The Effect of the Cyclin-Dependent Kinase Inhibitor Olomoucine on Cell Cycle Kinetics

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The effect of the cyclin-dependent (CDK) inhibitors olomoucine and roscovitine on cell kinetics was studied. To this end, nonsmall cell lung cancer (NSCLC) cell line MR65 and neuroblastoma cell line CHP-212 were pulse labeled with bromodeoxyuridine (BrdUrd) and chased in culture medium, to which various concentrations of olomoucine or roscovitine were added. A dose-dependent inhibition of the G1/S-phase and G2/M-G1 transitions was observed. Furthermore, S-phase progression was also inhibited in a dose-dependent manner. Similarly, roscovitine, another CDK inhibitor with a 10-fold higher efficiency for both CDK1 and CDK2 as compared to olomoucine, showed the same effects at a 10-fold lower concentration. At the highest tested doses both olomoucine (200 \( \mu \text{M} \)) and roscovitine (40 \( \mu \text{M} \)) induced a complete cell cycle block in both cell lines, paralleled by the appearance of apoptotic figures. In these cultures a decrease in CDK1 protein level was found as shown by Western blotting. Bivariate CDK1/DNA analysis confirmed these observations and showed that a subpopulation of cells with characteristics of apoptosis became CDK1 negative. The presented data suggest that cyclins and CDKs are involved at an important nodal point shared by pathways regulating cellular proliferation and apoptosis.

INTRODUCTION

The carefully ordered progression through the cell cycle is tightly controlled by the sequential formation, activation, and inactivation of a series of cell cycle regulating kinases [1, 2]. Key players in the regulation of the cell cycle are the cyclin-dependent kinases (CDKs), a family of serine/threonine kinases with several highly homologous members [3–6]. These CDKs are constantly expressed during the cell cycle, but their activity is tightly regulated by complex formation with cyclins, a class of proteins which also consists of several highly homologous members [5–7]. In contrast to CDKs, cyclins are periodically expressed during the different phases of the cell cycle [7, 8]. For instance, the mitotic cyclin B is expressed very late in the S-phase and during G2 and part of the M-phase, while cyclin E is expressed during late G1 and early S-phase of the cell cycle [9–13].

The complex formation per se does not result in activation of the kinase. The activity of each complex is regulated by phosphorylation and dephosphorylation of specific amino acid residues of the CDK/cyclin complex. For instance the cyclin B/CDK1 complex is turned on by phosphorylation of thr161 by the cyclin activating kinase (CAK, CDK7/cyclin H) [14, 15] and turned off by phosphorylation of thr14 (myt1 kinase) and tyr15 (wee1 and mik1 kinase), two residues located in the ATP-binding domain of the CDK1 kinase [16–19]. Activation occurs by dephosphorylation of the thr14 and tyr15 residues by the CDC25 phosphatase [20]. Both wee1 kinase and CDC25 phosphatase activities are under control of internal triggers and depend on completion of DNA synthesis. In this way the proper timing of activation of the CDK/cyclin complexes is ensured. The rapid activation of the CDK/cyclin complex is brought about through a self-amplification loop, resulting in phosphorylation and activation of the CDC25-C by CDK-cyclin B [20] and phosphorylation and inactivation of wee1. At the same time the active CDK/cyclin complex is involved in activating components of the cyclin-ubiquitin ligase pathway [21], resulting in its inactivation. This inactivation of the CDK/cyclin complexes is brought about by rapid proteolytic breakdown of the cyclins [22], mediated through the ubiquitin degradation pathway [21, 23, 24].

Several critical checkpoints exist in the cell cycle, all governed by different combinations of CDKs and cyclins [7]. In this way both the target specificity and the timing of activation of these complexes is regulated.
[25]. For instance, evidence exists that the CDK1/cyclin B complex is responsible for phosphorylation of the lamins during mitosis [26], which results in the dissociation of the nuclear lamina. On the other hand, the CDK4/cyclin D complex seems to govern the G1 transition by phosphorylating the RB gene product [27, 28]. The CDK2/cyclin E kinase plays an essential role in G1/S transition [10, 29]. Physical association of cyclin A with transcription factor E2F has been found, which implicates a role of CDK2/cyclin A complex in regulation of transcription [30, 31]. Furthermore, biochemical evidence exists for a role of the CDK2/cyclin A complex during DNA replication, since this complex is able to phosphorylate a replication factor in vitro [32]. Furthermore, immunocytochemical analysis revealed that cyclin A colocalizes with replication sites [33, 34].

Many putative target proteins have been identified for cyclin-dependent kinase proteins in cell-free systems [1, 2, 35]. However, little information exists on the in vivo targets of the CDK/cyclin complexes. Recently, intrinsic CDK inhibitors have been identified, such as p21 CIP1/WAF1 and others [36, 37]. The expression of, for instance, p21 is under the control of the p53 protein and functions to arrest the cells at the G1-phase of the cell cycle in response to DNA damage [38]. By influencing the transcription of these intrinsic inhibitors it would be possible to elucidate the role of these CDK/cyclin complexes in vivo and identify the target proteins that are affected by these complexes. An alternative approach would be to use extrinsic inhibitors of these kinases but so far the availability of specific inhibitors of these complexes has been limited.

Recently, it was shown that a compound isolated from Aspergillus terreus, name butyrolactone I, was able to inhibit several CDK/cyclin complexes in vitro and in vivo [39]. The authors showed that butyrolactone I was able to inhibit the phosphorylation of Rb both in vitro and in vivo. Recently, Vesely et al. [40] described a new purine derivative, olomoucine (2-(hydroxyethylamino)-6-benzylamino-9-methylpurine), which acts as a potent inhibitor of the CDK1/cyclin B and related kinases by competing for the ATP binding domain of the kinase [40, 41]. Of 35 protein kinases tested, olomoucine inhibited CDK2/cyclin A, CDK2/cyclin E, CDK1/cyclin B, CDK5/p35, and erk1. The related kinases CDK4/cyclin D and CDK6/cyclin D3 were not inhibited or were less sensitive to olomoucine. This new compound showed no effect on other kinases tested, such as protein kinases A or C, cyclic AMP- or cyclic GMP-dependent kinases, and many others, nor on several enzymes that are directly involved in DNA-synthesis, such as DNA topoisomerases and DNA polymerases at doses less than 1 mM.

In this study we investigated in detail the effect of olomoucine and roscovitine (an olomoucine analogue with 10-fold higher efficiency toward CDK1 and CDK2 and a similar efficiency toward erk1 and the other kinases tested) on the cell kinetics of two cell lines. To this end cells were pulse labeled with the thymidine analogue bromodeoxyuridine (BrdUrd) and chased in normal culture medium supplemented with various doses of inhibitor. Evidence was found for a dose-dependent inhibition of cell cycle phase transition rates in both cell lines. At the highest doses tested, both olomoucine and roscovitine induced an almost complete arrest of cells in their specific cell cycle phase, which ultimately resulted in apoptosis. No evidence was found for a cell cycle phase-specific induction of apoptosis by olomoucine or roscovitine.

**MATERIALS AND METHODS**

Cell lines. The human nonsmall cell lung cancer (NSCLC) cell line MR65, kindly supplied by Dr. Gropp (Philips Universitats Klinik, Marburg, Germany), was cultured in Eagle’s modified minimal essential medium (GIBCO, Paisley, Scotland), supplemented with 1% nonessential amino acid solution (GIBCO), 1% glutamine (Serva, Heidelberg, Germany; No. 22942), 1% HEPES (GIBCO), 10% heat-inactivated newborn calf serum (GIBCO, 021-6010M), and 0.1% gentamycin (AUV, Cuyck, The Netherlands). The human neuroblastoma cell line CHP-212 [42] was cultured in Dulbecco’s modified medium (GIBCO), supplemented with 1% glutamine (Serva), 10% heat-inactivated newborn calf serum, and 0.1% gentamycin (AUV).

Cells were harvested during exponential growth by trypsinization.

BrdUrd pulse chase labeling. The cell lines were pulse labeled with 10 μM BrdUrd (Serva, Heidelberg, Germany) for 30 min, rinsed twice in prewarmed PBS, and chased in prewarmed culture medium supplemented with 5 μM deoxythymidine.

CDK inhibitor treatment. Olomoucine and roscovitine were dissolved in DMSO and added to the cell cultures after BrdUrd pulse labeling, at various concentrations i.e., 10, 50, 100, and 200 μM for olomoucine and 1, 5, 10, 20, and 40 μM for roscovitine. The final DMSO concentration in the culture medium was 2%. Control cultures received 2% DMSO. At various time intervals cells were harvested by collecting both the floating as well as the adherent cells. Part of the cell suspension was centrifuged onto glass slides, while the remainder of the suspension was fixed in 70% ethanol at 4°C. Immunocytochemistry. Incorporated BrdUrd was detected as described previously [43]. Briefly, appropriately 10^6 ethanoltreated fixed cells were rinsed once in PBS and resuspended in 2 ml of 0.4 mg/ml pepsin in 0.1 N HCl (Serva, Heidelberg, Germany). After 30 min at room temperature cells were pelleted, resuspended in 2 N HCl, and incubated for another 30 min at 37°C. Cells were rinsed in 0.1 M borate buffer, pH 8.5, and PBS/BSA (1 mg/ml BSA in PBS). Appropriately diluted anti-BrdUrd antibody (clone IIB5, available from Euro-Diagnostica B.V., Arnhem, The Netherlands) was added to the cell pellet, resuspended in 100 μl PBS/BSA. After incubation for 1 h at room temperature, the cells were rinsed twice in PBS/BSA. For visualization, FITC-conjugated Fab fragments of rabbit anti-mouse Ig (DAKO A/S, Glostrup, Denmark) antibody were added to a 1:10 dilution. After incubation for 45 min at room temperature samples were rinsed twice in PBS/BSA and the cells were finally resuspended in 0.5 ml cold PBS supplemented with 100 μg/ml RNase (Serva) and 20 μg/ml propidium iodide (PI; Calbiochem, La Jolla, CA). The samples were allowed to stand for 15 min on ice in the dark before flow cytometric analysis. In the negative control the primary antibody was omitted.

For bivariate CDK1/DNA analysis cells were fixed in methanol for 5 min at −20°C. After rinsing the cells in PBS/BSA the resuspended
pellet was incubated for 1 h at RT with anti-cdk2 (SC-54, Santa Cruz Biotechnologies Inc., 1:20), followed by rinsing twice in PBS/BSA. Monodonal antibody binding was visualized by incubating the cells with FITC-conjugated Fab2 fragments of rabbit anti-mouse Ig (DAKO A/S, Glostrup, Denmark) antibody in a 1:10 dilution. After incubation for 45 min at room temperature samples were rinsed twice in PBS/BSA and the cells were finally resuspended in 0.5 ml cold PBS supplemented with 100 μg/ml RNase (Serva) and 20 μg/ml propidium iodide (PI; Calbiochem, La Jolla, CA). The samples were allowed to stand for 15 min on ice in the dark before flow cytometric analysis.

Cells, which were cytocrifuged onto glass slides, were fixed for 5 min in cold methanol at −20°C, rinsed in PBS, stained with PI (1 μg/ml in PBS) for 10 min at room temperature, and embedded in glycerol/DABCO/PI (9 parts of glycerol, 1 part of 0.2 M HCl, pH 8.0, 0.02% NaN3, and 2% DABCO (1,4-di-azobicyclo-(2,2,2)-octane, Merck, Darmstadt, Germany; no. 803456), pH 8.0) containing 0.5 μg/ml PI. The slides were covered with a coverslip and sealed with nail polish.

Flow cytometry. For flow cytometric analysis a FACSort (Becton–Dickinson, Sunnyvale, CA) equipped with a single Argon ion laser was used. Excitation was done at 488 nm, and the emission filters used were 515–545 BP (green; FITC), 572–588 BP (orange; PE), and 600 LP (red; PI). A minimum of 10,000 cells per sample were analyzed and data stored in list mode. FITC signals were recorded as logarithmic amplified data, while the PI signals were recorded as linear amplified data. For bivariate FITC/PI analysis no compensation was used.

Data analysis was performed with the standard Lysis and Cellfit software (Becton–Dickinson). As a standard procedure for all analyses, data were gated on pulse-processed PI signals to exclude doublets and larger aggregates.

For detailed cell kinetic analysis, five cell cycle compartments were identified as described by Higashikubo et al. [44]. Briefly, the following populations were quantified (see Fig. 1): (1) BrdU-positive undivided cells (N lu); (2) BrdU-positive divided cells (N ld); (3) BrdU-negative G2 cells (G2−); (4) BrdU-negative G1 cells (G1−); and (5) BrdU-negative G1 S cells (G1S−). From populations 1 and 5 the mean of dichroic mirrors and barrier filters. All scans were recorded in photon counting mode.

Photography. Phase contrast images of cell cultures were taken using an Axiovert 35 M microscope (Zeiss, Oberkochen, Germany), equipped with a Nikon camera. For this purpose an Agfa pan 25X black and white film was used.

Gel electrophoresis and immunoblotting. Cell samples were resuspended in 50 mM Tris–HCl, pH 7.4, containing 17% glycerol and 2 mM ATP and sonicated for 5 s at 0°C. Samples were centrifuged at 4°C and the supernatant and pellet fraction were split. These were boiled for 5 min in SDS-sample buffer and loaded onto denaturing 12% polyacrylamide gels [46]. The equivalent of approximately 106 cells was used per lane. Gels were run on the Mini-Protean II system (Bio-Rad Laboratories) for approximately 45 min at 200 V.

Immunoblotting was performed according to the method of Towbin et al. [47], using PVDF membranes (Schleicher and Schuell, Dassel, Germany). The blots were incubated with anti-cdk2 (sc-54, Santa Cruz Biotechnologies Inc.), washed with PBS, and subsequently incubated with a peroxidase-conjugated rabbit anti-mouse Ig antibody (DAKO A/S) to detect primary antibody binding. Peroxidase reactivity was detected by enhanced chemiluminescence (ECL-kit, Amer sham, Buckinghamshire, UK).

RESULTS

In a first series of experiments in which the cell lines MR65 and CHP212 were exposed to various concentrations of the CDK inhibitor 10 μM and harvested and 568 nm. Emission spectra were separated by the standard sets of dichroic mirrors and barrier filters. All scans were recorded in photon counting mode.
THE EFFECT OF OLOMOUCINE ON CELL CYCLE KINETICS

FIG. 2. Cytograms of bivariate BrdUrd/DNA analyses of MR65 cells pulsed with BrdUrd and chased for 10 h in medium supplemented with 0 (A), 50 (B), 100 (C), and 200 μM olomoucine (D). DNA content is shown on the ordinate and BrdUrd immunofluorescence on the abscissa. A clear dose-dependent inhibition of cell cycle traverse of the BrdUrd-positive and BrdUrd-negative cells can be seen. At a dose of 200 μM olomoucine the cells are completely blocked in the cell cycle and the appearance of cells in the sub G₁ region is noticeable.

At different time intervals, no obvious changes in cell cycle phase distributions were observed (data not shown). The cultures seem not to be blocked in a specific phase of the cell cycle by olomoucine. In order to investigate the cell cycle effects of this CDK inhibitor in more detail, the cell lines were pulse labeled with BrdUrd and chased in the presence of various concentrations of the drug. Figure 2 shows a series of bivariate BrdUrd/DNA cytograms of MR65 cells, chased for 10 h in the presence of 0, 50, 100, and 200 μM olomoucine. In contrast to the control culture (Fig. 2A), in which the BrdUrd-labeled cells have progressed to G₂/M and G₁ phase of the cell cycle, cells exposed to olomoucine lagged behind in the cell cycle in a dose-dependent manner. The number of BrdUrd-positive cells still in S-phase were higher with increasing dose of the inhibitor. Delayed cell cycle progression in the presence of olomoucine was also obvious when analyzing the number of cells occupying the BrdUrd-negative S-phase compartment. In the control cultures (Fig. 2A) cells which were originally in the G₁-phase of the cell cycle at the time of pulse labeling, have progressed through S-phase in contrast to the cultures exposed to olomoucine (Figs. 2B–2D). At the highest dose of olomoucine, i.e., 200 μM, the cells seem to be frozen in the cell cycle. Furthermore, both in the BrdUrd-positive and -negative compartment, cells with a sub-G₁ DNA content seem to accumulate.

For a more detailed analysis the number of cells in the various regions (R2–R6) of the scattergram were quantitated as outlined under Materials and Methods. Figure 3A shows the number of BrdUrd-negative G2/M-phase cells (R5) as a function of time for the various doses of olomoucine tested in MR65 cells. No dose or time-dependent differences in exit rate from this cell cycle compartment were observed up to a dose of 200 μM olomoucine, at which the exit rate from the G₂/M compartment declined. In contrast, for the BrdUrd-
positive cells, the exit rate from the G2/M compartment decreased in a dose-dependent fashion as is outlined in Fig. 3B. Similarly, the S-phase entry rate also decreased in a dose-dependent manner (Fig. 3C). No evidence is found for either a delayed entry of cells into G1- or S-phase in the presence of olomoucine, only the rate at which the cells progress is affected by the CDK inhibitor. This is also the case for the progression of cells through the S-phase of the cell cycle as depicted in Fig. 3D. At the highest dose of olomoucine tested; i.e., 200 μM the cells could be completely arrested in the S-phase.

The changes in cell cycle progression for both MR65 and CHP-212 cells were normalized and shown in Figs. 4A and 4B. Both cell lines showed similar dose-dependent kinetic changes. The main effect of olomoucine is on the exit rates from G1- and G2/M phase of the cell cycle, while the progression rate through the S-phase of the cell cycle was affected to a lesser extend. Incubation of MR65 cells with roscovitine showed exactly the same results. Figure 5 shows the differential dose-dependent effects of both olomoucine and roscovitine on G1-/S-phase transition and S-phase progression. However, the ID50 values for roscovitine were 5- to 10-fold lower as compared to olomoucine.

A contradictory finding for both CDK inhibitors was that the cells that were originally in the G2/M phase of the cell cycle at the time of BrdUrd administration progressed normally while the BrdUrd-positive cells showed a delayed G2/M phase transition rate. A possible explanation for this phenomenon could be the fact that it takes some time for the cells to acquire a sufficient high intracellular dose of the inhibitor to be effective. In order to test this hypothesis cell cultures were preincubated for 3 h with various doses of roscovitine prior to BrdUrd pulse labeling. The cultures were subsequently chased for 3 h and harvested. The decrease in the number of BrdUrd-negative G2/M phase cells was normalized as compared to the control cultures. As can be seen from Fig. 6 the decrease in the number of BrdUrd-negative G2/M-phase cells was less as the dose of roscovitine increased.

In order to evaluate the CDK levels in MR65 cells, incubated for various time periods in the presence of both inhibitors, cell lysates were prepared and subjected to polyacrylamide gel electrophoresis followed by Western blotting. For both olomoucine and roscovitine treated cells a decrease in total CDK1 protein levels were observed (Fig. 7). This decrease in the level of CDK1 protein was not due to a nonspecific effect of cell cycle inhibition since no decrease in protein levels was found when the culture were exposed to hydroxyurea. This loss was confirmed by bivariate CDK1/DNA analysis (Fig. 8A) in which increasing amounts of CDK1-negative cells were detected when cells were cultured in the presence of increasing amounts of roscovitine. Furthermore, the CDK1-negative cells showed characteristics of apoptosis, i.e., loss of DNA (Fig. 8A) and

**FIG. 3.** The effect of various doses of olomoucine (▲, 0; ●, 10; *, 50; ○, 100; and ▼, 200 μM) on cell cycle phase transitions in MR65 cells. A shows the percentage of BrdUrd-negative G2/M-phase cells (abscissa) versus time in the presence of olomoucine (ordinate). B depicts the fraction of undivided cells among the BrdUrd-positive population (abscissa) versus time in the presence of olomoucine (ordinate). C and D show the relative DNA content of the BrdUrd-negative G1/S-phase cells and BrdUrd-positive/undivided cells, respectively, as function of exposure to olomoucine. The exit rate from G2/M-phase, the entry rates to G1- and S-phase, and the rate of progression through the S-phase of the cell cycle are calculated from the linear portions of the graphs in A, B, C, and D, respectively.
The MR65 and CHP-212 cells showed different kinetics of induction of apoptosis at high olomoucine dose. Figure 10 shows that for MR65 cells the frequency of apoptotic cells gradually increased, with up to 50% apoptotic cells after 10 h at a dose of 200 μM olomoucine. In CHP-212 cultures the number of apoptotic figures increased rapidly after a 3-h lag time.

**DISCUSSION**

The molecular basis of activation and deactivation of CDKs is mainly elucidated in cell-free systems using purified components. In this way many target proteins are identified which are phosphorylated by different CDK/cyclin complexed in vitro [1, 2]. However, it is difficult to correlate events occurring on a molecular scale (e.g., activation) with those that occur on a cellular scale (e.g., the start of S-phase). Studies at the cellular level are largely hampered by the lack of specific inhibitors. Recently, Kitagawa et al. [39], Losiewicz et al. [49], and Vesely et al. [40] described specific inhibitors of certain CDKs (see review by Meijer [50]).

In the present study a detailed analysis of the cell cycle effects of olomoucine was performed. To this end Fig. 4. The dose-dependent effects of olomoucine on cell cycle kinetics of MR65 (A) and CHP-212 cells (B). Data of G2/M-exit rates of BrdUrd-negative (*), BrdUrd-positive cells (●), and S-phase (●) entry rates and S-phase progression (▲) are compared to untreated and control cultures after normalization. A clear dose-dependent decrease in the various traverse rates can be observed, except for the G2/M-exit rate as calculated from the BrdUrd-negative G2/M phase cells. This parameter is, however, affected by a dose of 200 μM olomoucine in the case of MR65 cells.

altered scatter characteristics (data not shown). Again no loss of CDK1 immunoreactivity was observed when cells were exposed to hydroxyurea (Fig. 8B).

When cell cultures, treated with highest dose of olomoucine or roscovitine, were examined microscopically, many cells showed membrane blebbing and detached from the culture plastic. To examine these cells in more detail, they were harvested at various time intervals and cytocentrifuged onto glass slides. The slides were stained with propidium iodide and the nuclear morphology was examined using confocal scanning laser microscopy. Figures 9A - 9D show phase contrast images of such MR65 cell cultures and confocal images of nuclear morphology of the corresponding cytocentrifuge preparations (Figs. 9E - 9H). With increasing time of exposure more cells showed membrane blebbing and detachment from the culture dish. Simultaneously, the frequency of cells showing chromatin aggregation, typical for apoptosis, increased. Some cells clearly showed chromatin condensation at the nuclear periphery, while in the majority of cells the nucleus was completely broken down and only fragments of condensed chromatin were seen.
FIG. 5. The dose-dependent effects of olomoucine (●) and roscovitine (●) on cell cycle kinetics of MR65 cells. Data of S-phase entry rates (A) and S-phase progression (B) are normalized compared to untreated control cultures after normalization. Roscovitine shows identical effects on cell cycle kinetics as compared to olomoucine at a 5- to 10-fold lower concentration.

The dose of inhibitor. Furthermore, the normalized data in Fig. 5 shows that S-phase progression is only affected in a modest way, indicating that the rate of increase in the relative DNA content of G1/S cells unlabelled with BrdUrd mainly reflects the G1/S transition alone. Similarly, it can be shown that the division of BrdUrd-positive cells mainly reflects the G2/M transition and is only affected to a limited extent by the rate of completion of S-phase.

The finding that roscovitine, an analogue of olomoucine having a 10-fold increased efficiency toward CDKs and a similar low efficiency toward erk1, showed exactly the same cell cycle effects, makes it very likely that the observed cell cycle effects are caused by direct inhibition of CDKs in vivo rather than by inhibition of MAP kinases. Taken together, the data presented here are consistent with the hypothesis of a direct effect of olomoucine and roscovitine on both CDK2 and CDK1, governing the G1- to S-phase and the G2/M- to G1-phase of the cell cycle, respectively [2, 7].

Interestingly, in both MR65 and CHP-212 cell lines a decreased S-phase progression rate was observed. Since this effect cannot be explained by a direct inhibition of DNA-polymerases by olomoucine (IC50s for these enzymes exceed 200 μM), a plausible explanation may be that CDKs play a direct role in DNA-synthesis. For example, Dutta et al. [32] reported that CDKs are able to phosphorylate RPA, a human cell DNA replication factor, and activate replication. In concordance with this finding, Vesely et al. [40] showed inhibition

FIG. 6. Relative decrease in the number of BrdUrd-negative G2/M phase cells. MR65 cells were exposed to 5, 10, and 20 μM Roscovitine 3 h prior to (solid bars) or immediately after (open bars) pulse labeling with BrdUrd (solid bars). The cell cultures were subsequently chased, harvested at different time intervals, and subjected to bivariate BrdUrd/DNA analysis. The number of BrdUrd-negative G2/M phase cells are quantified and the percentage decrease in numbers is expressed compared to untreated cultures.

FIG. 7. CDK1 expression in MR65 cells incubated in the presence of roscovitine. Cell lysates of exponentially growing cells (lane 1), cells treated for 7 h in the presence of 10 μM (lane 3), 25 μM (lane 4), and 50 μM (lane 5) roscovitine were separated by gel electrophoresis and transferred on PVDF membranes. Western blots were probed with anti-CDK1. A decrease in total CDK1 protein levels is evident in the cells treated with roscovitine. No change in CDK1 protein levels are observed in the hydroxyurea-treated culture (lane 2).
FIG. 8. Flow cytometric analysis of CDK1 expression in MR65 cells after 7-h exposure to 50 μM Roscovitine. (A) Bivariate CDK1/DNA analysis, showing DNA content (ordinate) versus CDK1 immunofluorescence (abscissa). Note the loss of DNA in the CDK1-negative cells. The line indicates the threshold for positive immunofluorescence and was set based on the negative control allowing less than 1% positive immunoreactivity. (B) Histograms of CDK1 immunofluorescence of control cultures (solid line), and cultures treated for 7 h with 50 μM Roscovitine (dotted line) and 0.2 mM hydroxyurea (striped line) treated cultures. The marker indicates the threshold for positive immunofluorescence and was set based on the negative control allowing less than 1% positive immunoreactivity.
exposure of cells to hydroxyurea did not result in decreased levels of CDK1 protein levels.

To date, data on the role of CDKs during apoptosis are inconclusive. On the one hand, many authors provide evidence for activation of CDKs during apoptosis. In a study of Meikrantz et al. [55] the appearance of condensed chromatin was accompanied by a 2- to 7-fold increase in cyclin A-associated histone H1 kinase activity, where both CDK1 and CDK2 were activated. The authors suggested that cyclin A targets activated CDK1 and CDK2 to substrates necessary for chromatin condensation and other morphological changes during both apoptosis and mitosis. The authors also suggest that the sensitizing effect of cycloheximide, which was shown to occur in the MR65 NSCLC cell line [56], would be due to its ability to increase cyclin A-associated kinase activity rather than to inhibit the synthesis of apoptosis-suppressing proteins. Hoang et al. [57] also provided evidence for the participation of cyclin A in myc-induced apoptosis. They showed that both in proliferating cells and cells undergoing apoptosis, the cyclin A (but not B, C, D1, and E) mRNA level was elevated in unsynchronized myc-overexpressing cells when compared with parental rat fibroblasts.

On the other hand, recent evidence suggests that PITSLRE kinase isoforms are cellular targets for caspase-3-mediated cleavage during TNF-induced apoptosis [63]. Furthermore, Yoshida et al. [64] showed evidence that staurosporine-induced apoptosis is not accompanied by intracellular activation of CDK1. Also, the observation made by Guagdago [60] seem to contradict a need for CDK activation during apoptosis. They showed that the appearance of cyclin A messenger RNA and protein in late G1, was dependent on cell adhesion. It is also known that prevention of substrate attachment leads to apoptosis by a process named anokis, a term derived from the Greek word for homelessness [61].

In the presented study apoptosis was induced by inhibiting CDKs. An explanation for the apparent discrepancies of many studies might be that activation or inhibition of CDKs per se is not associated with apoptosis, but untimely activation or deactivation of these kinases, implying that cyclins and CDKs are involved at an important nodal point shared by pathways regulating cellular proliferation and apoptosis. In line with this reasoning are the observations made by Lahtti et al., showing that ectopic expression of certain PIT-
SLRE kinase isoforms, distantly related members of the CDK1 gene family, can induce apoptosis. It is conceivable that timely activation of CDK complexes commits a cell to enter and complete the cell cycle and on the other hand inhibits the apoptotic program by modifying regulatory molecules. The differences observed in the induction of apoptosis between MR65 and CHP212 cells, while the kinetics of cell cycle interference are identical, are in line with such reasoning assuming differences in cellular composition of apoptosis regulatory molecules such as bc12 and bax.

In summary, the present study shows that the cell cycle effects of olomoucine and roscovitine, i.e., inhibition of the G1/S- and G2/M-phase transition as well as S-phase progression, are consistent with the assumed specific inhibition of CDK1 and CDK2. At high dose, olomoucine and roscovitine are able to completely block cell cycle progression and to induce apoptosis. The unique specificity of olomoucine and roscovitine for CDKs make these compounds useful new tools for studying cell cycle regulation at the cellular level.

REFERENCES


