studies have provided a blueprint against which the function of other potential Aurora inhibitors can be tested. Here, we show that reversine seems to inhibit preferentially the Aurora B kinase in a cell culture system, as it provides effects that are similar to those previously attributed to this kinase, including the inhibition of cytokinesis and the induction of polyploidy. We tested the antitumor effects of reversine on primary blood tumors and found that reversine has strong antitumor activity associated with good tolerability by normal cells. Finally, the determination of the crystal structure of the reversine-Aurora B complex reveals that reversine defines a novel class of Aurora kinase active site inhibitors.

## Materials and Methods

### Cell Culture and Treatment

HeLa cervical carcinoma cells, T47D breast cancer cells, and NT2 cells (all from American Type Culture Collection) were cultured in growth medium consisting of high-glucose DMEM supplemented with 2 mmol/L glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin (Invitrogen), and 10% (v/v) fetal bovine serum (FBS; HyClone). The human colon cancer cell line HCT116 (American Type Culture Collection) was maintained in McCoy's 5A modified medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin. A375 melanoma cells were grown in DMEM supplemented with 2 mmol/L glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin (Invitrogen), and 15% (v/v) FBS (HyClone). Promyelocytic leukemia cells HL60 were grown in RPMI with 2 mmol/L glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin (Invitrogen), and 10% (v/v) FBS (HyClone), whereas MCF-7 breast cancer cells were grown in MEM supplemented with 2 mmol/L glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, 1% nonessential amino acids (Invitrogen), and 10% (v/v) FBS (HyClone). Reversine prepared according to the published protocol (1) was dissolved in DMSO and 10 mmol/L stock solutions were stored at  $-20^{\circ}\text{C}$  until use.

### RNA Preparation and TaqMan Analysis

Total RNA was prepared with RNeasy midi kit (Qiagen). Human *Reelin* gene expression was measured 24, 48, and 72 h after the treatments using TaqMan chemistry with the ABI 7700 Prism Real-time PCR instrument (Applied Biosystems). Forward (400 nmol/L) and reverse (800 nmol/L) primers sequences were 5'-TGGGTGGAACCGAATAACAATT-3' and 5'-ACATGACGGCCAATA-TAAACAT-3' and the probe (200 nmol/L) sequence was 5'-CCCTCCTAACGCAGCACTAACCCG-3'. Data analysis was done using human glyceraldehyde-3-phosphate dehydrogenase as internal standard (Applied Biosystems).

### Fluorescence-Activated Cell Sorting Analysis

To monitor the differentiation of HL60,  $5 \times 10^5$  cells were harvested, washed twice with cold PBS, and incubated with antibody against human CD11b PE conjugated (Becton

Dickinson) for 30 min at room temperature. After incubation, the cells were washed twice with diluent solution (PBS containing 1% bovine serum albumin and 0.1%  $\text{NaN}_3$ ), fixed with 2% paraformaldehyde, and analyzed on a FACSCalibur flow cytometer (Becton Dickinson). The fluorescence intensity of the viable cells (identified by their light scattering characteristics) was analyzed using CellQuest software and represented as percentage of  $\text{CD11b}^+$  cells.

To measure cell death, we did Annexin V analysis on HCT116 tumor cell line. The cells were treated with the drug for 0 and 24 h then were washed with ice-cold PBS and incubated in a binding buffer [125 mmol/L NaCl, 25 mmol/L HEPES (pH 7.5), 1% bovine serum albumin] with Annexin V/propidium iodide (1 µg/0.2 µg/mL) in a 100 µL volume for 30 min in dark on ice; 0.5 mL binding buffer was added and cells were analyzed by flow cytometry using a Becton Dickinson FACS CANTO.

### Cell Cycle Analysis

HCT116 and HL60 cells were incubated with either 5 µmol/L reversine or DMSO 0.01%. Cells were harvested and fixed in 70% ethanol overnight. After double washing with PBS, cells were labeled with cell cycle staining reagent PBS, 0.1% Triton X-100, 200 µg/mL DNase-free RNase, and 25 µg/mL propidium iodide (Invitrogen) and incubated at room temperature in the dark for 30 min. DNA content was analyzed using FACSCalibur (Becton Dickinson).

### Immunofluorescence

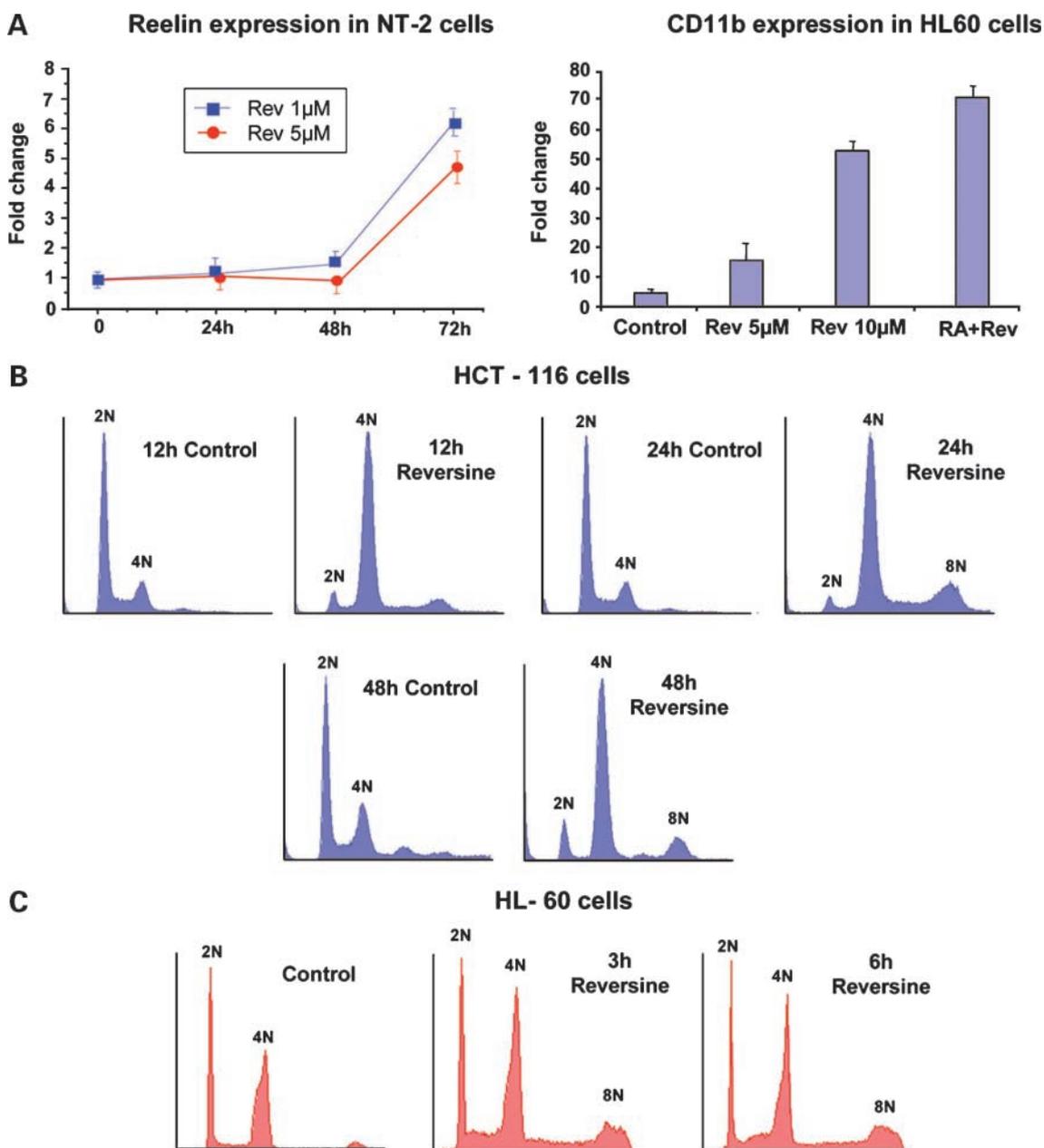
HCT116 cells ( $5 \times 10^4$ ) were cultured on poly-lysine (Sigma)-coated coverslips. For analysis of histone H3 phosphorylation, cells were fixed in 4% paraformaldehyde for 10 min at room temperature and then permeabilized with PBS/0.1% Triton X-100 for 5 min at room temperature. Cells were incubated with the primary antibody anti-Ser<sup>10</sup>P-H3 (5–10 µg/mL; Upstate) followed by a FITC conjugate antibody to rabbit IgG (1:3,000; Sigma) and then counterstained with 0.5 µg/mL 4',6-diamidino-2-phenylindole.

### Live Cell Imaging

The video light microscopy conditions used in this study have been described previously (9).

### Western Blot Analysis

Cells were rinsed three times with cold PBS and harvested at the time indicated. Cells were then lysed in 50 mmol/L Tris-HCl (pH 8.0) containing 150 mmol/L NaCl, 1% NP-40, 2 µg/mL aprotinin, 1 µg/mL pepstatin, 2 µg/mL leupeptin, and 1 mmol/L  $\text{Na}_3\text{VO}_4$ . The cell lysate was centrifuged at  $13,000 \times g$  for 30 min at  $4^{\circ}\text{C}$  and the pellet was discarded. Protein concentration was determined by Bradford assay. Equal amounts of extracted proteins (80 µg) were loaded and separated on 4% to 12% SDS-PAGE gels and then transferred onto a nitrocellulose membrane (Schleicher & Schuell Biosciences) using a semidry transfer (Invitrogen). Immunodetection was done using the following primary antibodies: anti-cyclin B1 (1:200; Santa Cruz Biotechnology) and anti-Ser<sup>10</sup>P-H3 (1:500; Upstate). For cyclin B1 analysis, cells were synchronized in G<sub>1</sub>-S phase according to Harrington et al. (11).



**Figure 1.** Reversine induces cell differentiation. **A, left**, TaqMan analysis of reelin mRNA in NT2 cells treated with 1 and 5  $\mu$ mol/L reversine for 24, 48, and 72 h showed that reelin mRNA levels increased significantly after 72 h of treatment. Reelin expression was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase gene. HL60 exposed to 5  $\mu$ mol/L reversine for 3 days showed an increased expression of the differentiation marker CD11b compared with control cells. The effect of reversine was additive to that of retinoic acid at 10  $\mu$ mol/L (**A, right**). **B** and **C**, reversine treatment causes accumulation of cells with  $\geq 4N$ . **B**, HCT116 cells were treated with 5  $\mu$ mol/L reversine for 12, 24, and 48 h, whereas HL60 cells were treated for 3 and 6 h. DNA content was assessed by flow cytometry analysis of cells labeled with propidium iodide. Fluorescence-activated cell sorting analysis revealed that reversine caused accumulation of cells with  $>4N$  DNA.

#### ***In vitro* Kinases Assay**

Kinase activity was measured in an *in vitro* assay using a range of concentrations of reversine according to Upstate protocols (Kinase Profilers Assay Protocols) and Invitrogen.

#### **Crystallization and Structure Determination**

The conditions for expression, purification, and structure determination of Aurora B<sup>60-361</sup>:INCENP<sup>790-847</sup> have been

described (12). Crystals obtained by microseeding were harvested in 50  $\mu$ L mother liquor. A 10 mmol/L stock of reversine (2.5  $\mu$ L) dissolved in pure DMSO was added in steps to give a final concentration of 500  $\mu$ mol/L. After a  $\geq 2$ -h incubation,  $\sim 20$   $\mu$ L cryobuffer [50 % PEG400, 100 mmol/L bis-Tris-propane (pH 6.5), 2 mmol/L TCEP] was added in 10- to 15-min steps, at which point crystals

were flash frozen. X-ray diffraction data from single crystals were collected at beam-line ID14-1 at the European Synchrotron Radiation Facility. Data processing was carried out with DENZO/SCALEPACK (13). For subsequent calculations, we used the CCP4 suite (Collaborative Computational Project, 1994). MR was carried out with MolRep (CCP4) using Aurora B as search model (PDB code 1OL5). Model building was carried out with Coot (14).

#### Analysis of Cell Proliferation and Viability

Cell viability of different tumor cell lines was assessed using ATPlite 1step (Perkin-Elmer). Briefly,  $2 \times 10^4$  cells for each well were plated in a 96-well plate in presence of crescent quantity of reversine. After 72 h, the plates were recovered and 100  $\mu$ L ATPlite solution was added to each well. The plates were shaken for 2 min at 700 rpm and luminescence was measured using EnVision Multilabel plate reader (Perkin-Elmer). Each sample was analyzed in triplicate.

#### Colony Formation Analysis

CFU cells ( $1.5 \times 10^5$ ) from acute myeloid leukemia (AML) patients, from healthy donors (donors' sample are from Cambrex), and from patients in clinical remission were resuspended in H44100 methylcellulose semisolid medium supplemented with Iscove's modified Dulbecco's medium with FBS (30%, v/v), 10 ng/mL granulocyte-macrophage colony-stimulating factor, 2 mmol/L glutamine, and 1% bovine serum albumin (all products are from StemCell Technologies). Reversine and VX-680 were added at increasing concentrations. The culture dishes were placed in a fully humidified (>95%) atmosphere at 5% CO<sub>2</sub> and

incubated for 14 days at 37°C before counting colonies. DMSO was used as negative control. The cultures were plated in triplicate, and after the indicated time, colonies with >20 cells were scored using an inverted microscope (Nikon USA).

#### Statistical Analysis

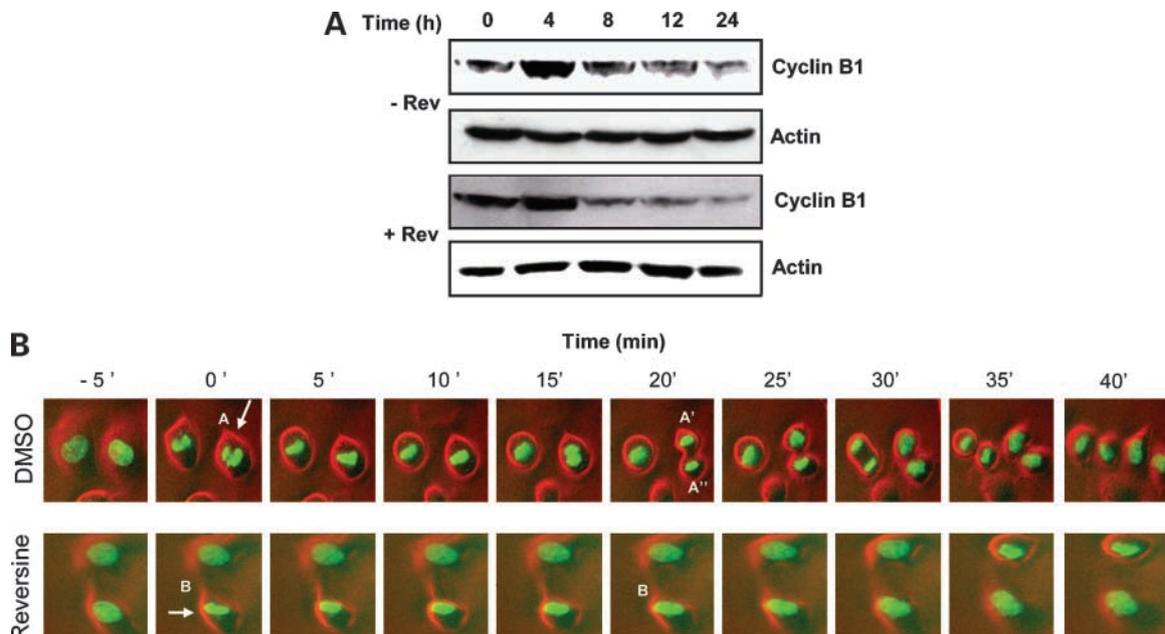
Statistical analysis was done by ANOVA test using Origin 6.1 software for Windows (Microcal Software). The IC<sub>50</sub> values were calculated using SigmaPlot software for Microsoft Office (Systat Software).

## Results

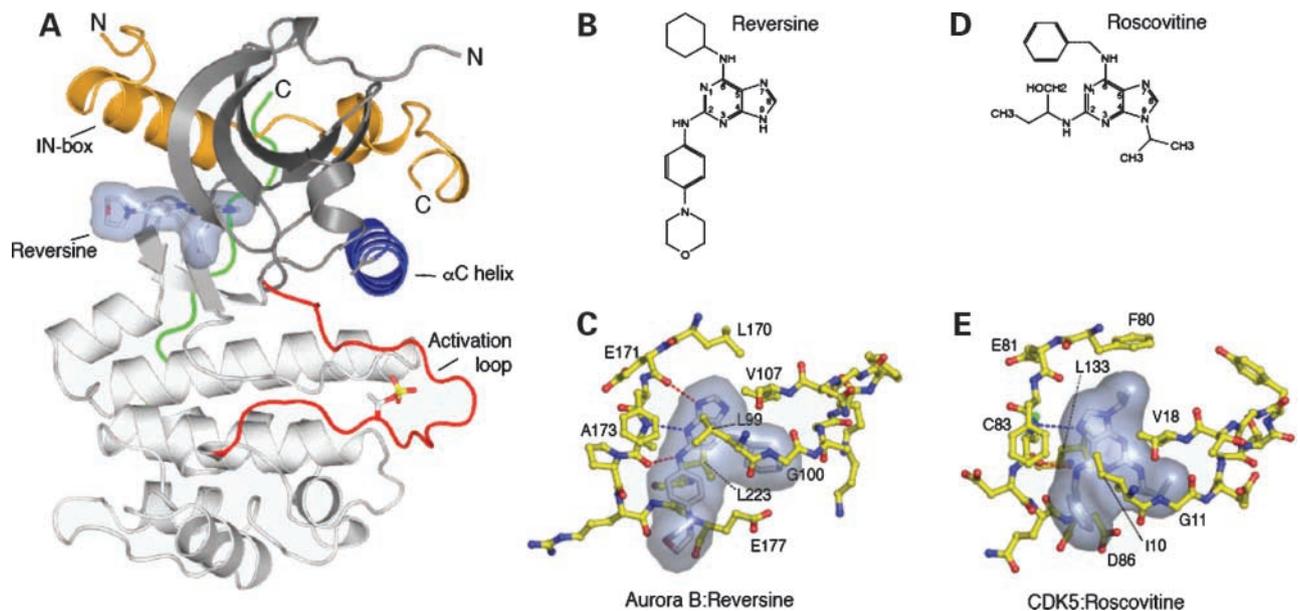
#### Effect of Reversine on Cellular Differentiation

The mouse model myoblast cell line C2C12 differentiates into characteristic multinucleated myotubes on serum withdrawal. As originally shown by Chen et al. (1), we verified that reversine induces dedifferentiation in C2C12 mouse muscle cells (data not shown). We also observed that reversine has an effect on the differentiation of other nonmuscle cell lines, for instance, on the human embryonal carcinoma cell line NT2. Treated NT2 showed elevated level of reelin, a neuronal marker selectively expressed in GABAergic neurons (3), as assessed by TaqMan analysis after treatment with 1 and 5  $\mu$ mol/L reversine for 24, 48, and 72 h. After 3 days of 1 or 5  $\mu$ mol/L reversine treatment, reelin mRNA levels had significantly increased (Fig. 1A, left).

The induction of cell differentiation was also observed in HL60, a human promyelocytic leukemia cell line. Reversine treatment induces the expression of the adhesion molecule



**Figure 2.** Reversine impairs cytokinesis. **A**, Western blot analysis of cyclin B1 showed that expression peaked and subsequently decreased in both reversine-treated and control cells, indicating that the exit from mitosis was not blocked. **B**, time-lapse experiments in presence of reversine were done on HeLa cells stably expressing histone H2B-GFP. The cells were treated with DMSO (control) or 5  $\mu$ mol/L reversine (6 h after release from thymidine block) and followed by time-lapse microscopy. Reversine-treated cells were able to initiate mitosis (white arrows). However, whereas control cells (see cell "A") normally divided them in to two daughter cells (cell A'-cell A''), reversine-treated cells (see cell "B") did not separate resulting in polyploid cells.



**Figure 3.** Structure of reversine complex. **A**, Aurora B<sup>60-361</sup> consists of the NH<sub>2</sub>-terminal small lobe (gray), a COOH-terminal helical large lobe (white), and a short COOH-terminal extension (green). The activation loop (also known as T-loop or activation segment; red) is important for catalytic activation and substrate recognition. A conserved threonine residue in this loop is phosphorylated as part of the activation mechanism. The  $\alpha$ C helix (blue) is also important for the catalytic activity of Aurora B. INCENP<sup>790-847</sup> (orange) crowns the small lobe of Aurora B, stabilizing an active conformation of the kinase. Reversine, shown in ball-and-stick and surrounded by a semitransparent surface, occupies the ATP-binding pocket at the interface between small and large lobes. **B**, molecular structure of reversine. **C**, ball-and-stick representation of the interaction of reversine with selected residues of Aurora B. Red, oxygen atoms; blue, nitrogen atoms; green, sulfur atoms; white, carbon atoms in reversine; yellow, carbon atoms in Aurora B. A semitransparent molecular surface of reversine is shown. Dashed lines, hydrogen bonds.

CD11b, frequently employed as a maturation marker for HL60 cells in response to drug-induced differentiation (15). As shown in Fig. 1A (right), reversine induced a significant increase of the number of CD11b<sup>+</sup> cells compared with control cells and its effect was additive to the effect of retinoic acid, which is commonly used to induce terminal differentiation of leukemic cells, increasing the number of CD11b<sup>+</sup> cells from 52% (only retinoic acid) to 73% (retinoic acid and reversine). Taken together, these data show that reversine influences the process of cell differentiation in a cell type-specific manner.

#### Reversine Induces Polyploidy

Previous studies reported that the control of the cell cycle is tightly associated to cellular differentiation (for instance, see ref. 16). For this reason, we decided to investigate the effects of reversine on cell cycle progression in different cell lines. Cell cycle analysis by flow cytometry indicated that HCT116 cells (human colon carcinoma) treated with 5  $\mu$ mol/L reversine for 12, 24, and 48 h underwent profound modifications of their ploidy. In the absence of reversine, cells showed a normal cell cycle profile, with a majority of cells having a 2N DNA content (Fig. 1B). In contrast, after 12 h of reversine treatment, the majority (~80%) of cells had doubled their DNA content (4N). This was indicative of a possible G<sub>2</sub>-M arrest or, alternatively, a failure in cytokinesis that resulted in an enrichment of 4N interphase population. This second interpretation was reinforced by the observation that

longer reversine treatments of HCT116 cells resulted in DNA endo-reduplication and in the accumulation of cells with DNA content greater than 4N (Fig. 1B). Similar effects on the cell cycle were also observed in other cell lines, including HL60 (Fig. 1C) and C2C12 and NT2 cells (data not shown).

These results suggest that, on treatment with reversine, at least a fraction of the near-diploid HCT116 and HL60 cells fail to complete cytokinesis and become polyploid. To reinforce our evidence in favor of this interpretation, we monitored in HCT116 cells the levels of cyclin B1, a protein whose levels peak during the G<sub>2</sub> and M phases of the cell cycle. On release from a single thymidine G<sub>1</sub>-S arrest in the presence or absence of reversine, a similar timing of accumulation and subsequent degradation of cyclin B1 was observed (Fig. 2A). This result indicates that HCT116 cells enter and exit mitosis with normal timing, lending credit to the hypothesis that the 4N population that accumulates in the presence of reversine (Fig. 2B) consists of a 4N G<sub>1</sub> population.

Cell cycle profiles similar to those of HCT116 and HL60 cells treated with reversine have been observed previously on treatment of A549 and HME cells with ZM447439, an Aurora kinase inhibitor (17). Partial rather than complete endo-reduplication of these cells in the presence of the Aurora inhibitors can be probably ascribed to the presence of a functional p53 pathway and to the activation of a so-called tetraploidy checkpoint, whose function requires p53

(17). Consistently, HeLa cells and other p53-negative cells undergo massive endo-reduplication and apoptosis in the presence of ZM447439 or Hesperadin, another Aurora inhibitor (10, 17).

The most prominent defect observed in cells treated with ZM447439 or Hesperadin is a failure to complete cytokinesis, which results in the subsequent accumulation of polyploid cells. To test whether reversine inhibited cytokinesis, we filmed HeLa cells stably transfected to express a fluorescent version of histone H2B as they were undergoing mitosis in the presence of reversine. In the absence of reversine, most cells completed mitosis in 25 to 30 min, with a negligible number of failures in cytokinesis. Conversely, in the presence of reversine, most cells failed to complete cytokinesis and exited mitosis without dividing. Thus, it appears that, in cells treated with reversine, polyploidy is a consequence of impaired cytokinesis, strongly suggesting the possibility that reversine impairs the function of Aurora kinases or other kinases involved in cell cycle progression and cytokinesis (Fig. 2B).

#### Reversine Inhibits Aurora Kinases

To gain an insight on the ability of reversine to inhibit protein kinases, we tested its effects on the activity of 26 kinases, the majority of which are involved in cell cycle

regulation and cytokinesis (Table 1). Interestingly, we found that reversine is a potent *in vitro* inhibitor of Aurora A and B and is also an inhibitor of Aurora C kinase. As shown in Table 1, Aurora A and B activities were inhibited by 80% and Aurora kinase C by 55%, already at a concentration of 0.5  $\mu\text{mol/L}$ , whereas no inhibition or only modest inhibition was observed on others kinases tested. In a second round of experiments, we determined the  $\text{IC}_{50}$  of reversine on Aurora kinase A to be 150 nmol/L (Upstate assay conditions) and 400 nmol/L (Invitrogen assay conditions), whereas Aurora kinase B and C  $\text{IC}_{50}$  were 500 and 400 nmol/L, respectively (Invitrogen assay conditions). In the light of the data published by Chen et al. (18), we also determined that the  $\text{IC}_{50}$  on MEK1 is  $>1.5 \mu\text{mol/L}$  and that the  $\text{IC}_{50}$  on muscle myosin (an analogue of nonmuscle myosin II) is 350 nmol/L (data not shown).

#### Crystal Structure of Reversine in the Active Site of Aurora B

In light of the interest of Aurora kinase inhibitors as anticancer therapeutics, we next investigated the structural bases of the interaction of reversine with Aurora B. For this, we resorted to previously described crystals of the Aurora B:INCENP complex from *Xenopus laevis* (12). The crystals contain a tight complex of residues 60 to 361 of *X. laevis*-Aurora B (Aurora B<sup>60-361</sup>) and residues 790 to 847 of *X. laevis*-INCENP (INCENP<sup>790-847</sup>), the so-called IN-box. The IN-box is sufficient to activate Aurora B; therefore, the crystallized complex represents an active form of Aurora B (12). The structure of the Aurora B-reversine complex was determined at a resolution of 1.7 Å (Supplementary Table S1).<sup>3</sup> There was excellent electron density for reversine in the ATP-binding pocket of Aurora B. The latter is a large cleft situation at the interface of the small and large lobes of the kinase (Fig. 3A). Stacking interactions with the side chains of Ala<sup>120</sup>, Leu<sup>170</sup> (the "gatekeeper" residue), Ala<sup>173</sup>, Leu<sup>99</sup>, and Leu<sup>223</sup> hold the purine ring of reversine in place (Fig. 3B and C). Furthermore, the NH group at position 9 of the purine ring of reversine donates a hydrogen bond to the carbonyl of Glu<sup>171</sup>, near the hinge surrounding the ATP-binding pocket (Fig. 3C). The cyclohexyl group of reversine overlaps precisely with the position of the ribose group of ADP, which can be predicted based on the structure of the ADP complex of the Aurora B-related kinase Aurora A (19).

#### Reversine Inhibits H3 Phosphorylation

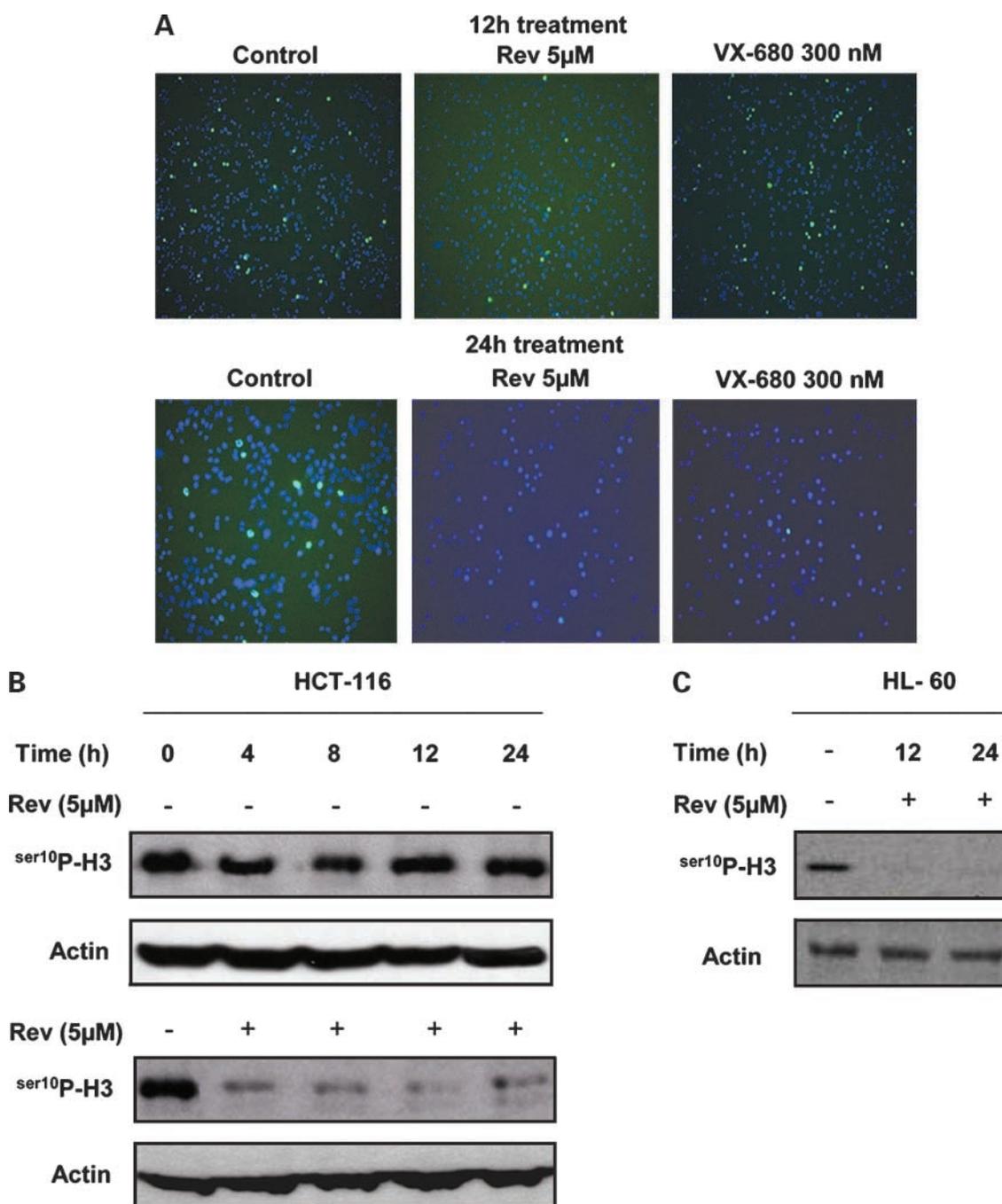
To confirm that Aurora kinases are also *in vivo* targets of reversine, we examined the effects of reversine on the phosphorylation of a well-known Aurora target, histone H3 (20). After treating HCT116 cells with 5  $\mu\text{mol/L}$  reversine for 12 and 24 h, we assessed histone H3 phosphorylation on Ser<sup>10</sup> (Ser<sup>10</sup>P-H3) by immunocytochemistry. Figure 4A shows that Ser<sup>10</sup>P-H3 was significantly reduced after 12 h of reversine treatment and had completely disappeared after 24 h of treatment. As a positive control, HCT116 cells

**Table 1. *In vitro* kinase assay**

Kinase	Reversine	
	0.5 $\mu\text{mol/L}$	1 $\mu\text{mol/L}$
Aurora A	20	12
Aurora B	25	13
Aurora C	55	20
CDK1/cyclin B	95	89
CDK2/cyclin A	94	78
CDK2/cyclin E	87	78
CDK3/cyclin E	86	84
CDK6/cyclin D3	91	81
CDK7/cyclin H/MAT1	84	80
CDK9/cyclin T1	83	69
CHK1	88	70
GSK3 $\alpha$	95	91
GSK3 $\beta$	102	108
MAPK1	106	109
MAPK2	106	91
MEK1	83	69
PDK1	75	62
PKA	76	47
PKB $\alpha$	120	127
PKB $\beta$	102	96
PKB $\gamma$	96	94
PKC $\beta$ II	93	97
PKC $\delta$	80	83
Plk3	113	112
SAPK2a	92	93
SAPK2b	93	92

NOTE: The Table shows the activity (expressed as a percentage) of a panel of protein kinases tested with an *in vitro* assay using 0.5 and 1  $\mu\text{mol/L}$  of reversine.

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



**Figure 4.** Reversine inhibits histone H3 phosphorylation on Ser<sup>10</sup>. **A**, HCT116 cells were treated with 5  $\mu$ mol/L reversine or 300 nmol/L VX-680 for 12 h (top) and 24 h (bottom). Histone H3 phosphorylation on Ser<sup>10</sup> (green) was assessed by immunocytochemistry. Total population was visualized by 4',6-diamidino-2-phenylindole staining (blue). **B** and **C**, HCT116 and HL60 cells were treated with 5  $\mu$ mol/L reversine at different time points. Western blot analysis of Ser<sup>10</sup>P-H3 was assessed to confirm the data of immunocytochemistry. Actin was used as loading control.

were treated with 300 nmol/L VX-680, another Aurora kinase inhibitor (11). Confirming the results of immunocytochemistry, Western blot analysis on HCT116 protein extracts revealed a marked reduction of Ser<sup>10</sup>P-H3 protein expression (Fig. 4B) after only 4 h treatment with reversine. The levels of Ser<sup>10</sup>P-H3 protein remained low also after 24 h

of reversine treatment, indicating effective inhibition of Aurora kinases. Similar results were obtained with HL60 cells (Fig. 4C).

#### Effect of Reversine on Primary AML Cells

The results presented above indicate that reversine is a potent inhibitor of Aurora kinases in both *in vitro* and in

cell-based assay. These results, combined to the ability of reversine to induce tumor cell differentiation, are particularly interesting in light of the notion that Aurora kinases have been identified as promising targets for anticancer drugs. Because Aurora kinases are essential for cell proliferation, we examined the proliferation of a panel of tumor cell lines in the presence of reversine. Cells were incubated with inhibitors for 72 h, and viability was evaluated by measuring the ATP content as luminescence expressed in counts/s. As shown in Fig. 5A, we found that reversine effectively blocks tumor cell proliferation. In Fig. 5B, we also show that reversine induces cell death.

To investigate the effects of reversine on primary human tumor samples, we did a blast colony formation assay analyzing 10 bone marrow samples from AML patients and 6 bone marrow samples from healthy donors or patients on remission. Bone marrows were seeded in cytokine-supplemented semisolid culture medium in the presence of graded concentrations of reversine and VX-680, an alternative Aurora kinase inhibitor that has recently entered in phase II clinical trials (11, 21). The compounds inhibited the growth of colonies with  $IC_{50}$  from 30 to 550 nmol/L for reversine and from 30 to 770 nmol/L for VX-680, showing a similar efficacy to arrest the growth of blasts. Moreover, when we did the same assay on bone marrows from healthy donors or on patients in remission to assess the toxicity of the two compounds, we found a statistically significant difference between reversine and VX-680. The  $IC_{50}$  values of reversine were in the range 175 to 780 nmol/L, whereas the  $IC_{50}$  values of VX-680 were in the range 45 to 80 nmol/L, suggesting a less toxic effect of reversine on normal cells and a selective action on AML cells (Fig. 5C; Supplementary Tables S2 and S3).<sup>3</sup>

## Discussion

Ding et al. originally identified a 2,6-disubstituted purine, named reversine, as a molecule that induces dedifferentiation of murine myoblasts into multipotent progenitor cells (1). Recently, it has been shown that reversine enhances adipocyte differentiation by inducing the expression of adipogenic marker genes (22). In addition, human fibroblasts treated with reversine are able to differentiate in muscle cells both *in vitro* and *in vivo* (2). In our conditions, reversine promotes cell differentiation causing the differentiation of neuronal NT2 cells and human promyelocytic leukemia cell line HL60 (Fig. 1A). Hence, reversine promotes both processes of differentiation and reversal of differentiation depending on the cell type. The ability of reversine to differentiate some tumor cells, such as leukemic cells, suggests its possible use as an anticancer agent.

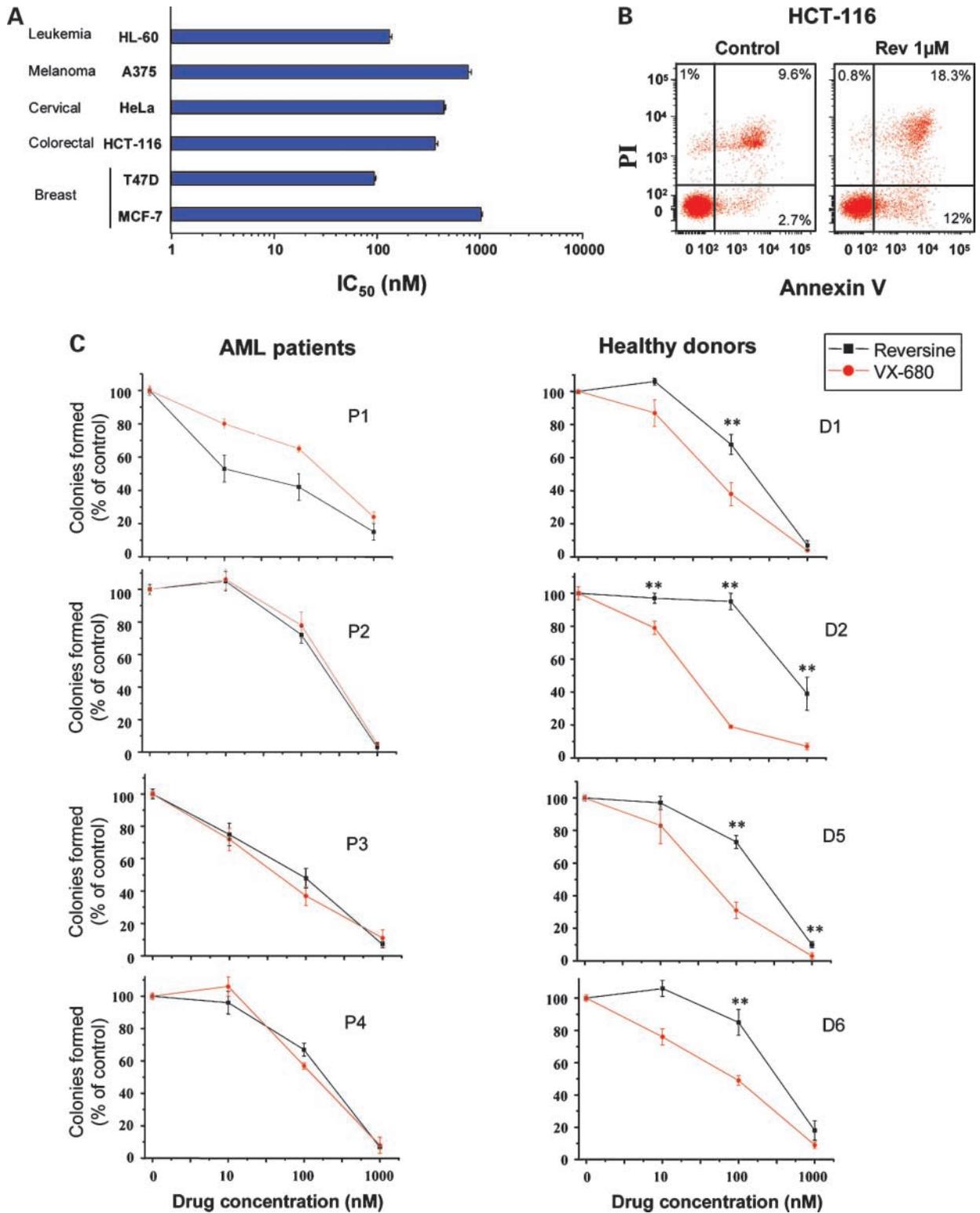
Chen et al. (18) recently reported that reversine-treated cells accumulate with  $G_2$ -M DNA content. These authors interpreted this observation as an indication of cell cycle arrest before mitosis. However, we found that reversine impaired the last phases of mitosis, cytokinesis, resulting in the formation of polyploid cells (Fig. 1B and C). This interpretation was confirmed by time-lapse video micros-

copy in HeLa cells, showing that reversine-treated cells round up and cycle rapidly through mitosis, exiting without completing abscission and therefore accumulating as tetraploid  $G_1$  cells. We also measured the timing of cyclin B1 accumulation and degradation, which was found to be largely normal. Specifically, HCT116 cells synchronized in  $G_1$ -S phase and then treated with reversine showed a peak of cyclin B1 expression followed by a subsequent decrease whose timing was indistinguishable from that of untreated cells (Fig. 2A).

In summary, the accumulation of 4N cells is not due to a premitotic arrest but is the result of the inhibition of cytokinesis by reversine. Despite an apparently similar efficacy toward Aurora A, B, and C in our *in vitro* assays, the phenotype from addition of reversine to dividing cells phenocopies the RNA interference-mediated depletion of the Aurora B kinase. The effects from inhibiting the Aurora A kinase in human cells have been recently understood and consist in the formation of monopolar spindles due to a failure in centrosome maturation and separation (6). Although we did not visualize the state of mitotic spindles in reversine-treated cells, we can exclude these effects because most cells undergoing mitosis in the presence of reversine appear to form a metaphase plate before anaphase. Thus, reversine seems to act predominantly as an Aurora B inhibitor in our experimental conditions and in the drug range used. This behavior is closely reminiscent of that previously described for Hesperadin, a compound that show similar potency against Aurora A and B *in vitro* but that seems to predominantly target Aurora B *in vivo* (9). However, in the light of the recent demonstration that reversine is probably also an inhibitor of nonmuscle myosin II (ref. 18; direct evidence of that is lacking, but reversine inhibits muscle myosin kinase, a close analogue of nonmuscle myosin II), we have to consider the possibility that also this activity might contribute to the cytokinesis failure.

It has been shown that the inhibition of Aurora kinases is a valid approach to cancer therapy (23), holding the potential to induce regression of a different range of tumor types. The inhibitor of Aurora kinases VX-680 has recently entered phase II clinical trials for cancer. It blocks cell proliferation and is able to inhibit colonies formation of primary leukemic cells (11, 21). The exact mechanism of toxicity of Aurora inhibitors remains to be elucidated. Aurora inhibitors target cytokinesis, a fundamental aspect of the mitotic cell division process, and their usage results in the production of a polyploid progeny. Toxicity might arise because fresh polyploid cells that also contain supernumerary centrosomes (a consequence of failure of cytokinesis) will undergo a highly aberrant subsequent mitosis, characterized by the presence of a multipolar spindle, and resulting in the producing of a nonviable progeny (7, 24).

Reversine potently inhibited the proliferation of a wide variety of tumor cell line with  $IC_{50}$  values from ~100 to 1,000 nmol/L and induces cell death (Fig. 5A and B). However, in addition to test the effect of reversine on immortalized cell lines, we analyzed the consequences of



**Figure 5.** Reversine blocks proliferation of multiple tumor cell types. **A**, proliferation of a panel of tumor cell line was assessed by ATP content after 72-h treatment with reversine. **B**, fluorescence-activated cell sorting analysis for the Annexin V confirms that reversine treatment (1 μmol/L) induces cell death after 24 h. **C**, clonogenic survival of AML samples from 10 different patients was assessed after 14 d of exposure to reversine and VX-680. In the panel, 4 of 10 patients and 4 of 6 donors tested are shown. On healthy donors (*D1* and *D2*) and on patients in remission (*D5* and *D6*), the effect of reversine is less toxic of VX-680. Values observed in reversine-treated samples were statistically different from those obtained in the corresponding sample cultured in presence of VX-680. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . Percentage of colonies formed in treated cells relative to untreated cultures is shown.

compound treatment on primary human tumor samples. Reversine strongly inhibits the colony formation of primary leukemic cells from patients with AML as the inhibitor VX-680 (Fig. 5C).

An important observation concerned the selectivity of reversine toward tumor cells. We verified that reversine is more effective on cancer samples than on samples from healthy donors. Specifically, reversine was less potent on normal cells than VX-680 as indicated by a comparison of the IC<sub>50</sub> values of reversine and VX-680 on the bone marrow of healthy donors. Therapeutic selectivity is one of the most relevant variables in cancer chemotherapy, and the design of therapeutic strategies or drugs to preferentially kill malignant cells, minimizing harmful effects to normal cell, represents a significant goal. Altogether, our observations raise important expectations on reversine as a possible anticancer drug.

To our knowledge, reversine represents a new class of Aurora kinase inhibitors. As a purine-based compound containing substituents at positions 2 and 6 of the purine ring, reversine binds to the active site of the Aurora B kinase according to a scheme that is closely reminiscent of the mode of binding of the 2,6-disubstituted purine NU6086 to the active site of CDK2. The mode of binding of 2,6-disubstituted purines is distinct from that of 2,6,9-trisubstituted purines. The orientation of the purine ring in the Aurora B active site is incompatible with the presence of a substituent at position 9, as this would clash with the kinase main chain near Glu<sup>171</sup>. Indeed, the available structures of 2,6,9-trisubstituted purine inhibitors in the active site of kinases show that the presence of substituents at position 9 forces the “flipping” of the purine ring about its long axis. For instance, this is the mode of binding of roscovitine (Fig. 3D and E) to the active site of cyclin-dependent kinase 5 (PDB ID code 1UNL). In the future, it will be important to perform structure-activity relationship studies to create additional variants of reversine that adapt even more selectively to the Aurora active site.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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