N-&-N1, a new class of cell death-inducing kinase inhibitors derived from the purine roscovitine

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Abstract
Cyclin-dependent kinases (CDKs) and their regulators show frequent abnormalities in tumors. Ten low molecular weight pharmacologic inhibitors of CDKs are currently in clinical trials against various cancers, including the 2,6,9-trisubstituted purine (R)-roscovitine (CYC202/Seliciclib). We here report the characterization of N-&-N1, a bioisoster of roscovitine displaying improved antitumoral properties. N-&-N1 shows exquisite selectivity for CDKs, with 2- to 3-fold enhanced potency compared with (R)-roscovitine. Inhibition of retinoblastoma protein phosphorylation and RNA polymerase II Ser2 phosphorylation in neuroblastoma SH-SY5Y cells exposed to N-&-N1 indicates that N-&-N1 is able to inhibit CDKs in a cellular context. N-&-N1 also down-regulates the expression of RNA polymerase. Cocrystal structures of N-&-N1 and (R)-roscovitine in complex with CDK2/cyclin A reveal that both inhibitors adopt similar binding modes. A competitive assay shows that, compared with (R)-roscovitine, N-&-N1 has reduced affinity for Erk2 and pyridoxal kinase. N-&-N1 triggers cell death in a panel of diverse cell lines. Cell death is accompanied by events characteristic of apoptosis: cytochrome c release, activation of effector caspases, and poly(ADP-ribose) polymerase cleavage. Induction of p53 and p21cip1 and down-regulation of the Mcl-1 antiapoptotic factor were also observed. Studies in mice show that N-&-N1 has pharmacokinetics properties similar to those of (R)-roscovitine. Altogether, these results show that analogues of (R)-roscovitine can be designed with improved antitumor potential. [Mol Cancer Ther 2008; 7(9):2713–24]

Introduction
Since their initial discovery in yeast (1) and the identification of a first homologue in man (2), cyclin-dependent kinases (CDK) have been the object of considerable work with the aim of understanding their regulatory mechanisms and functions in controlling the various steps of the cell division cycle (3). CDKs have also been the targets of numerous molecular screening studies designed to identify low molecular weight pharmacologic inhibitors with potential use against various human diseases derived from abnormal cell cycle regulation (review in ref. 4). These include cancers (5) but also diseases associated with nontumoral proliferations such as various renal diseases (6) and inflammation (7). Over 120 CDK inhibitors have been identified and characterized (8–10). To our knowledge, 10 CDK inhibitors are currently undergoing clinical evaluation as anticancer drugs: flavopiridol (Alvocidib; ref. 5), (R)-roscovitine (CYC202/Seliciclib; refs. 11, 12), R547 (13, 14), SNS-032 (15), PD-0332991 (16, 17), AZD5438 (18, 19), ZK 304709 (20, 21), AG-024322, AT7519, and P276-00 (21).

Among these, (R)-roscovitine was initially identified in a CDK1/cyclin B screen (22) and proven to be one of the first selective CDK inhibitors. Its selectivity has been extensively studied using either panels of purified kinases (≥152 kinases tested; refs. 23–25), affinity chromatography on immobilized roscovitine (25, 26), a quantitative competition assay (27), and a yeast three-hybrid screen (28). Altogether, these methods have provided a rather precise view on the targets of (R)-roscovitine. The main targets are CDK1, CDK2, CDK5, CDK7, and CDK9 (CDK4, CDK6, and CDK8 being poorly if at all sensitive to roscovitine). Yet, a few other kinases are sensitive at higher concentrations of roscovitine, such as casein kinase 10ε/ε, DYRK1A, and Erk1/2. Affinity chromatography revealed an unexpected interacting protein, pyridoxal kinase (PDXK; refs. 25, 26). The pharmacology of (R)-roscovitine has been extensively studied in mice,
rat, and human (reviewed in ref. 12). Pharmacokinetic studies of (R)-roscovitine revealed a rather short half-life [30 min in rat (29, 30), 1.19 h in mice (31–34), and 2.5 h in man (35, 36)]. Several metabolites have been identified, the major ones result from either rapid loss of the isopropyl group (M1), one or more oxidations (M2-M7), or conjugation of a glucose residue (M8). COOH-(R)-roscovitine M3 (inactive on CDKs) is the most abundant metabolite (37). Phase I clinical studies revealed limited toxicity of roscovitine (36), but both preclinical studies using various animal tumor models and phase I/II clinical trials revealed that high doses of (R)-roscovitine were required to generate antitumor activities (9, 36, 38).

These high doses of (R)-roscovitine might constitute a potentially limiting factor in the development of (R)-roscovitine as an anticancer agent. In an effort to identify new roscovitine analogues with enhanced selectivity, reduced toxicity, and overall increased antitumor potency, we did some detailed molecular modeling and generated new (R)-roscovitine biososters. We here report on the improved biological properties of one of these, N-&-N1.

**Materials and Methods**

**Chemistry**

(R)-roscovitine was synthesized as described previously (12, 26) and kindly provided by Dr. Hervé Galons and Nassima Oumata (Université René Descartes).

N-&-N1 and N-&-N2 were synthesized by Florence Popowycz, Gyu Fournet, and Benoît Joseph (Université de Lyon).

**Crystallography**

Expression, Purification, and Co crystallization of Human CDK2/Cyclin A with N-&-N1 or (R)-Roscovitine. Thr160-phospho-CDK2/cyclin A was purified as described previously (39) and concentrated to 13 mg/mL in 40 mmol/L HEPES (pH 7.0), 200 mmol/L NaCl, and 0.01% monothioglycerol. The protein solution was incubated 20 min on ice with N-&-N1 (2 mmol/L) or (R)-roscovitine (2 mmol/L). Before setting up the hanging drop crystallization trials, the protein solution was filtered through a 0.2 μm filter unit. The reservoir solution contained 0.6 to 0.8 mol/L KCl, 0.9 to 1.2 mol/L (NH₄)₂SO₄, and 100 mmol/L HEPES (pH 7.0). Orthorhombic crystals grew within a few weeks at 4°C. Crystals were briefly cryoprotected in 8 mol/L sodium formate before being cryoциcooled in liquid nitrogen. The crystal structure of phospho-CDK2/cyclin A with (R)-roscovitine will be described in details elsewhere (40).

**X-ray Crystallography Data Collection and Processing, Structure Solution, and Refinement.** Data were collected from a single crystal on ESRF ID14-EH-2 beam line at 100 K. Data processing and integration were carried out using MOSFLM and SCALA (41). The structure in complex with the N-&-N1 inhibitor was solved by molecular replacement with MOLREP using a well-refined structure of phospho-CDK2/cyclin A as the search model. The asymmetric unit contained two CDK2/cyclin A heterodimers. The presence of the inhibitor was confirmed by strong electron density at the ATP site after rigid body refinement of the structure. The structure refinement to 2.7 Å, the inhibitor model building, and the library generation for the N-&-N1 inhibitor were done using the CCP4 software suite (41). Alternate cycles of rebuilding in Coot (42) and refinement in Refmac (43) were carried out to obtain the final model. Data processing and refinement statistics are presented in Supplementary Table S1.

**Affinity Chromatography on Roscovitine-Sepharose Beads**

**Buffers.** Homogenization buffer: 60 mmol/L β-glycerophosphate, 15 mmol/L p-nitrophenylphosphate, 25 mmol/L MOPS (pH 7.2), 15 mmol/L EGTA, 15 mmol/L MgCl₂, 1 mmol/L DTT, 1 mmol/L sodium vanadate, 1 mmol/L NaF, 1 mmol/L phenylphosphate, 0.1% NP-40, and protease inhibitor cocktail. Bead buffer: 50 mmol/L Tris (pH 7.4), 5 mmol/L NaF, 250 mmol/L NaCl, 5 mmol/L EDTA, 5 mmol/L EGTA, 0.1% NP-40, 10 μg/mL leupeptin, aprotinin, and soybean trypsin inhibitor, and 100 μmol/L benzamidine.

**Preparation of Extracts.** Pork brains were obtained from a local slaughterhouse and directly homogenized and processed for affinity chromatography or stored at −80°C before use. Tissues were weighed, homogenized, and sonicated in homogenization buffer (2 mL/g material). Homogenates were centrifuged for 10 min at 14,000 × g at 4°C. The supernatant was recovered, assayed for protein content (Bio-Rad protein assay), and immediately loaded batchwise on the affinity matrix.

**Affinity Chromatography of Roscovitine-Interacting Proteins.** (R)-roscovitine was immobilized on Sepharose beads as described (25). Just before use, 10 μL packed roscovitine beads were washed with 1 mL bead buffer and volume was adjusted to 1,200 μL with this buffer. The brain extract supernatant (3 mg total protein) supplemented with various concentrations of roscovitine or N-&-N1 was then added; the tubes were rotated at 4°C for 30 min. After a brief spin at 10,000 × g and removal of the supernatant, the beads were washed four times with bead buffer before addition of 45 μL of 2× Laemmli sample buffer. Following heat denaturation for 3 min, the bound proteins were analyzed by SDS-PAGE and Western blotting or silver staining as described below.

**Electrophoresis and Western Blotting**

**Antibodies.** Some antibodies were obtained from commercial sources: anti-CDK5 C-8 (Santa Cruz; 1:500; 1 h) and

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

9 Jane Endicott, unpublished results.

6 Popowycz et al., submitted for publication.
anti-Erk1/2 (Sigma; 1:4,000; 1 h). Anti-PDXK was generated by Eurogentec Europe (Double XP program). Two rabbits were immunized with a mix of two human CDK internal peptides: LLAWTHKPNNLK (amino acids 241-253) and LRMOVSKRDIEDPEI (amino acids 291-305). The resulting antiseraum (1:500; overnight) cross-reacts with PDXK from a variety of species including mouse, rat, porcine, monkey, and human.

Electrophoresis, Western Blotting, and Silver Staining.

The proteins bound to the roscovitine matrix were separated on 10% NuPAGE precast Bis-Tris polyacrylamide mini-gels (Invitrogen) with MOPS SDS running buffer followed by immunoblotting analysis or silver staining using an Amersham SDS-PAGE silver staining kit. For immunoblotting, proteins were transferred to 0.45 µm nitrocellulose filters (Schleicher & Schuell). These were blocked with 5% low-fat milk in TBS-Tween 20, incubated for 1 h or overnight with antibodies, and analyzed by enhanced chemiluminescence (Amersham).

Protein Kinase Assays

Buffers. Buffer A: 10 mmol/L MgCl$_2$, 1 mmol/L EGTA, 1 mmol/L DTT, 25 mmol/L Tris-HCl (pH 7.5), and 50 µg heparin/mL. Buffer C: 5 mmol/L EGTA, 15 mmol/L NaF, 1 mmol/L Na$_3$VO$_4$, 1 mmol/L Na$_2$P$_2$O$_7$, 25 mmol/L MOPS (pH 7.2), 5 mmol/L EGTA, 15 mmol/L MgCl$_2$, 1 mmol/L DTT, 1 mmol/L sodium vanadate, and 1 mmol/L phenylphosphate.

Kinase Preparations and Assays.

Kinase activity was assayed in buffer A or C at 30°C for a fixed period of time (in the absence of inhibitors). Controls were carried out with appropriate dilutions of DMSO.

CDK1/cyclin B (M-phase starfish oocytes, native), CDK2/cyclin A, CDK2/cyclin E, CDK5/p25, and CDK7/cyclin H (human, recombinant) were prepared as described previously (44). Their kinase activity was assayed in buffer C, with 1 mg histone H1/mL, in the presence of 15 µmol/L ATP, 3,000 Ci/mmol; 10 mCi/mL) in a final volume of 30 µL. After 30 min incubation at 30°C, 25 µL aliquots of supernatant were spotted onto 2.5 x 3 cm pieces of Whatman P81 phosphocellulose paper, and, 20 s later, the filters were washed five times (for at least 5 min each time) in a solution of 10 mL phosphoric acid/1 L water. The wet filters were counted in the presence of 1 mL ACS scintillation fluid (25).

CDK9/cyclin T (human, recombinant, expressed in insect cells) was assayed as described for CDK1/cyclin B but using a pRB fragment (amino acids 773-928; 3.5 µg/assay) as a substrate.

GSK-3α/β (porcine brain, native) was assayed as described for CDK1 but in buffer A and using a GSK-3-specific substrate (GS-1: YRRAVPPPSLHRSSPHQSpEDEEE; pS stands for phosphorylated serine; ref. 25). GS-1 was synthesized by Millegen.

CDK1 (porcine brain, native) was assayed as described for CDK1 but using the CK1-specific peptide substrate RKKHAAIGpSAYSITA (Millegen; ref. 45).

Erk2 (rat, recombinant) was assayed as described for CDK1 but using the specific substrate Ets1 (amino acids 1-138) in buffer A.

DYRK1A (rat, recombinant, expressed in Escherichia coli as a glutathione S-transferase fusion protein) was purified by affinity chromatography on glutathione-agarose and assayed as described for CDK1/cyclin B using myelin basic protein as a substrate.

Cell Biology

Antibodies and Chemicals. AcDEVDaf and Q-VD-OPh were purchased from MPbiomedicals. Cell Titer 96 containing the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent and CytoTox 96 kits were purchased from Promega. The protease inhibitor cocktail was from Roche. Unless otherwise stated, the nonlisted reagents were from Sigma.

Monoclonal antibodies against actin and p21(CIP1) were from Calbiochem. Monoclonal antibodies against cytochrome c and retinoblastoma protein (Rb) were purchased from BD Biosciences. Polyclonal antibody against phospho-Ser$^{259}$/Thr$^{262}$-Rb was provided by Biosource.

Polyclonal antibodies against RNA polymerase II and phospho-Ser$^5$-RNA polymerase II were supplied by Covance Research Products. Polyclonal antibodies against Mcl-1 and poly(ADP-ribose) polymerase (PARP) or monoclonal antibody against p53 were obtained from Santa Cruz Biotechnology.

Cell Lines and Culture Conditions. SH-SY5Y human neuroblastoma (kindly provided by Dr. Jacint Boix) and Huh7 human hepatocarcinoma cell lines were grown in DMEM (Invitrogen). The HCT116 human colorectal carcinoma cell line was grown in McCoy’s 5a medium (American Type Culture Collection). The Fl rat biliary epithelial cell line (kindly provided by Dr. Christiane Guillouzo) was grown on Williams’ medium E (Invitrogen) supplemented with 2 mmol/L I-glutamine (Lonza). The HEK 293 human embryonic kidney and MDA-MB-231 human breast adenocarcinoma (kindly provided by Dr. Jan Mester) cell lines were grown in MEM (Invitrogen). Human foreskin primary fibroblasts (kindly provided by Dr. Gilles Ponzio) were grown in DMEM supplemented with 2 mmol/L I-glutamine and 20 mmol/L HEPES. All media were supplemented with antibiotics (penicillin-streptomycin; Lonza) and 10% volume of FCS (Invitrogen). Cells were cultured at 37°C with 5% CO$_2$. Drug treatments were done on exponentially growing cultures at the indicated time and concentrations. Control experiments were carried out using appropriate dilutions of DMSO.

Cell Death and Cell Viability Assessments.

Cell viability was determined by measuring the reduction of MTS. Cell death was determined by measuring the level of lactate dehydrogenase activity released on cell lysis. Both procedures have been described previously in detail (46).

Caspase Assay. Caspase activity was measured by determining the fluorescence released from the AcDEVDaf synthetic substrate after its direct addition to the
culture medium, detergent lysis, and incubation at 37°C. This method is devised to be applied to 96-multiwell plates. It allows kinetic determinations of caspase activation and the characterization of multiple drugs simultaneously (47).

**Electrophoresis and Western Blotting.** Cells were resuspended, lysed for 30 min at 4°C in homogenization buffer, and sonicated. After centrifugation (14,000 rpm for 15 min at 4°C), the protein concentration was determined in the supernatants by the Bradford protein assay (Bio-Rad). To study cytochrome c release from mitochondria, a 0.05% digitonin cytosolic extraction was done (46).

Following heat denaturation for 5 min, proteins were separated by 10% or 7% NuPAGE precast Bis-Tris or Tris-acetate polyacrylamide mini gel electrophoresis system (Invitrogen) with MOPS SDS (all but cytochrome c, RNA polymerase II, and phospho-Ser²-RNA polymerase II Western blots), MES SDS (cytochrome c), or Tris-acetate SDS (RNA polymerase II and phospho-Ser²-RNA polymerase II) running buffer depending on protein size. Proteins were transferred to 0.45 μm nitrocellulose filters (Schleicher & Schuell). These were blocked with 5% low-fat milk in TBS-Tween 20, incubated for 1 h with antibodies (anti-actin; 1:2,000) or overnight at 4°C (cytochrome c; 1:500), Rb (1:500), phospho-Rb (1:500), RNA polymerase II (1:500), phospho-Ser²-RNA polymerase II (1:500), Mcl-1 (1:500), PARP (1:500), p53 (1:1,000), and p21CIP1 (1:1,000), and analyzed by enhanced chemiluminescence (Amersham).

**Figure 1.** (R)-roscovitine, N-&-N1, and N-&-N2: structure, kinase, and cell survival inhibitory activities. A, structure of the three molecules. Red, nitrogen, which occupies a different position. B, tridimensional structure of the three molecules where the surface is color coded according to electrostatic potential. Geometry optimization was carried out by MOE (Chemical Computing Group) and MMFF94 force field. Calculations of partial charges were calculated based on MMFF94 in MOE and visualization by Vida II software package (http://www.eyesopen.com; technical realization: Sebastian Rohrer). Red, negative; blue, positive. C, effects of the three molecules on ten protein kinase targets. Compounds were tested at various concentrations in the kinase assays as described in Materials and Methods. IC₅₀ values were calculated from the dose–response curves and are reported in μmol/L. D, effects of the three molecules on the survival of various cell lines. (R)-roscovitine and its two analogues were tested at various concentrations for their effects on seven different cell lines: cell survival was estimated 48 h after the addition of each compound using the MTS reduction assay. IC₅₀ values were calculated from the dose–response curves and are reported in μmol/L. Average ± SE of two independent measurements done in triplicates.
Pharmacokinetics in Mice

Chemicals, instrumentation, chromatographic conditions, and calibrations are described in the Supplementary Material.

Animals. The protocols for the animal experiments were approved by the Karolinska Institute Animal Ethical Committee and designed according to the care and use of laboratory animals guidelines established by this committee. The drug was dissolved in DMSO: saline (10:90, v/v). Twenty BALB/c female mice received i.p. doses of N-&-N1 (25 mg/kg body weight) and two mice were killed at each time point. Blood samples were collected by cardiac puncture in heparinized tubes 10, 20, 30, 45, and 60 min and 2, 3, 4, 6, and 7 h after administration. Two control mice received the drug vehicle alone, and plasma samples were also collected as above. Blood samples were centrifuged, immediately after collection, at 3,000 × g for 5 min at 4°C and the plasma was transferred to other tubes. Methanol (200 µL) was added to 100 µL plasma, mixed for 15 s, and centrifuged for 15 min at 4,000 × g to precipitate plasma proteins, supernatants were collected, and all samples were frozen at −20°C directly after preparation until further analysis.

Calculations and Statistics. The peak areas in the chromatograms were plotted versus the corresponding nominal concentrations of the standards and the standard

Figure 2. Targets of (R)-roscovitine and N-&-N1. Porcine brain extracts were loaded for 30 min on (R)-roscovitine beads in the presence of increasing concentrations of (R)-roscovitine (A) or N-&-N1 (B). After extensive washing, the bound proteins were analyzed by SDS-PAGE followed by silver staining (left) and Western blotting (right) using antibodies directed against Erk2, PDXK, and CDK5.
curve was calculated by linear regression. N-&-N1 concentrations in the mice plasma and in the control samples were calculated from the obtained curves. All values are presented as mean ± SD.

**Pharmacokinetics.** N-&-N1 concentration-time data were adjusted to a two-compartment open model using Gauss-Newton criteria. Variables including the distribution volume of the central compartment, the elimination rate constant, the plasma maximum concentration, and the microconstants were estimated. The clearance and distribution volume at the steady state were calculated from the primary variables. The plasma concentration versus time curve were calculated from the model-derived variables and the elimination half-lives were calculated from the slope of the terminal phase of elimination. The pharmacokinetic modeling was done using WinNonlin version 5.2.

**Results**

**Synthesis of and Kinase Inhibition by Two Roscovitine Analologues**

Two compounds closely related to roscovitine were synthesized, N-&-N1 and N-&-N2 (GP0210 and GP0212, respectively, at Greenpharma; Fig. 1A). We reasoned that a slight shift of the nitrogen from position 9 in roscovitine to position 5 (N-&-N1) or position 4 (N-&-N2) equivalents should lead to modest modifications in the electrostatic potential (Fig. 1B). However, these modifications might result in significant differences in the interaction with biological targets. (R)-ros covitine, N-&-N1, and N-&-N2 were tested on the major roscovitine targets (CDK1/cyclin B, CDK2/cyclin A, CDK2/cyclin E, CDK5/p25, CDK7/cyclin H, CDK9/cyclin T, CK1γ/δ, Dyrk1A, and Erk2) and the closely related GSK-3α/β (Fig. 1C). N-&-N1 was found to be significantly more active on all CDKs than either (R)-ros covitine or N-&-N2. Compared with (R)-ros covitine, N-&-N1 was three to five times more active on CK1 and GSK-3α/β, but both were roughly equipotent on Erk2. Whether enhanced activity on CK1 and GSK-3 has consequences on the cell cycle/apoptosis effects of N-&-N1 remains to be investigated.

As an alternative method to compare the interaction of roscovitine targets with N-&-N1, we used a competitive assay with roscovitine immobilized on Sepharose beads (25). Porcine brain extracts were loaded on roscovitine beads in the presence of increasing concentrations of either (R)-ros covitine or N-&-N1 in the brain extract before and during incubation with the (R)-ros covitine beads. The effects of this competition on the binding of several protein targets were analyzed by electrophoresis of the bound proteins followed by silver staining and Western blotting (Fig. 2). Results showed that, in contrast to (R)-ros covitine (Fig. 2A), N-&-N1 only weakly competed for binding of Erk2 and PDXK (Fig. 2B). However, N-&-N1 was a slightly better competitor than (R)-ros covitine for CDK5. This suggests that N-&-N1 has a slightly higher affinity for CDK5 than (R)-ros covitine does but that its interaction with PDXK and Erk2 is much weaker. These observations were confirmed when similar experiments were carried out using extracts of SH-SY5Y cells (data not shown). In other words, N-&-N1 appears to have increased selectivity toward CDKs.

**N-&-N1-CDK2/Cyclin A Cocrystal Structure**

The molecular interactions of N-&-N1 and (R)-ros covitine with CDK2 was investigated by cocrystallization of each inhibitor with CDK2/cyclin A (Supplementary...
Fig. S3). A full description of the roscovitine-CDK2/cyclin A cocrystal structure will be presented elsewhere (40). Both inhibitors occupy the CDK2 ATP-binding site and make two hydrogen bonds to the CDK2 backbone within the hinge sequence that links the two lobes of the kinase. For both inhibitors, the amine group that bridges between the fused heterocycles and the benzyl moiety is engaged in a hydrogen bond with CDK2 Leu 83 main chain carbonyl. A second hydrogen bond between the inhibitors and CDK2 is made via the main chain amine of Leu 83 and the common nitrogen atom of the isopropyl-pyrazole ring of N-\&-N1 and the isopropyl-imidazole ring of roscovitine. The two inhibitors are in interaction with neighboring water molecules. Based on the two crystal structures, it is difficult to rationalize the increased inhibitory activity of N-\&-N1 compared with (R)-roscovitine (Fig. 1C) as their binding modes derived from the cocrystal structure are almost exactly identical.

**N-\&-N1 Induces Apoptotic Cell Death**

We next compared the three compounds for their ability to induce cell death in neuroblastoma SH-SY5Y cells as measured with a MTS reduction assay (Figs. 1D and 4A and B). Because MTS reduction is occasionally observed under conditions different from cell death, we also used an independent cell death assay, the lactate dehydrogenase release assay (Fig. 4C). Dose-response curves showed that (R)-roscovitine and N-\&-N2 displayed similar activities while N-\&-N1 was consistently more potent in terms of concentration required to reduce cell survival (MTS reduction; Fig. 4A and B) or in terms of cell death (lactate dehydrogenase release; Fig. 4C). To confirm that the enhanced induction of cell death by N-\&-N1 was not a specific property of SH-SY5Y cells, we tested (R)-roscovitine, N-\&-N1, and N-\&-N2 on seven human cell lines (Fig. 1D). With the exception of human fibroblasts, all cell lines were more sensitive to N-\&-N1 (average IC50 = 5.2 \( \mu \)mol/L) than to (R)-roscovitine (average IC50 = 28.3 \( \mu \)mol/L) or N-\&-N2 (average IC50 = 25.0 \( \mu \)mol/L). The (S)-enantiomer of N-\&-N1 was as potent as the (R)-enantiomer (data not shown).

**Figure 4.** Effects of (R)-roscovitine, N-\&-N1, and N-\&-N2 on SH-SY5Y cells. A, SH-SY5Y cells were exposed for 24 h to increasing concentrations of the three compounds. Cell survival was estimated by the MTS reduction assay and is expressed in percentage of survival in untreated cells. Average ± SE of at least four independent experiments with three independent measurements per experiment. B, time-course experiment: SH-SY5Y cells were treated with 5 or 50 \( \mu \)mol/L of each compound for 3, 6, 12, 24, 36, or 48 h. Cell survival was assessed by the MTS procedure. Points, mean of two independent experiments with at least three independent measurements per experiment; bars, SE. C, an experiment similar to that presented in A was done, but lactate dehydrogenase release was assayed 24 h after the addition of the compounds. Average ± SE of two independent experiments with three independent measurements per experiment. D, SH-SY5Y cells were exposed to 5 or 50 \( \mu \)mol/L of each compound for 24 h and proteins were resolved by SDS-PAGE followed by Western blotting with antibodies directed against phospho-Rb, Rb, Mcl-1, and actin.
Cell death was accompanied by dose-dependent activation of effector caspases (Fig. 5A and B). N-&-N1 was most potent at inducing caspases activation when compared with (R)-roscovitine or N-&-N2. Both (R)-roscovitine and N-&-N1 induced a dose-dependent release of mitochondrial cytochrome c (Fig. 5C) and induction of PARP (poly(ADP-ribose) polymerase) cleavage (Fig. 5D). Both compounds triggered induction of p53 and transient induction of p21 CIP1 (Fig. 6A). Finally, (R)-roscovitine or N-&-N1 inhibited, in a dose-dependent manner, the phosphorylation of RNA polymerase II on Ser 2, a site typically phosphorylated by CDK9/cyclin T (Fig. 6B, top). However, the total amount of RNA polymerase II was also reduced, although not completely (Fig. 6B, bottom). Down-regulation of Mcl-1 was induced more potently by N-&-N1 than by (R)-roscovitine (Fig. 6C, top).

**Pharmacokinetics of N-&-N1**

An essential condition to further investigate roscovitine analogues as potential antitumor agents is that their pharmacokinetics be similar to or better than those of (R)-roscovitine. To conduct the N-&-N1 pharmacokinetic studies, a simple high-performance liquid chromatography-UV method for the determination of N-&-N1 in human plasma was thus developed and validated (Supplementary Material). Plasma proteins were precipitated with methanol (1:2, v/v). The chromatographic separation of N-&-N1 in plasma was carried out using a reverse-phase Zorbax SB C18 column with tetrahydrofuran:phosphoric acid 0.1% in water (16:84, v/v) as the mobile phase. The lower limit of drug quantification was 100 ng/mL using 200 nL plasma. The calibration curve was linear over the range 1,000 to 5,000 ng/mL ($r^2 > 0.9987$). The intrabatch and interbatch accuracy ranged from 3.4% to 12.3% and the variation coefficients were no more than 10.3% for both intrabatch and interbatch precision over the calibration curve range. The absolute recovery of N-&-N1 from plasma was >80%. N-&-N1 showed consistent short-term, long-term, and freeze-thaw stability in human plasma. This analytical method was validated and was shown to provide enough selectivity, sensitivity, and stability for pharmacokinetic studies according to the bioanalytical guidelines mentioned by Shah et al. (48, 49).

The pharmacokinetics of N-&-N1 were then investigated in mice and found to fit a two-compartment open model. The elimination half-life was biphasic with rapid
distribution (9 min) and an elimination half-life of ~1 h (Fig. 7A). The variables obtained from both series were very similar. The pharmacokinetic variables are listed in Fig. 7B. The pharmacokinetic variables of N-&-N1 were essentially similar to those (in mice) of other CDK inhibitors from the 2,6,9-trisubstituted purine family such as olomoucine ($t_{1/2} = 58$ min) and roscovitine ($t_{1/2} = 72$ min; refs. 29, 33).

Discussion
Despite numerous investigations, the use of CDK inhibitors against cancers has met only modest success. However, lessons learned from the early inhibitors and recent progress in our understanding of CDKs and their regulation suggest that new inhibitors may be designed with better anticancer potential (21).

In this investigation, we report on the biological properties of N-&-N1 and N-&-N2, two bioisosters of (R)-rosocovitine. In all investigated aspects, N-&-N2 behaved similarly to (R)-rosocovitine. In contrast, when compared with (R)-rosocovitine, N-&-N1 was found to display improved biological properties in terms of ability to inhibit various kinase targets and to induce apoptotic death in tumor cell lines.

N-&-N1 was roughly three to five times more potent than (R)-rosocovitine at inhibiting CDK1, CDK2, CDK5, and CDK9. N-&-N1 was three to four times more active on CK1 and GSK-3a/b. In contrast, N-&-N2 and (R)-rosocovitine were equipotent on all kinases tested (with the exception of CK1 and GSK-3a/b, where N-&-N2 was 2- to 3-fold more potent). In an attempt to understand the better inhibitory potency of N-&-N1 on CDKs, both N-&-N1 and (R)-rosocovitine were cocrystallized with active CDK2/cyclin A.

Figure 6. Alteration of some key proteins by (R)-rosocovitine and N-&-N1 in SH-SYSY cells. SH-SYSY cells were exposed for 24 h to increasing concentrations of the two compounds and proteins were resolved by SDS-PAGE followed by Western blotting with antibodies. A, both compounds triggered sustained up-regulation of p53 and transient induction of p21CIP1. B, (R)-rosocovitine or N-&-N1 inhibited, in a dose-dependent manner, the phosphorylation of RNA polymerase II on Ser², a site typically phosphorylated by CDK9/cyclin T (top), but also the expression of total RNA polymerase II (bottom). C, (R)-rosocovitine or N-&-N1 induce the down-regulation of the survival protein Mcl-1. Equal loading of samples was verified by assessing the level of actin (bottom).
Pharmacokinetics of N-&-N1 in mice. A, the pharmacokinetics of N-&-N1 were fitted a two-compartment open model. The elimination half-life was biphasic with rapid distribution and an elimination half-life of 2.8 ± 0.3 h under plasma concentration curve. Solid line, two-compartment model; filled triangle, experimental time points ± SD. Each time point includes two animals. B, pharmacokinetics parameters. Abbreviations: AUC, area under plasma concentration curve; Cl, clearance; C\textsubscript{max}, maximum reached concentration.

Figure 7. Pharmacokinetics of N-&-N1 in mice. A, the pharmacokinetics of N-&-N1 were fitted a two-compartment open model. The elimination half-life was biphasic with rapid distribution and an elimination half-life of 2.8 ± 0.3 h under plasma concentration curve. Solid line, two-compartment model; filled triangle, experimental time points ± SD. Each time point includes two animals. B, pharmacokinetics parameters. Abbreviations: AUC, area under plasma concentration curve; Cl, clearance; C\textsubscript{max}, maximum reached concentration.

(Fig. 3). Resolution of the cocrystal structures revealed that both molecules occupy essentially the same site in the same orientation. No explanation for a more favorable interaction of N-&-N1 with CDK2 can be proposed. One possibility is that subtle differences between the two molecules in their electrostatic potential might be sufficient to alter the kinetics of binding of the inhibitors to and their release from the catalytic site of the kinase. Alternatively, the structural differences between (R)-roscovitine and N-&-N1 might be perturbing the pKa of the heterocyclic nitrogens, thus altering the strengths of hydrogen bonds that they make. Finally, details of electron distribution around the heterocycles may also modulate the strength of the aromatic-aliphatic interactions that sandwich the inhibitors between NH\textsubscript{2} and COOH-terminal kinase lobes.

Kinase selectivity studies (Fig. 1C) did not reveal major differences in selectivity between (R)-roscovitine and N-&-N1. We investigated the possibility of altered selectivity toward other targets using an indirect affinity chromatography approach on immobilized (R)-roscovitine (Fig. 2) and increasing concentrations of either free N-&-N1 or free (R)-roscovitine. This competition-based assay revealed that, compared with (R)-roscovitine, N-&-N1 appears to have a much reduced affinity for Erk2 and PDAX. This point is of importance as interaction of (R)-roscovitine with PDAX may dilute its effects in a cellular context by reducing the amount of (R)-roscovitine that is free to interact with the CDK targets. Reduced binding to PDAX, as observed with N-&-N1, is expected to lead to enhanced biological effects at lower concentrations, with most of the N-&-N1 targeting CDKs directly, rather than being diverted to PDAX.

Indeed, N-&-N1 has been found to induce cell death with better efficacy (5.4-fold lower IC\textsubscript{50}, average of six cell lines) than (R)-roscovitine (Fig. 1D), although this effect may also result from different cell penetrance by the two compounds. In contrast, N-&-N2 displayed the same potency as (R)-roscovitine. Various variables of the induced cell death were analyzed: MTS reduction, lactate dehydrogenase release, p53 and p21 induction, inhibition of phosphorylation of RNA polymerase II on its CDK9 site, down-regulation of RNA polymerase II, down-regulation of the survival factor Mcl-1, cytochrome c release, activation of caspases, and PARP cleavage. N-&-N1 was consistently more potent than (R)-roscovitine and N-&-N2 at inducing all these physiological/biochemical events.

We believe that the enhanced apoptosis-inducing effects of N-&-N1 over (R)-roscovitine can be accounted for by the combination of two factors: (a) an overall increase in the inhibitory potency of N-&-N1 on its CDK and GSK3-β targets but also of some other kinase targets and (b) a reduced interaction of N-&-N1 with two ‘‘off-targets’’ proteins, Erk2 and PDAX, resulting in a higher availability of the inhibitor to interact with CDKs. However, we cannot rule out the existence of an unidentified key target of N-&-N1. This hypothesis will be addressed directly using affinity chromatography on immobilized N-&-N1. Although we have no information on this, we cannot rule out the possibility that cell permeability of N-&-N1 is higher than that of (R)-roscovitine and N-&-N2. Similarly, the intracellular distribution of all three compounds is unknown and different intracellular localization of the drug might result in different biological consequences. Finally, we have no information on the cellular stability of N-&-N1, (R)-roscovitine, and N-&-N2, and subtle differences might translate into major differences in biological effects.

As a last step in our studies on N-&-N1 and (R)-roscovitine, we thought it was important to establish a reliable assay for N-&-N1 to investigate its pharmacokinetics. Poor pharmacokinetics would counteract the increased biochemical/cell biology potential of N-&-N1 as an improved roscovitine. Detailed pharmacokinetics revealed that N-&-N1 behaves, at least in mice, exactly like (R)-roscovitine (Fig. 7). We are thus now in good position to analyze the comparative antitumor properties of N-&-N1 and (R)-ros covitine. Preliminary experiments carried out in xenografted nude mice reveal significantly enhanced antitumor activity of N-&-N1 (data not shown).

Altogether, these results show that it is possible to generate (R)-ros covitine analogues with improved bioactivity. Their chemical similarity to (R)-ros covitine allows us...
to reasonably hope for similar biological, toxicologic, and pharmacodynamic properties. Yet, the subtle chemical differences introduced in N-& N1 result in significant improvement in kinase inhibitory properties and enhanced selectivity (by reduced interaction with off-targets) and consequently improved cellular effects. These modest modifications may contribute to the design of second roscovitine analogues, allowing enhanced antitumoral activity with lower quantities of drug, thereby circumventing one of the limitations of (R)-roscovitine (36).

Disclosure of Potential Conflicts of Interest

P. Bernard: patent holder. The other authors disclosed no potential conflicts of interest.

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References

7. Rossi AG, Sawatzky DA, Walker A, et al. Cyclin-dependent kinase inhibitors enhance the resolution of inflammation by promoting inflamma-
13. Chu XJ, DePinto W, Bartkovitz D, et al. Discovery of (4-amino-2-(1-methanesulfonyl)piperidin-4-ylamino)pyrimidine-5-yl[i][2,3-difluoro-6-methox-
thiazole inhibitors of cyclin-dependent kinase 2. N-[5-5-11,1-dime-
dynamic study of the effects of the cyclin-dependent kinase-inhibitor AZD5438 on cell cycle markers within the buccal mucosa, plucked scalp hairs and peripheral blood mononucleocytes of healthy male volunteers. Cancer Chemother Pharmacol 2007;60:479 –88.
30. Vita M, Meurling L, Pettersson T, Cruz-Siden M, Siden A, Hassan M. Analysis of roscovitine using novel high performance liquid chromatogra-
37. McClue SJ, Stuart I. Metabolism of the trisubstituted purine cyclin-dependent kinase inhibitor Seliciclib (R-roscovitine) in vitro and in vivo. Drug Metab Dispos 2008;36:561–70.
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