

Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5

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Cyclin-dependent kinases (cdk) play an essential role in the intracellular control of the cell division cycle (cdc). These kinases and their regulators are frequently deregulated in human tumours. Enzymatic screening has recently led to the discovery of specific inhibitors of cyclin-dependent kinases, such as butyrolactone I, flavopiridol and the purine olomoucine. Among a series of C2, N⁶, N9-substituted adenines tested on purified cdc2/cyclin B, 2-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine (roscovitine) displays high efficiency and high selectivity towards some cyclin-dependent kinases. The kinase specificity of roscovitine was investigated with 25 highly purified kinases (including protein kinase A, G and C isoforms, myosin light-chain kinase, casein kinase 2, insulin receptor tyrosine kinase, c-src, v-abl). Most kinases are not significantly inhibited by roscovitine. cdc2/cyclin B, cdk2/cyclin A, cdk2/cyclin E and cdk5/p35 only are substantially inhibited (IC₅₀ values of 0.65, 0.7, 0.7 and 0.2 μM, respectively). cdk4/cyclin D1 and cdk6/cyclin D2 are very poorly inhibited by roscovitine (IC₅₀ > 100 μM). Extracellular regulated kinases erk1 and erk2 are inhibited with an IC₅₀ of 34 μM and 14 μM, respectively. Roscovitine reversibly arrests starfish oocytes and sea urchin embryos in late prophase. Roscovitine inhibits *in vitro* M-phase-promoting factor activity and *in vitro* DNA synthesis in *Xenopus* egg extracts. It blocks progesterone-induced oocyte maturation of *Xenopus* oocytes and *in vivo* phosphorylation of the elongation factor eEF-1. Roscovitine inhibits the proliferation of mammalian cell lines with an average IC₅₀ of 16 μM. In the presence of roscovitine L1210 cells arrest in G1 and accumulate in G2. *In vivo* phosphorylation of vimentin on Ser55 by cdc2/cyclin B is inhibited by roscovitine. Through its unique selectivity for some cyclin-dependent kinases, roscovitine provides a useful anti-mitotic reagent for cell cycle studies and may prove interesting to control cells with deregulated cdc2, cdk2 or cdk5 kinase activities.

Keywords: cell cycle; cyclin-dependent kinase; kinase inhibitor; purine; anti-tumor agent.

Regulation of the cell division cycle has been the object of numerous studies during the last few years (see reviews in [1–5]). Among cell cycle regulators, cyclin-dependent kinases (cdk) appear to play a central role in the initiation and ordering of cell cycle events (reviews in [6–9]). Cdk proteins are constituted of a catalytic subunit, the prototype of which is cdc2, and a regulatory subunit (cyclin). Eight human cdk proteins have been described so far: cdk1 (= cdc2), cdk2–cdk8. With the exception of cdk3, for which the regulatory cyclin has not yet been identified [10], all these proteins are regulated by the transient association with one member of the cyclin family: cyclin A (cdc2, cdk2), B1–B3 (cdc2), C (cdk8), D1–D3 (cdc2, cdk2, cdk4, cdk5, cdk6), E (cdk2), H (cdk7). Each step of the cell cycle is

thought to be regulated by such cdk complexes: G1/S transition (cdk2/cyclin E, cdk3/unknown cyclin, cdk4/cyclin D1–D3, cdk6/cyclin D1, cdk8/cyclin C), S phase (cdk2/cyclin A), G2 (cdc2/cyclin A), G2/M transition (cdc2/cyclin B, cdk7/cyclin H). Other cdc2-related kinases and cyclins have been sequenced which await identification of their regulatory partners and of their cell cycle regulatory functions. Cdk proteins are finely regulated by post-translational (phosphorylation/dephosphorylation) modifications, transient associations with other proteins and intracellular translocations. The regulators include activators (cyclins, cdk7/cyclin H, cdc25 phosphatases), the interacting subunits p9^{CKShs} and p15^{cdk-BP}, natural inhibitors (p16^{INK4A}, p15^{INK4B}, p18^{INK4C}, p27^{KIP1}, p57^{KIP2}, p21^{cip1}) and inhibiting enzymes (wee1, mik1, myt1, KAP) (reviewed in [8, 9]). The crystal structures of cdk2 [11] and of cdk2/cyclin A [12] have been recently determined.

Human tumour development is associated with numerous alterations of cdk proteins and their regulators (reviews in [13, 14]): overexpressions of cyclins D and E in a large variety of tumours, overexpression of cdc2, oncogenicity of the D cyclins, oncogenicity of cyclin A in a human hepatic cancer, oncogenicity of the cdc25 phosphatase, abnormal temporal expression of

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Abbreviations. cdc, cell division cycle; cdk, cyclin-dependent kinase; erk1 and erk2, extracellular regulated kinase 1 and 2; 1MeAde, 1-methyladenine; MPF, M-phase promoting factor; [Ser(P)⁵⁵]vimentin, vimentin phosphorylated on Ser55; NCI, National Cancer Institute (USA).

cyclins in human tumour cell lines, presence of INK4-resistant cdk4 in some tumours, high frequency deletions and mutations of INK4 in human tumours cell lines and primary tumours, transcriptional regulation of p21^{cip1} by the tumour suppressor p53, etc.

The frequent deregulation of cdk proteins and their regulators in cancer and the therapeutic potential of natural inhibitors [15] has stimulated an active search for chemical cdk inhibitors. A few years ago we set up a simple screening test using affinity-purified p34^{cdc2}/cyclin B^{cdc13} as a target [16]. The enzyme activity is assayed, in the presence of potential inhibitors, using histone H1 and [³²P]ATP. Using this or very closely related methods, five types of cdk inhibitors have been described so far: a) staurosporine [16, 17], b) butyrolactone I [18–20], c) flavopiridol [21–23], d) suramin [24] and e) olomoucine [25–28]. The chemical, enzymatic and cellular properties of these compounds have been reviewed [29, 30].

The purine olomoucine displays a quite narrow selectivity: among 35 kinases tested, it only inhibits cdc2, cdk2, cdk5, and erk1 to a lesser extent [27]. The position of olomoucine in the ATP binding pocket of cdk2 has been determined by analysis of an olomoucine/cdk2 co-crystal [31]. Interestingly the purine rings of olomoucine and ATP are orientated in a totally different manner. Recently, olomoucine was found to stimulate massive apoptosis in cells which have been arrested in G2 by the use of DNA-damaging agents [32]. In contrast cdk inhibitors (olomoucine and flavopiridol) display anti-apoptotic activity in post-mitotic neuronally differentiated PC12 cells and sympathetic neurons [33]. These encouraging results led us to evaluate other C2, N⁶, N9-substituted adenines as potential cdk inhibitors [34]. We here report the biochemical properties and cellular effects of a novel inhibitor, 2-(R)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine (roscovitine). The molecular interaction of roscovitine with cdk proteins is described in the preceding paper [34].

EXPERIMENTAL PROCEDURES

Chemicals. Sodium orthovanadate, 1-methyladenine (1MeAde), EGTA, EDTA, Mops, glycerol 2-phosphate, dithiothreitol, sodium fluoride, nitrophenyl phosphate, leupeptin, aprotinin, soybean trypsin inhibitor, benzamidine, histone H1 (type III-S), myelin basic protein, casein, protamine sulfate, isopropyl β-D-thiogalactopyranoside, CNBr-activated Sepharose 4B, Luria-Bertani broth base, glutathione and glutathione-agarose beads were obtained from Sigma Chemicals.

Roscovitine was synthesized by one of us (Havlicek et al., unpublished) and dissolved as a 10–50 mM stock solution in dimethylsulfoxide (Me₂SO). It was diluted to 5–10 mM in Me₂SO just prior to use in aqueous buffers. Final Me₂SO concentration in the reaction mixture was less than 1% (by vol.). [³²P]ATP (PB 168) was obtained from Amersham.

Retinoblastoma protein (conjugated to glutathione S-transferase) was expressed in bacteria and purified on glutathione-Sepharose beads as previously described [27, 35].

Monoclonal anti-PSTAIRES antibodies (raised against the EGVSTAIRESLLKEGGC peptide) were generously donated by Dr M. Yamashita (Sapporo). 4A4 monoclonal antibodies (raised against the SLYSSS(P)PGGAYC peptide) recognizing [Ser(P)⁵⁵]vimentin were provided by Drs N. Inagaki and M. Inagaki (Tokyo).

Buffers. Homogenization buffer: 60 mM glycerol 2-phosphate, 15 mM *p*-nitrophenyl phosphate, 25 mM Mops pH 7.2, 15 mM EGTA, 15 mM MgCl₂, 1 mM dithiothreitol, 1 mM sodium vanadate, 1 mM NaF, 1 mM phenyl phosphate, 10 μg leu-

peptin/ml, 10 μg aprotinin/ml, 10 μg soybean trypsin inhibitor/ml and 100 μM benzamidine.

Buffer C: homogenization buffer but 5 mM EGTA, no NaF and no protease inhibitors.

Preparation of starfish M-phase oocyte extracts. For large-scale oocyte extracts preparations, gonads were removed from ripe *Marthasterias glacialis* and incubated with 10 μM 1MeAde in Millipore-filtered natural sea water until spawning; by that time, all the oocytes had entered the M phase. Oocytes were then removed from the incubation medium by centrifugation, directly frozen in liquid nitrogen and kept at -80°C [27, 36]. M-phase oocytes were homogenized in homogenization buffer at a ratio of 2 g oocytes/ml buffer. After a 45-min centrifugation at 100000 g, the supernatant was recovered and directly used for affinity-chromatography purification of the p34^{cdc2}/cyclin B kinase on p9^{CKShs1}-Sepharose beads as described [27, 35].

Enzymes. Kinases activities were assayed at 30°C in buffer C (unless otherwise specified). Blank values were subtracted from the data and activities calculated as molar amount of phosphate incorporated in protein acceptor during a 10-min incubation. Controls were performed with appropriate dilutions of Me₂SO. In a few cases, phosphorylation of the substrate was assessed by autoradiography after SDS/PAGE.

p34^{cdc2}/cyclin B was purified from M-phase starfish (*M. glacialis*) oocytes by affinity chromatography on p9^{CKShs1}-Sepharose beads, from which it was eluted by free p9^{CKShs1} as described above [35–37]. It was assayed with 1 mg histone H1/ml, in the presence of 15 μM [³²P]ATP (3000 Ci/mmol; 1 mCi/ml) in a final volume of 30 μl [27]. After a 10-min incubation at 30°C, 25-μl aliquots of supernatant were spotted onto pieces (2.5×3 cm) of Whatman P81 phosphocellulose paper, and, after 20 s, the filters were washed five times (for at least 5 min each time) in a solution of 10 ml phosphoric acid/1 water. The wet filters were transferred into 6-ml plastic scintillation vials, 5 ml ACS (Amersham) scintillation fluid was added and the radioactivity measured in a Packard counter. The kinase activity was expressed as molar amount of phosphate incorporated in histone H1 during a 10-min incubation or as a percentage of maximal activity.

p33^{cdk2}/cyclin A and p33^{cdk2}/cyclin E (kindly provided by W. Harper) were reconstituted from extracts of sf9 insect cells infected with various baculoviruses. Cyclins A and E were fusion proteins with glutathione S-transferase and the complexes were purified on glutathione-agarose beads. Kinase activities were assayed with 1 mg/ml histone H1, in the presence of 15 μM [³²P]ATP, during 10 min, in a final volume of 30 μl, as described for the p34^{cdc2}/cyclin B kinase.

p33^{cdk5}/p35 (provided by J. Lew and J. H. Wang) was purified from bovine brain [38], excluding the Mono S chromatographic step. The active fractions from the Superose 12 column were pooled and concentrated to a final concentration of approximately 25 μg enzyme/ml. The kinase was assayed with 1 mg/ml histone H1 in the presence of 15 μM [³²P]ATP, during 10 min in a final volume of 30 μl, as described for the p34^{cdc2}/cyclin B kinase.

p33^{cdk4}/cyclin D1 was obtained from insect cell lysates (provided by W. Harper). Cdk4 was a fusion protein with glutathione S-transferase and the active complex was purified on glutathione-agarose beads. Its kinase activity was assayed with purified retinoblastoma protein (complexed with glutathione-S-transferase) in the presence of 15 μM [³²P]ATP, in a final volume of 30 μl. After a 15-min incubation, 30 μl 2×Laemmli sample buffer was added. The phosphorylated substrate was resolved by 10% SDS/PAGE and analysed by autoradiography by overnight exposure to Hyperfilm MP and densitometry.

p33^{cdc6}/cyclin D2 (provided by M. Meyerson) was obtained from insect cell lysates [39]. It was assayed with purified retinoblastoma protein (complexed with glutathione-S-transferase) in the presence of 15 μ M [γ -³²P]ATP in a final volume of 30 μ l. After a 30-min incubation, 30 μ l 2 \times Laemmli sample buffer was added. The phosphorylated substrate was resolved by 10% SDS/PAGE and analysed by autoradiography by overnight exposure to Hyperfilm MP and densitometry.

MAP kinase erk1 [40] (tagged with glutathione-S-transferase; cloned from a human HepG2 library; provided by D. Charrest and S. L. Pelech), was expressed in bacteria, purified on glutathione-agarose beads and assayed with 1 mg myelin basic protein/ml in the presence of 15 μ M [γ -³²P]ATP as described above for the p34^{cdc2}/cyclin B kinase. His-tagged erk1 and erk2 (provided by M. Cobb) were activated *in vitro* by mitogen-activated protein kinase kinase, purified (Ni-affinity and Mono Q) and assayed as described above during 10 min in a final volume of 30 μ l.

Protein kinase C isoforms were purified from baculovirus-infected sf9 insect cells and assayed with 1 mg/ml protamine sulfate in the presence of 15 μ M [γ -³²P]ATP, during 10 min at 30°C, in a final volume of 30 μ l. Phosphorylated protamine sulfate was recovered on Whatman P81 phosphocellulose paper as described for the cdc2 kinase.

The catalytic subunit of cAMP-dependent protein kinase (provided by S. Lohmann), purified from bovine heart, was assayed with 1 mg histone H1/ml, in the presence of 15 μ M [γ -³²P]ATP as described for the p34^{cdc2}/cyclin B kinase.

cGMP-dependent protein kinase (provided by F. Hofmann), purified to homogeneity from bovine tracheal smooth muscle, was assayed with 1 mg histone H1/ml, in the presence of 15 μ M [γ -³²P]ATP as described for the p34^{cdc2}/cyclin B kinase.

Casein kinase 2 (provided by L. Pinna) was isolated from rat liver cytosol and assayed with 1 mg casein/ml and 15 μ M [γ -³²P]ATP. The substrate was spotted on Whatmann 3MM filters and washed with 10% (mass/vol.) trichloroacetic acid.

Myosin light chain kinase, purified from chicken gizzard (provided by T. J. Lukas and M. Watterson) was assayed in the presence of 100 nM calmodulin, 100 μ M CaCl₂, 50 mM Hepes, 5 mM MgCl₂, 1 mM dithiothreitol and 0.1 mg BSA/ml at pH 7.5 using a synthetic peptide based on the smooth-muscle myosin light-chain phosphorylation site (KKRPQRATSNVFAM, 50 μ M) and in the presence of 15 μ M [γ -³²P]ATP, in a final volume of 50 μ l. Incorporation of radioactive phosphate was monitored on phosphocellulose filters as described above.

ASK- γ , a plant homologue of GSK-3, was expressed as a glutathione-S-transferase fusion protein in *Escherichia coli* [41] and purified on glutathione-agarose. ASK- γ kinase was assayed, for 10 min at 30°C, with 5 μ g myelin basic protein, in the presence of 15 μ M [γ -³²P]ATP, in a final volume of 30 μ l. The phosphorylated myelin basic protein was recovered on Whatman P81 phosphocellulose paper as described for the p34^{cdc2}/cyclin B kinase.

Insulin receptor tyrosine kinase domain (CIRK-41) [42] was overexpressed in a baculovirus system and purified to homogeneity (provided by H. Y. L. Tung). Its kinase activity was assayed, for 10 min at 30°C, with 5 μ g Raytide (Oncogene Sciences), in the presence of 15 μ M [γ -³²P]ATP, in a final volume of 30 μ l. The phosphorylated Raytide was recovered on Whatman P81 phosphocellulose paper as described for the p34^{cdc2}/cyclin B kinase.

c-src kinase was purified from infected Sf9 cells. The v-abl kinase was expressed in *E. coli* and affinity purified on IgG-Affigel 10. Both kinases (provided by Dr Helmut Mett) were assayed for 10 min at 30°C, with 5 μ g Raytide, in the presence of 15 μ M [γ -³²P]ATP, in a final volume of 30 μ l. The phosphory-

lated Raytide was recovered on Whatman P81 phosphocellulose paper as described for the p34^{cdc2}/cyclin B kinase.

Electrophoresis and western blotting. Proteins bound to p9^{CKShst1}-Sephacrose beads (starfish cdc2) or crude extracts (L1210) were denatured with 2 \times Laemmli sample buffer. Samples were run on 10% SDS/polyacrylamide gels. Proteins were transferred from the gel to a 0.1- μ m nitrocellulose sheet (Schleicher & Schüll) in a milliblot-SDE system (Millipore) for 30 min at 2.5 mA/cm² in transfer buffer. Subsequently, the filter was blocked with 5% low fat milk in 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20 (Tris/NaCl/Tween-20) for 1 h. The filter was then washed with (Tris/NaCl/Tween-20) and incubated for 1 h with the first antibodies (anti-PSTAIR, 1:2000; anti-[Ser(P)⁵⁵] vimentin, 1 mg/ml). After four washes (one of 20 min three of 5 min) with (Tris/NaCl/Tween-20), the nitrocellulose sheet was treated for 1 h with horseradish-peroxidase-coupled secondary antibodies diluted in (Tris/NaCl/Tween-20) (1:1000). The filter was then washed five times (one of 20 min, four of 5 min) with (Tris/NaCl/Tween-20) and analysed by enhanced chemiluminescence with ECL detection reagents and hyperfilm MP.

Sea urchin embryos. Sea urchins (*Sphaerechinus granularis*) were collected by diving in Brittany and kept in running sea water until use. Shedding of gametes was induced by injection of 0.2 ml 0.2 M acetylcholine. Sperm was collected dry and kept undiluted at +4°C. Eggs were collected in Millipore-filtered natural sea water (NSW). They were washed once with NSW and resuspended as a 10% (by vol.) suspension. To facilitate fertilisation membrane elevation, 0.1% (mass/vol.) glycine was added to the egg suspension prior to fertilisation. Sperm was diluted just before insemination (1 drop dry sperm/5 ml NSW; 1 drop of this dilution/10 ml egg suspension). At 2–3 min after sperm addition, the eggs were checked for successful fertilisation (100% in all experiments) and excess sperm was removed by washing the eggs once with NSW. All experiments were performed at +18°C. Roscovitine was added 60 min after fertilisation. Embryos were scored for cleavage under the microscope (Nomarski phase contrast).

Starfish oocyte maturation. Starfish (*Marthasterias glacialis*) oocytes were prepared, maturation induced and p34^{cdc2} analysed as previously described [43]. The level of tyrosine phosphorylation of p34^{cdc2} was analysed by western blotting with anti-PSTAIR antibodies after SDS/PAGE of the p9^{CKShst1}-affinity-bound proteins, as described above.

Activity of M-phase promoting factor (MPF) and DNA synthesis in *Xenopus* egg extracts. *Xenopus* egg extracts and demembrated *Xenopus* sperm nuclei were prepared as described previously [44, 45] and stored in liquid nitrogen until required. For MPF inhibition, extracts of eggs undergoing mitosis were supplemented with 25 mM phosphocreatine, 5 μ g/ml creatine phosphokinase and demembrated sperm nuclei (final concentration: 2 ng DNA/ μ l extract). After 1.5 h at 21°C, roscovitine was added at various concentrations. MPF inhibition was then monitored by assessing the percentage of sperm nuclei that had been assembled into interphase nuclei, possessing a complete phase-dense nuclear envelope. For DNA synthesis assays, extracts from metaphase-arrested eggs were supplemented with 25 mM phosphocreatine, 5 μ g/ml creatine phosphokinase, 250 μ g/ml cycloheximide, [α -³²P]-dATP at 50 μ Ci/ml. Demembrated sperm nuclei were added to a final sperm concentration of 4 ng DNA/ μ l extract. Roscovitine was added after 20 min DNA synthesis was assessed by releasing extract into interphase by the addition of 0.3 mM CaCl₂ and measuring the total amount of [α -³²P]dATP incorporation after 3 h by trichloroacetic acid coprecipitation [44].

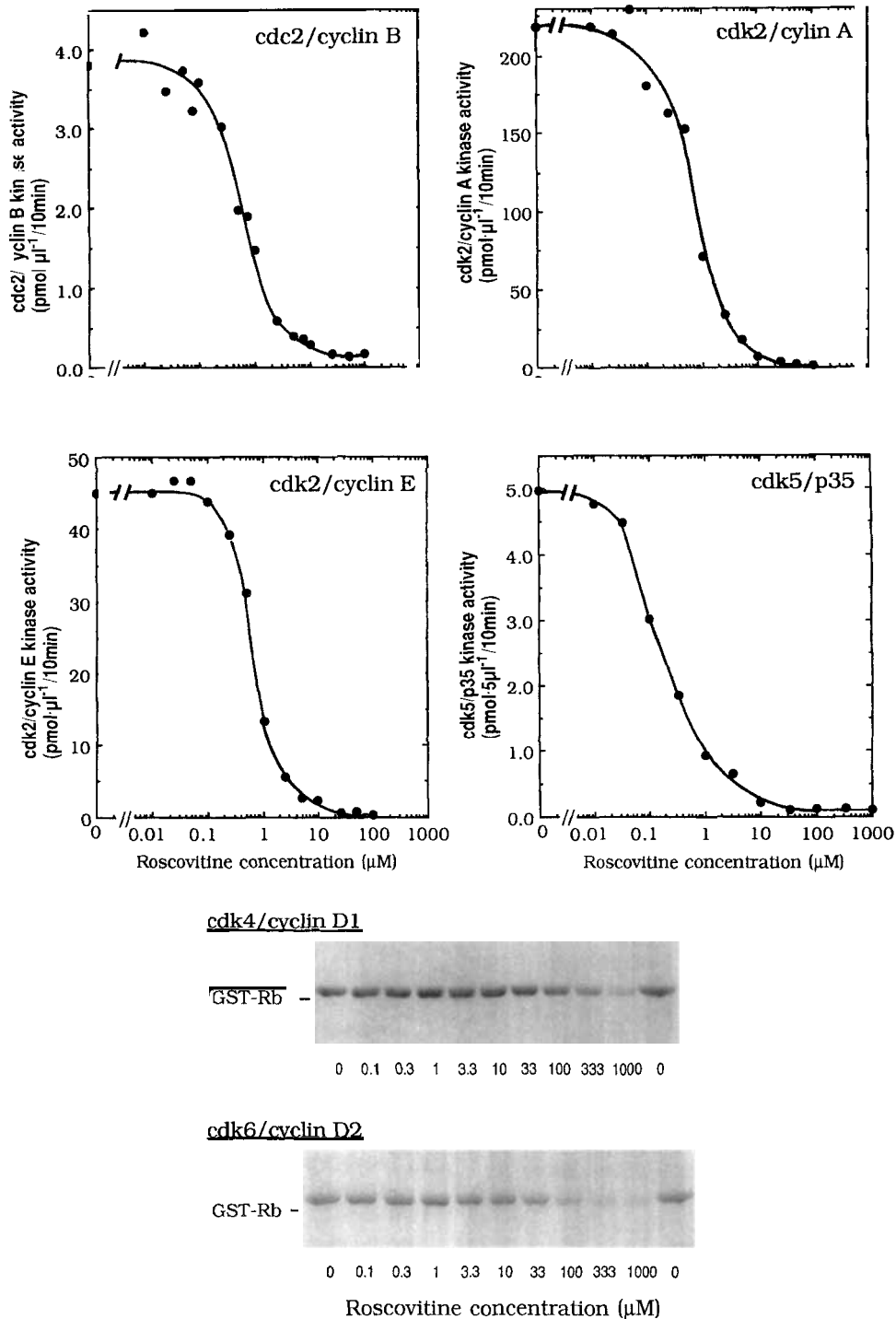


Fig. 1. Roscovitine dose/response curves for p34^{cdc2}/cyclin B, p33^{cdk2}/cyclin A, p33^{cdk2}/cyclin E, p33^{cdk5}/p35, p33^{cdk4}/cyclin D1 and p40^{cdk6}/cyclin D2 protein kinases. Enzyme activities were assayed as described under Experimental Procedures in the presence of increasing concentrations of roscovitine. Gels are also shown for p33^{cdk4}/cyclin D1 and p40^{cdk6}/cyclin D2 protein kinases; GST-Rb, retinoblastoma protein conjugated to glutathione *S*-transferase.

Xenopus laevis oocytes. *X. laevis* oocytes were prepared as described previously [46]. Various concentrations of roscovitine were added 4 h before addition of 1 μM progesterone. The percentage of germinal vesicle breakdown was recorded 8 h later. In another set of experiments, oocytes were treated with 0, 50 or 100 μM roscovitine prior to 1 μM progesterone. *In vivo* phosphorylation of elongation factor 1 subunits β , γ and δ was assessed after immunoprecipitation of the whole complex [46] in ³²P-labelled oocytes.

National Cancer Institute (NCI) disease-oriented *in vitro* screen. 60 human tumour cell lines comprising nine tumour types [47] were cultured for 24 h prior to a 48-h continuous exposure to 0.01–100 μM roscovitine. A sulforhodamine B protein assay was used to estimate the cytotoxicity.

L1210 cell culture. L1210 cells taken from exponentially growing cultures in RPMI-1640 medium supplemented with 10% foetal calf serum, penicillin and streptomycin, were counted using a hemocytometer, seeded at 5×10^4 cells/ml in

Table 1. IC₅₀ values for roscovitine and olomoucine added to various purified enzymes. Enzyme activities were assayed as described in Experimental Procedures, in the presence of increasing concentrations of roscovitine and olomoucine. IC₅₀ values were calculated from the dose/response curves. When no inhibitory effect was observed (–), the highest concentration tested is given in parentheses. Some data for olomoucine was originally presented by Vesely et al. [27]. GST-, conjugated to glutathione S-transferase.

Enzyme	IC ₅₀ for	
	roscovitine	olomoucine
	μM	
cdc2/cyclin B	0.65	7
cdk2/cyclin A	0.7	7
cdk2/cyclin E	0.7	7
cdk4/cyclin D1	> 100	>1000
cdk5/p35	0.16	3
cdk6/cyclin D3	> 100	> 250
GST-erk1	30	30
erk1	34	50
erk2	14	40
Protein kinase C α	> 100	>1000
Protein kinase C β1	> 100	>1000
Protein kinase C β2	> 100	>1000
Protein kinase C γ	100	800
Protein kinase C δ	>1000	>1000
Protein kinase C ε	> 100	>1000
Protein kinase C η	> 100	930
Protein kinase C ζ	>1000	>1000
cAMP-dependent protein kinase	>1000	>2000
cGMP-dependent protein kinase	–(1000)	>2000
Myosin light-chain kinase	90	>1000
Casein kinase 2	–(1000)	>2000
ASK-γ (plant GSK-3)	220	130
Insulin-receptor tyrosine kinase	70	400
c-src	250	–(1000)
v-abl	>1000	–(100)

tissue-culture 96-wells plates in the presence or absence of various concentrations of roscovitine or olomoucine and incubated at 37°C under 5% CO₂. For reversion of the roscovitine effect, L1210 cells cultured two days in the presence or absence of roscovitine were washed in phosphate-buffered saline to remove any trace of the drug, counted and reseeded in fresh medium containing no drug. Cell growth was monitored daily using the microculture tetrazolium assay [48]. Cell cycle analysis was performed on cells that were fixed in ethanol, treated with 100 μg/ml RNase and stained with propidium iodide [49]. We used a Coulter EPICS Elite flow cytometer for acquisition and the Multicycle software (Phoenix Flow systems) for analysis of the data. All assays were performed in triplicate and experiments repeated at least twice.

In vivo phosphorylation of vimentin. To investigate *in vivo* phosphorylation of vimentin by cdc2 kinase, cells were treated or not with 60 μM roscovitine for 48 h prior to exposure to 10 ng colcemid/ml for another 2 h. Cell extracts were then run on 10% SDS/PAGE and western blots performed using the 4A4 antibodies. These antibodies cross-react with cdc2-phosphorylated vimentin, but not with vimentin phosphorylated by other kinases nor with unphosphorylated vimentin [50].

RESULTS

In the preceding paper [34], we have described the identification of roscovitine and its molecular interaction with cdk2.

Here we describe an investigation into its enzymatic selectivity and cellular effects.

Kinase specificity of the roscovitine inhibitory effect. Roscovitine was tested against various cdk proteins (Fig. 1; Table 1). IC₅₀ values for cdc2/cyclin B, cdk2/cyclin A, cdk2/cyclin E and cdk5/p35 are respectively 0.65, 0.7, 0.7 and 0.16 μM (Fig. 1). When compared with olomoucine, roscovitine thus showed a 10-fold higher efficiency towards its cdc2 and cdk2 targets, a 20-fold higher efficacy towards cdk5. As observed for olomoucine [27], roscovitine had a very limited effect on the cdk4/cyclin D1 and cdk6/cyclin D2 kinases (IC₅₀ values >100 μM) (Fig. 1). This lack of effect was confirmed with cdk4 from several independent sources (data not shown). Under identical assay conditions, p16^{INK4A} inhibited cdk4/cyclin D1 and cdk6/cyclin D2 (data not shown).

Roscovitine was tested on a variety of highly purified kinases (Table 1). Kinase activities were assayed with appropriate substrates (histone H1, myelin basic protein, casein, etc.), with 15 μM ATP and in the presence of increasing concentrations of roscovitine. IC₅₀ values were calculated from the dose/response curves (Table 1). Most kinases were poorly or not inhibited. Although roscovitine displayed a 10-fold higher efficacy towards its cdk targets when compared with olomoucine, both compounds displayed a similar inhibitory action on erk1 and erk2 (Table 1, Fig. 2). A 40-fold higher roscovitine concentration was needed to inhibit erk1 similarly to cdc2 (20-fold for erk2). The selectivity of roscovitine was therefore about 10-fold higher than that of olomoucine.

Cell cycle effects of roscovitine: oocytes and embryos. The cellular effects of olomoucine have been described recently [28]. We selected a few cellular models to investigate the cellular effects of roscovitine (Figs 3–8).

Prophase-arrested starfish oocytes were treated with various concentrations of roscovitine prior to exposure to 1MeAde, the hormone which triggers the prophase/metaphase transition [51]. Roscovitine inhibited nuclear envelope breakdown with an IC₅₀ of 5 μM (Fig. 3A). IC₅₀ for olomoucine is 30 μM [27]. As previously observed with olomoucine, roscovitine slowed down, but did not inhibit, the *in vivo* tyrosine dephosphorylation of p34^{cdc2} as monitored by its change of electrophoretic mobility with antibodies against PSTAIRE (Fig. 3B). In other words, it did not inhibit the activation of cdc2, but its activity. p34^{cdc2} tyrosine dephosphorylation is catalysed by the cdc25 phosphatase and normally precedes the cdc2 kinase activation at the G2/M transition [43]. The cdc2 kinase phosphorylates and hyperactivates the cdc25 phosphatase [52–54]. In addition, the cdc2 kinase phosphorylates and inactivates the wee1 kinase [55–59]. These positive feedback loops may thus have been interrupted by roscovitine at the cdc2 kinase level, resulting in a slowed down dephosphorylation.

Roscovitine was next tested on mitotic sea urchin embryos. When added 60 min after fertilisation, roscovitine was found to arrest cells in late prophase in a dose-dependent manner (Fig. 4A; IC₅₀ of 10 μM). A large nucleus could be observed in the roscovitine arrested eggs (Fig. 4B). This arrest was completely reversible: following washing with sea water, the eggs re-entered mitotic cycles and developed into normal plutei (data not shown). This was observed even with highest concentration of roscovitine, 100 μM. At 100 μM, olomoucine only slows down the prophase/metaphase transition, without arresting cells in prophase [28].

The effects of roscovitine were next assessed using *Xenopus* egg extracts [44]. Mitotic egg extracts were incubated with roscovitine and sperm chromatin. In roscovitine concentrations

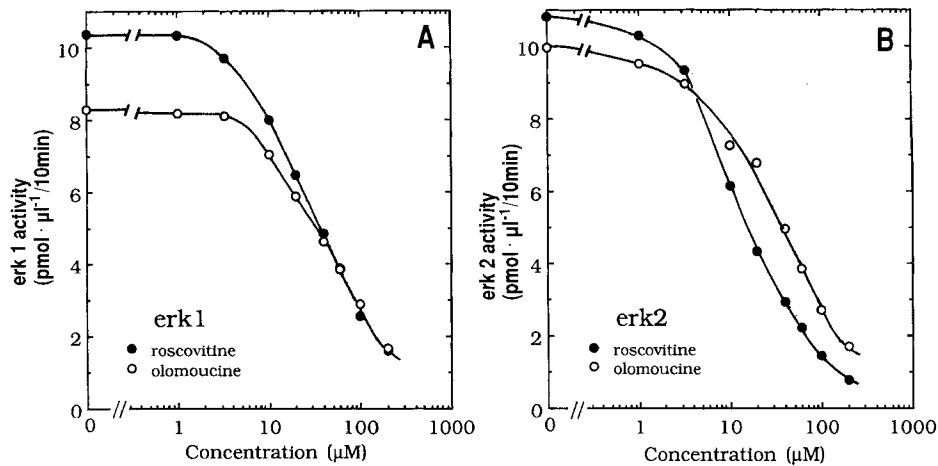


Fig. 2. Effects of roscovitine and olomoucine on erk1 and erk2. Enzyme activities were assayed as described under Experimental Procedures in the presence of increasing concentrations of roscovitine.

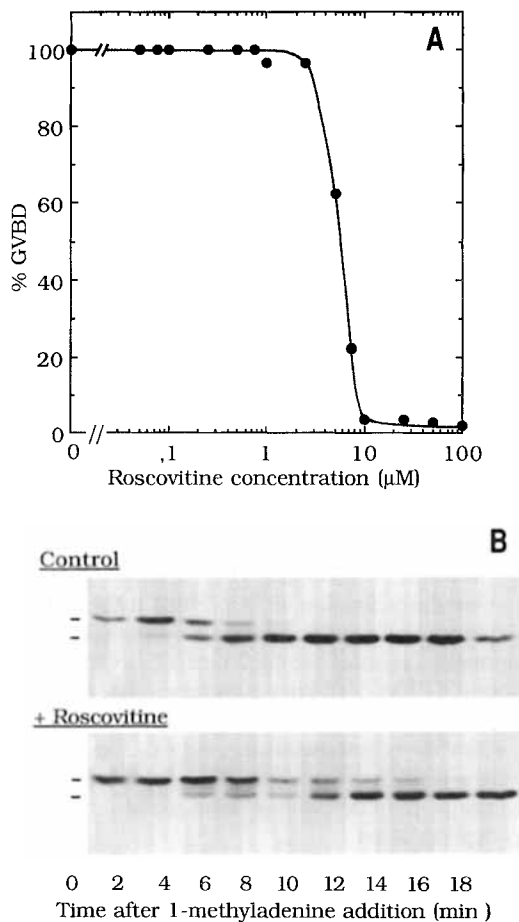


Fig. 3. Effects of roscovitine on starfish oocyte maturation and on *in vivo* tyrosine dephosphorylation of p34^{cdc2}. G2-arrested oocytes were treated for 15 min with increasing concentrations of roscovitine prior to addition of 1 μM 1-MeAde; after 30 min the percentage of germinal vesicle breakdown (GVBD) was scored (A). (B) Starfish oocytes were treated or not (control) with 10 μM roscovitine for 15 min prior to addition of 1 μM 1-MeAde at time zero. Extracts prepared at various times were loaded on p9^{CKShs1}-Sepharose beads. The bound proteins were resolved by SDS/PAGE prior to immunoblotting with antibodies against PSTAIRE. The immunoblots at the level of p34^{cdc2} are presented. The upper, phosphorylated, and lower, dephosphorylated, forms of p34^{cdc2} are detected.

ranging over 0–5 μM chromosomes appeared highly condensed and nuclear envelopes broke down. At 10 μM roscovitine and above, interphase nuclei remained with partially decondensed chromatin and an intact nuclear envelope, showing that MPF activity was inhibited (Fig. 5; IC₅₀ of 5 μM). Inhibition of DNA synthesis was assessed as described for olomoucine [27]: roscovitine and sperm chromatin were added to a metaphase-arrested egg extract. The extract was then released into interphase by addition of CaCl₂ [44, 45] and, 3 h later, total DNA synthesis was measured by [α -³²P]dATP incorporation in trichloroacetic-acid-precipitable material. Replication was inhibited by roscovitine with an IC₅₀ of 15 μM (Fig. 5).

Prophase-arrested *Xenopus* oocytes were simultaneously treated with various concentrations of roscovitine prior to progesterone addition. A dose-dependent inhibition of oocyte maturation was observed (Fig. 6A; IC₅₀: 25 μM). The effect of roscovitine on the *in vivo* activity of the cdc2 kinase was estimated on the phosphorylation of the elongation factor subunits. This cdc2 substrate is phosphorylated on its γ subunit (Thr230) and on its δ subunit (Thr122 and an unidentified Ser) [46]. The whole elongation factor 1 complex was immunoprecipitated from ³²P-labelled oocytes (prophase-arrested, progesterone-treated, progesterone- and roscovitine-treated), resolved by SDS/PAGE and autoradiography. Roscovitine inhibited the phosphorylation of eEF-1 γ and δ (Fig. 6B).

Cell cycle effects of roscovitine: mammalian cell lines. Roscovitine (0.01–100 μM; 48-h exposure) was tested on the NCI disease-oriented *in vitro* screen, i.e. 60 human tumour cell lines comprising nine tumour types (leukemia, non-small cell lung cancer, colon cancer, central nervous system cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, breast cancer). All cell lines displayed a similar sensitivity to roscovitine. The average IC₅₀ was 16 μM (60.3 μM for olomoucine [28]). No correlation was observed between the sensitivity of cell lines to roscovitine and the presence of wild-type or mutated p53. The COMPARE analysis method showed that the effects of roscovitine and flavopiridol were comparable.

We next investigated the effects of roscovitine on the growth of the L1210 cell line (Figs 7 and 8). Cells were grown in the presence of various roscovitine concentrations and their growth monitored after two and three days. A clear-cut dose-dependent inhibition of growth was observed (Fig. 7A). The curves were essentially the same after two and three days of culture (data not shown) as observed in the NCI *in vitro* screen. Roscovitine was

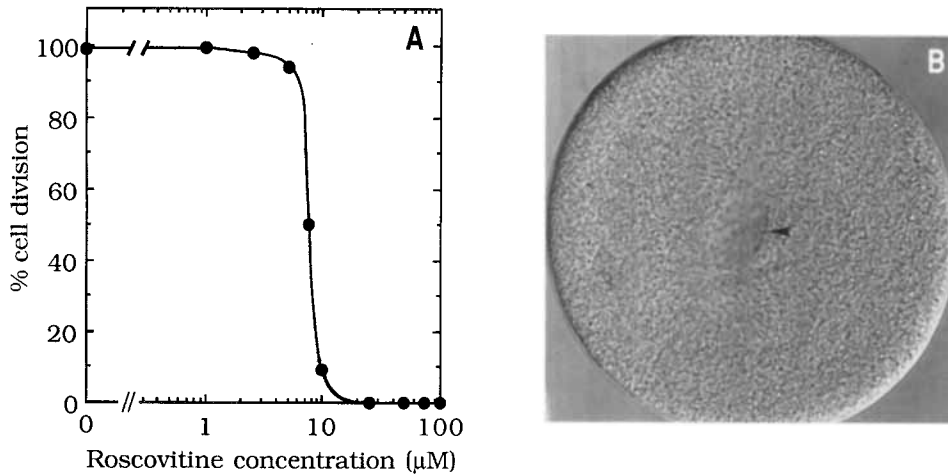


Fig. 4. Effects of roscovitine on sea urchin embryos mitotic cycle. Eggs were fertilised at time zero and various concentrations of roscovitine were added 60 min later. The percentage of divided embryos was monitored 120 min after fertilisation (A). Embryos were arrested in late prophase with an exceptionally large nucleus (B).

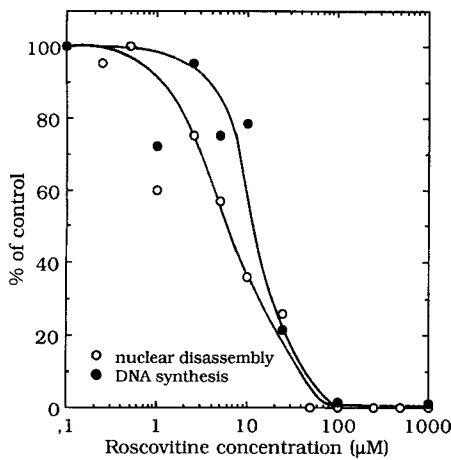


Fig. 5. Effects of roscovitine on *in vitro* DNA synthesis and MPF activity. Two different titrations were performed on *Xenopus* extracts (see text): (○) inhibition of MPF (percentage of assembled nuclei) and (●) inhibition of DNA synthesis.

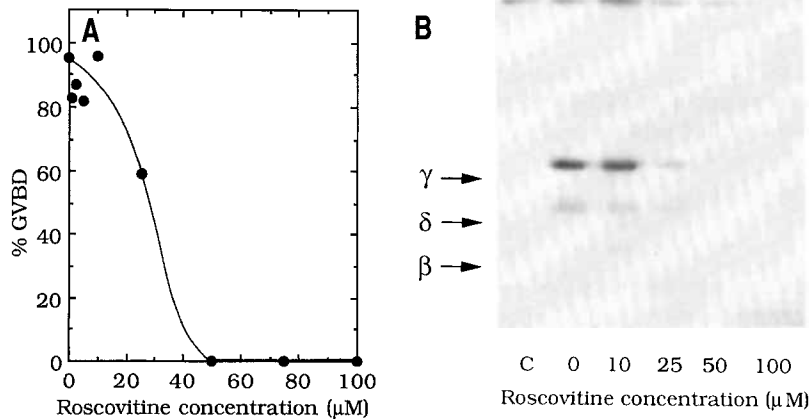


Fig. 6. Roscovitine inhibits *Xenopus* oocyte maturation and the *in vivo* phosphorylation of eEF-1 γ and δ . (A) Oocytes were exposed to progesterone and treated with increasing concentrations of roscovitine. The percentage of germinal vesicle breakdown (GVBD) was scored. (B) *In vivo* phosphorylation of eEF-1 γ and δ , immunoprecipitated from ^{32}P -labeled oocytes (control, progesterone-treated, progesterone + roscovitine-treated).

approximately fourfold more efficient at inhibiting cell growth when compared to olomoucine (IC_{50} : 40 μM and 160 μM , respectively). Although most cells were viable ($96 \pm 2\%$ Trypan blue exclusion) following a 48-h treatment with 60 μM roscovitine, they remained irreversibly arrested, even after extensive washing (data not shown). Cells exposed to 120 μM roscovitine died rapidly. The effects of roscovitine on cell cycle distribution was next investigated by flow cytometry. At 60 μM roscovitine, cells remained arrested in G1 and accumulated in G2 (Fig. 7B).

In an effort to identify the *in vivo* molecular target of roscovitine, we made use of the 4A4 monoclonal antibodies described by Tsujimura et al. [50]. These antibodies very specifically recognize [Ser(P)⁵⁵]vimentin, but not unphosphorylated vimentin, nor vimentin phosphorylated by various kinases (cAMP-dependent protein kinase, protein kinase C, Ca^{2+} -calmodulin-dependent protein kinase). The Ser55 residue of vimentin is specifically phosphorylated by the *cdc2* kinase as cells enter mitosis [50]. Cells were treated or not with 60 μM roscovitine for 48 h and then exposed to 10 ng colcemid/ml for another 2 h. Total proteins were extracted and resolved by SDS/PAGE prior to western blotting with 4A4 antibodies (Fig. 8). The non-treated cells arrested in metaphase and accumulated *cdc2*-phosphory-

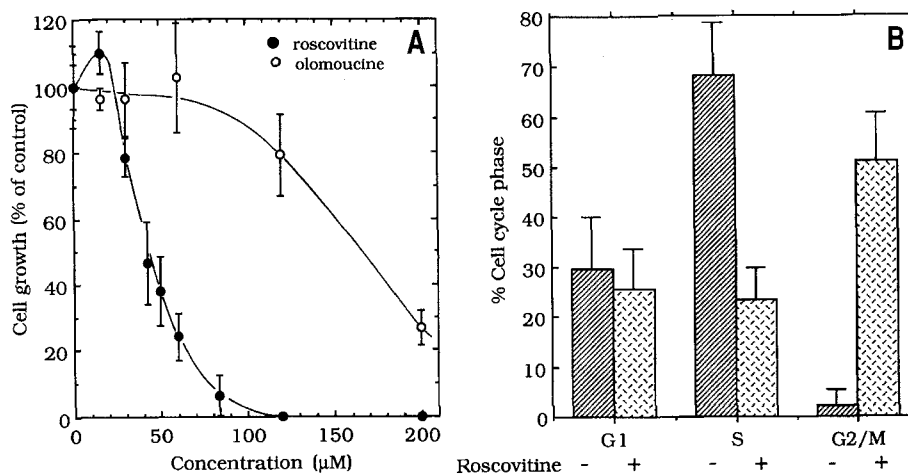


Fig. 7. Roscovitine inhibits the growth of L1210 cells and arrests cells in G2/M. (A) L1210 cells were exposed to various concentrations of roscovitine (●) or olomoucine (○). Their growth was monitored after two days and is presented as a percentage of the growth of control untreated cells (mean \pm SD). (B) Cells were first grown for 48 h in the absence or presence of 60 μ M roscovitine. The cell cycle distribution was then analysed as described in Experimental Procedures; data is presented as mean \pm SD.

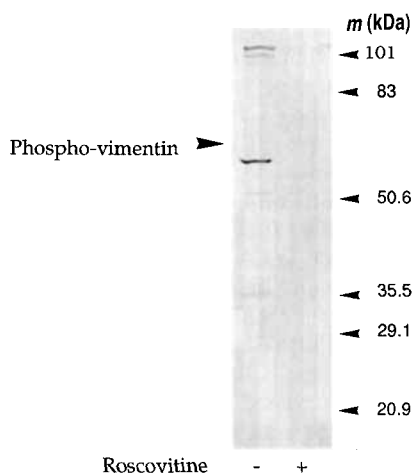


Fig. 8. Roscovitine inhibits *in vivo* phosphorylation of vimentin on cdc2-specific sites. L1210 cells were grown for 48 h in the absence or presence of 60 μ M roscovitine. They were then exposed to 10 ng colcemid/ml for another 2 h. Cells were then pelleted, frozen in liquid nitrogen and their proteins resolved by SDS/PAGE and analysed by western blotting using 4A4 monoclonal antibodies. These antibodies specifically recognize [Ser(P)⁵⁵]vimentin, i.e. vimentin phosphorylated only by cdc2/cyclin B.

lated vimentin. In contrast, roscovitine-treated cells did not display any cdc2-phosphorylated vimentin, showing that cdc2 had in fact been inhibited *in vivo*, and that cells were arrested prior to metaphase.

DISCUSSION

In this paper we describe the enzyme inhibitory properties and cellular effects of roscovitine, a novel, highly efficient and selective inhibitor of some cyclin-dependent kinases. Roscovitine belongs to a family of C2, N⁶, N9-substituted adenines which interact with the ATP-binding site of cdk [27, 31, 34].

Kinase specificity of inhibition by roscovitine. Among 25 kinases tested, only some members of the cdk family are inhibited by sub-micromolar concentrations of roscovitine. cdk5 appears to be the most sensitive cdk to roscovitine and olomoucine

(Table 1, Fig. 1). On the basis of their sensitivity to C2, N⁶, N9-substituted adenines, cdk proteins can be divided in two subfamilies, cdc2/cdk2/cdk5 (sensitive to roscovitine and olomoucine) and cdk4/cdk6 (poorly sensitive to roscovitine and olomoucine). This division is also apparent with two other inhibitors, staurosporine and butyrolactone I [29, 30]. The relative insensitivity of cdk4 and cdk6 may be related to the relative evolutionary distance of cdk4/cdk6 from cdc2/cdk2/cdk5 [60]: the cdc2 sequence displays 65%, 57%, 44% and 47% identity with cdk2, cdk5, cdk4 and cdk6, respectively, while cdk4 and cdk6 share 71% identity. Understanding the reasons behind the differential sensitivity of cdc2/cdk2/cdk5 and cdk4/cdk6 to inhibitors will certainly be helpful to design cdk4/cdk6-specific inhibitors. Surprisingly, the effects of roscovitine on erk1 and erk2 are quite similar to those of olomoucine (Table 1, Fig. 2). As roscovitine is 10-fold more active on cdk than olomoucine, this inhibitor presents increased selectivity and should allow the distinction between cdk- and erk-dependent events. Furthermore this information may help in designing erk-selective inhibitors. Two enzymes, myosin light-chain kinase and insulin-receptor tyrosine kinase were inhibited by roscovitine concentrations below 100 μ M, in contrast to olomoucine.

Cellular effects of roscovitine: G2/M and G1/S arrest. As illustrated with starfish and *Xenopus* oocytes and sea urchin embryos, roscovitine inhibits the prophase/metaphase transition in the micromolar range. This effect was also observed with higher concentrations of olomoucine [27, 28]. Accumulation of cells in late G2 was also observed in tobacco BY-2 cells (Planchais et al., unpublished) and with L1210 cells. The most straightforward interpretation for these observations is that the *in vivo* target for roscovitine is cdc2/cyclin B. This is further supported by a) the *in vitro* effects of roscovitine on MPF activity in *Xenopus* cell-free extracts, b) the *in vivo* reduced phosphorylation of elongation factor 1 in *Xenopus* oocytes treated with roscovitine, c) the *in vivo* phosphorylation (cdc2-specific sites) of vimentin in L1210 cells suppressed by roscovitine. The possibility that roscovitine effects can be accounted for by inhibition of erk protein is unlikely since roscovitine appears to be more efficient than olomoucine in its cellular effects but not in its effects on erk. Accumulation of cells in prophase following release from an aphidicolin block and further treatment with olomoucine/roscovitine has been successfully used to synchronize cells in this late part of the cell cycle [61].

Roscovotine is also able to arrest cells in late G1 as shown *in vitro* with *Xenopus* cell-free extracts, in L1210 cells and in tobacco BY-2 cells (Planchais et al., unpublished). We believe that it does so by inhibiting cdk2/cyclin E and cdk2/cyclin A. Cyclin A expression is a marker of for late G1 phase in cell proliferation. Cyclin A mRNA induction by Myc in fibroblasts was recently shown to be inhibited by roscovotine, p21 and p27 [62].

Roscovotine acts on many cells at higher concentrations than it does on purified enzymes. This could be due to poor permeability, unfavourable compartmentalisation, active metabolism into inactive compounds, presence of unidentified binding proteins, high concentrations of cellular ATP, or a combination of these factors. In addition, as cdc2/cyclin B activates its own ubiquitin-dependent proteolysis [63, 64], partial inhibition of cdc2 may in fact stabilize the residual kinase activity, thereby reducing the cellular effects of a cdk inhibitor.

Cdk inhibitors and apoptosis. Efficient anti-tumour drugs are thought to act by triggering apoptosis in the target tissue [65–68]. The relationship between cell cycle and apoptosis is rather complex but is starting to be unraveled (reviewed in [68–71]). Multiple pathways lead to apoptosis, some of which require active cdc2-cdk2/cyclin A [72] or cdc2/cyclin B [73, 74], while others do not apparently require any cdk [74–77]. Depending on the apoptosis-inducing agonist, T-cell hybridoma A11 cells enter programmed cell death by a cdk-dependent (anti-CD3) or cdk-independent (dexamethasone) pathway [74]. Mitotic PC12 cells undergo cell death when exposed to olomoucine or flavopiridol, while differentiated PC12 cells are protected from cell death by the same inhibitors [33]. Myc-induced apoptosis is insensitive to roscovotine, p21 and p27 [62].

Apoptosis can be induced either in G1 or in G2. Following DNA damage by various chemicals, ultraviolet irradiation or X-rays, some cells arrest in G1 and a p53-dependent apoptotic pathway is induced [78–80]. In other situations, cells arrest in G2/M in response to DNA-damaging agents, X-rays or taxol and a p53-independent apoptotic pathway is activated [78, 80–83]. This pathway may be extremely important in the therapy of tumours showing loss of active p53. Olomoucine greatly stimulates p53-independent apoptosis in cells which have been arrested in G2 by DNA-damaging agents such as mitoxantrone or *cis*-platinum [32]. The apoptosis-inducing effects of roscovotine and olomoucine are clearly p53-independent. Cdc2 inhibitors may thus enhance the therapeutic effects of currently used anti-tumour agents, particularly in p53-mutated cells. In this context, the cdk inhibitor flavopiridol is entering phase I studies as an anti-tumour agent.

Cdk inhibitors and the nervous system. Roscovotine and olomoucine may contribute to reduce the hyperphosphorylation of *tau* observed during Alzheimer's disease. The brain of patients afflicted by this disease contains intracellular deposits of abnormally phosphorylated *tau* (reviewed [84]). The relationship between abnormal *tau* phosphorylation and the development of Alzheimer's disease is still unclear. However many of the *tau* phosphorylation sites have been identified [85]. Some of these sites are phosphorylated by cdk5/p35 (reviewed [86]), a brain-specific cdk which is particularly sensitive to roscovotine.

It was recently found that olomoucine and flavopiridol prevent neuronal cell death induced by neurotrophic factor withdrawal (nerve growth factor-differentiated PC12 cells and sympathetic neurons). In contrast, these drugs promote apoptosis of mitotic PC12 cells [33]. We therefore anticipate that cdk inhibitors may be useful in the therapy of some nervous system diseases.

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