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Effects of olomoucine, a selective inhibitor of cyclin-dependent kinases, on cell cycle progression in human cancer cell lines

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We have studied the effects of olomoucine, a selective inhibitor of cdk2, cdc2 and MAP kinase, on the rate of proliferation and the cell cycle progression in human cancer cells in culture. Olomoucine inhibited the growth of the KB 3-1, MDA-MB-231 and Evsa-T cell lines in a concentration-dependent manner, with EC_{50} values of 45, 75 and 85 μM, respectively. Incubation of exponentially growing KB 3-1 cells in the presence of olomoucine led to an increased proportion of cells in G_1 phase after 24 h or more of incubation. Olomoucine failed to rapidly affect the phosphorylation of the Rb tumor-supressor gene product. However, [^{3}H]thymidine incorporation into the cell DNA was rapidly inhibited. We show that this inhibition is due, at least in part, to the diminution of thymidine entry into the cells. Suprisingly, all these cell lines, when synchronized at the G_1/S interface and relaxed in the presence of olomoucine, progressed unhindered through the S phase. Under these conditions, the G_2 phase transit was markedly retarded but not prevented. Insufficient permeability of the cell membrane to olomoucine may explain the low activity of the drug.

Key words: Cell cycle, cdk, nucleoside transport, olomoucine, pRb.

Introduction

Cyclin-dependent kinases (cdk) are sequentially activated throughout the cell cycle, and their activities are indispensable for the completion of the G_1 phase as well as for DNA replication and mitosis. Therefore, selective inhibitors of these enzymes are among choice targets for the development of novel anticancer drugs which may be effective against cells resistant to standard therapies. Olomoucine [2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine] is a recently described compound with a highly selective inhibitory activity directed towards cdk, the only other protein kinase known to be strongly inhibited by olomoucine being mitogen-activated protein kinase (MAPK); its mechanism of action is competitive inhibition of ATP binding. Olomoucine did not inhibit any of the other enzymes tested, including protein phosphatases.[1] In numerous cellular models, this compound has been shown to inhibit proliferation of cells in culture as well as meiosis in germ cells.[2] Since olomoucine inhibits the activities of cdk2 and cdc2 but not cdk4 (nor cdk6), it would be expected to block at least two essential events in the cell division cycle: replication of chromosomal DNA (reported to require the activity of cdk2)[3,4] and mitosis (dependent on cdc2).[5] Such actions of olomoucine have indeed been observed in several models including mammalian and plant cells.[2] Inhibition of MAPK can be expected to prevent the resumption of the cell division cycle in postmitotic cells. In contrast, the late G_1 phase progression need not be obstructed as cdk4, an enzyme supposed to phosphorylate pRb (the product of the RB1 tumor suppressor gene) in mid-G_1 (see reviews by Sherr[6] and Weinberg[7]), is not inhibited by this drug. Thus, by the combined use of cell synchonization and exposure to olomoucine, it should be possible to analyze the causal links between the activities of several of the key protein kinases implicated in cell cycle progression. In this work, we have explored the effects of olomoucine on the cell cycle of human cancer cell lines in culture.

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Materials and methods

Chemicals

Hydroxyurea and sodium butyrate were products of Sigma (St Louis, MO). [^{3}H]Thymidine was from

Amersham (Les Ulis, France). Olomoucine was a gift from L Meijer (CNRS, Station Biologique, Roscoff, France). Other chemicals were purchased from usual commercial sources.

Cell lines, culture and synchronization

The human epidermoid cancer cell line KB 3-1, and the breast carcinoma cell lines MDA-MB-231 and Evsa-T were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS).

To synchronize the cells at the G_1/S interface, they were incubated in complete medium containing 2.5 mM hydroxyurea for 15 h. Relaxation from the inhibitor allowed the synchronized cells to progress through the cell cycle. To obtain synchronization in early G_1, G_1/S cells were incubated for 24 h with lovastatin (20 μM; gift of Merck, Sharp and Dohme).[8]

Flow cytometry

The distribution of cells among the different phases of the cell cycle was evaluated by flow cytometry analysis of the DNA content. Cells ($1-2 \times 10^6$) were harvested from Petri dishes by trypsin, fixed by ethanol (70%) and stored at 4°C. For analysis, they were centrifuged, suspended in phosphate-buffered saline (PBS) 0.1% Triton X-100 (500 000 cells/ml), treated with RNase (1 mg/ml) and propidium iodide (20 mg/ml) for 30 min at room temperature, and then analyzed at a flow rate of about 500 cells/s in an orthocytofluorograph 50H (Ortho Diagnostic, Westwood, MN).[9]

Entry of [^{3}H]thymidine

Cells were incubated with 2 μCi/ml [^{3}H]thymidine (20 Ci/mmol)/ml during pulses of 30 min. Subsequently the cells were placed on ice, rapidly washed two times with cold PBS and lysed during 10 min in PBS plus 0.5% Nonidet P-40. The solubilized radioactivity was measured by liquid scintillation counting.

[^{3}H]Thymidine incorporation into DNA

Cells were introduced with 2 μCi/ml [^{3}H]thymidine/ml during pulses of 30 min. The incubation was terminated by acidification with 1 M ascorbic acid (3 drops/ml). The cells were fixed with 5% trichloroacetic acid, solubilized in 0.1 N NaOH and the incorporated radioactivity was determined by liquid scintillation counting.

Western blot analysis

The procedure followed was as described[10] except that aprotinin (Sigma; 0.1 trypsin inhibitor unit/ml) was the only protease inhibitor used. Protein quantification was carried out with the BioRad DC Protein Assay (BioRad, Ivry-sur Seine, France). Samples of 80 μg of protein were loaded per lane onto a 6% polyacrylamide gel. After electrophoresis, the portion of the gel covering the region between 90 and 130 kDa was transferred onto a nylon membrane and revealed using ECL detection (Amersham) by an anti-Rb rabbit polyclonal IgG (C-15, directed against the C-terminal 15 amino acids of the human Rb protein; Santa Cruz, Santa Cruz, CA) followed by anti-rabbit horseradish peroxidase-conjugated antibody (Amersham).

Assay of thymidine kinase activity

The kinase activity assay was carried out as described[11] with minor modifications. Exponentially growing cells were harvested in PBS, collected in microcentrifuge tubes and lysed by ultrasonication in Nonidet P-40 reagent. Reaction mixtures containing 40 μl of reaction buffer (75 mM Tris–HCl, pH8, 30 mM NaF, 5.4 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 160μM thymidine, 2 mM sodium vanadate) plus olomoucine at different concentrations and 20 μl of [^{3}H]thymidine (40 μCi/ml, 24 Ci/mmol) was prepared and supplemented with 5 mM ATP. Then 20 μl of the cell lysate was added and the samples were incubated at 20°C for 10 min. (We have verified that up to 10 min, the kinetics of thymidine phosphorylation was linear.) At the end of the incubation the samples were boiled for 2 min and transferred to ice. After centrifugation, 20 μl of the supernatants was spotted onto individual 2.5 cm discs of DE81 cellulose, left to dry and washed three times in ammonium formate (1 mM) for 30 min at room temperature. The filters were transferred to 20 ml scintillation vials and extracted for 30 min with 1 ml of 100 mM HCl before addition of 9 ml of liquid scintillation fluid. The radioactivity of the samples was determined by scintillation counting.

Incorporation of ^{35}S-labeled amino acids

The cells were seeded in 24-well boxes (5×10^4 cells/well). On the next day, the cells were washed two times with PBS and incubated for 2 h in DMEM without Cys and Met, in the presence of ^{35}S-labeled Cys and Met (1 μCi/ml; ICN Pharmaceuticals, Orsay, France). The cells were fixed with 5% trichloroacetic acid, solubilized in 0.1 N NaOH and the incorporated radioactivity was determined by liquid scintillation counting.

Results

Effect of olomoucine on the growth and cell cycle distribution

Olomoucine inhibited the cell proliferation rate in a dose-dependent manner (Figure 1). The inhibition of cell proliferation was similar between days 1 and 4; the data shown represent the cell numbers at the end of the experiment (day 4). The KB 3-1 cells were more sensitive than the MDA-MB-231 and Evsa-T: the EC_{50} values were 45 μM for KB 3-1, and 75 and 85 μM for the MDA-MB-231 and Evsa-T, respectively.

The inhibition of cell proliferation by olomoucine was not a consequence of an immediate block of cells in any particular phase of the cell cycle. After 24 h of culture of the KB 3-1 cells with 100 μM olomoucine, a majority of cells accumulated in the G_1 phase and the fraction of cells with intermediary DNA contents (S phase cells) was diminished but not eliminated (Figure 2). This observation could mean that olomoucine inhibited the G_1/S transition (i.e. initiation of DNA synthesis) as well as the continuation of chromosomal DNA replication and mitosis. Our previous data[2] showing that olomoucine inhibited the incorporation of [^{3}H]thymidine with EC_{50} values of the order of 30 μM were compatible with this interpretation. To test further the possible effect of olomoucine in the S and G_2 phases, we have carried out experiments with cell populations synchronized at the G_1/S interface by incubation with hydroxyurea.

Surprisingly, the results showed that after relaxation from hydroxyurea in the presence of 100 μM olomoucine the cells accomplish their DNA replication normally (Figures 3 and 4). This was true for all three cell lines studied. On the other hand, the G_2 phase transit was retarded by olomoucine, particularly in the MDA-MB-231 (and Evsa-T, data not shown) cells and to a lesser extent in the KB 3-1 cells.

Since in cell-free conditions olomoucine inhibits the activity of cdk2 (an enzyme reported to be necessary for DNA replication), as well as that of

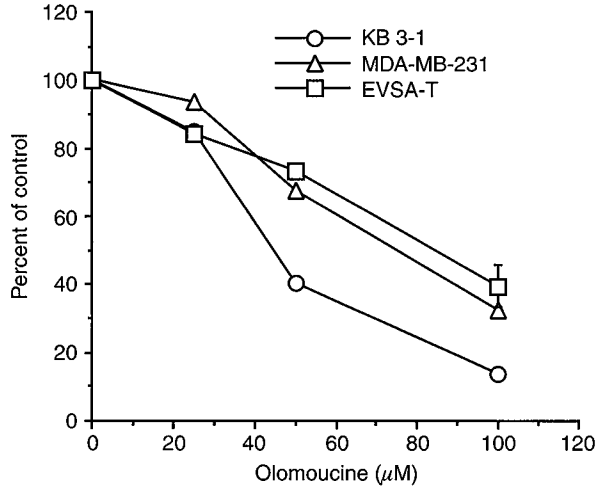

Figure 1. Inhibition of cell proliferation by olomoucine. KB 3-1, MDA-MB-231 and Evsa-T cells were seeded in 24-well boxes at 2×10^4 cells/well. On the next day, olomoucine was added at the concentrations indicated. The data shown are based on the cell number per well on day 4 after the addition of olomoucine and are expressed as percentages of the control (non-treated cells). Means of duplicates ± range are shown.

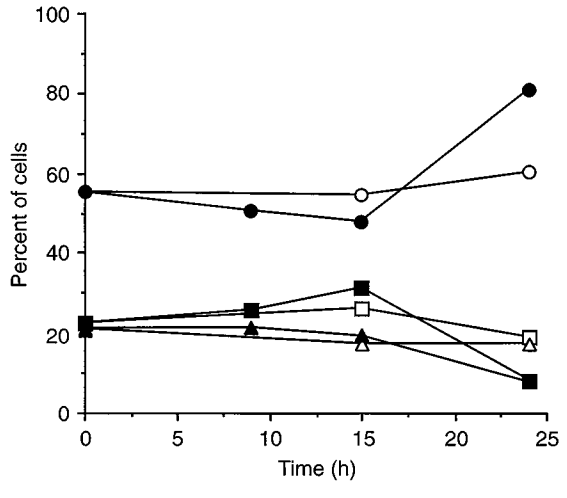

Figure 2. Effect of olomoucine on the cell cycle. KB 3-1 cells were seeded at 10^6 cells in 100 mm Petri dishes. On the next day, 100 μM olomoucine was added (solid symbols) or not (open symbols) and at the times indicated the cells were harvested for analysis by flow cytometry. The data represent the percentage of cells in G_1 phase (circles), S phase (squares) and G_2/M phases (triangles).

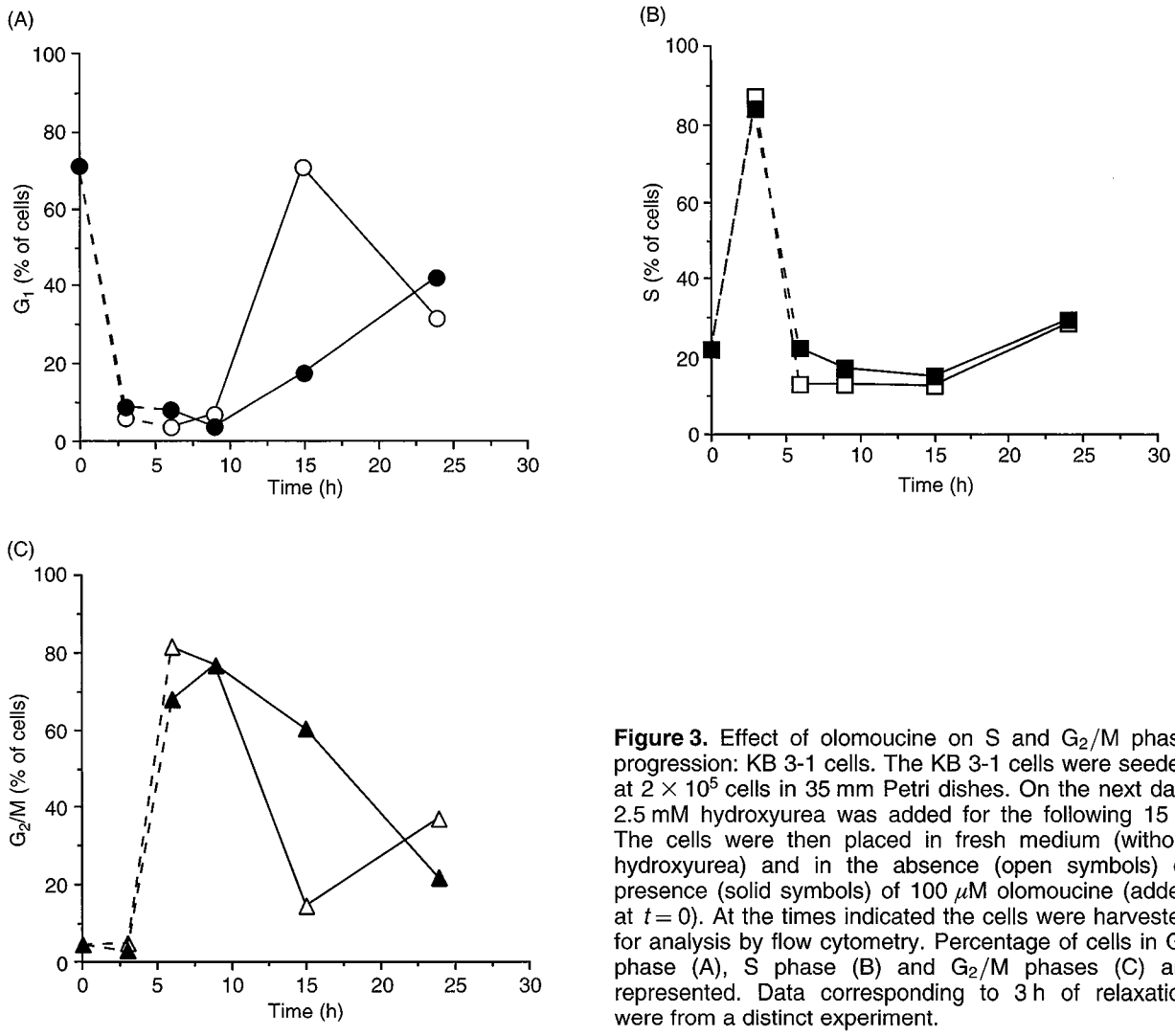

Figure 3. Effect of olomoucine on S and G_2/M phase progression: KB 3-1 cells. The KB 3-1 cells were seeded at 2×10^5 cells in 35 mm Petri dishes. On the next day, 2.5 mM hydroxyurea was added for the following 15 h. The cells were then placed in fresh medium (without hydroxyurea) and in the absence (open symbols) or presence (solid symbols) of 100 μM olomoucine (added at $t = 0$). At the times indicated the cells were harvested for analysis by flow cytometry. Percentage of cells in G_1 phase (A), S phase (B) and G_2/M phases (C) are represented. Data corresponding to 3 h of relaxation were from a distinct experiment.

cdc2 (necessary for mitosis), the explanation of our results may lie in the insufficient rate of entry of this compound into the cells. A direct measurement of cdk activities in cells exposed to olomoucine is not informative: after lysis, olomoucine bound to cdk dissociates and its concentration in the assay buffer is not comparable with that in the intact cell. To appreciate the effect of the drug *in vivo*, we have used an intracellular target of the activities of these enzymes, the Rb protein. The state of phosphorylation of Rb depends on the activities of cdk[6,7] and is a result of a rapid dynamic equilibrium as the phosphatase activity ensures a short half-life of phosphate residues (30–45 min).[12] The differently phosphorylated forms of Rb can be readily distinguished on the basis of their electrophoretic mobilities.[13,14] Rb was hyperphosphorylated in exponentially growing MDA-MB-231 cells (Figure 5, lane 1) as well as in G_2/M cells (10 h after relaxation from G_1/S arrest by hydroxyurea). The phosphorylation state of Rb was not affected by incubation with 100 μM olomoucine for as long as 24 h (Figure 5, lane 4), in support of the hypothesis that olomoucine fails to strongly inhibit cdk activities under these conditions. In this experiment, a majority (67%) of cells were still in G_2 phase after 24 h in the presence of olomoucine following relaxation from hydroxyurea-induced G_1/S arrest. Even a partial inhibition of (for instance) cdc2 activity could account for the observed lengthening of the G_2 phase (*cf.* Figure 2).

In comparison, the presence of lovastatin (24 h) led to an accumulation of cells in the G_1 phase, accompanied by an augmented proportion of hypophosphorylated Rb (Figure 5, lane 3).

In other experiments, an increased proportion of hypophosphorylated Rb was detected after a 48 h

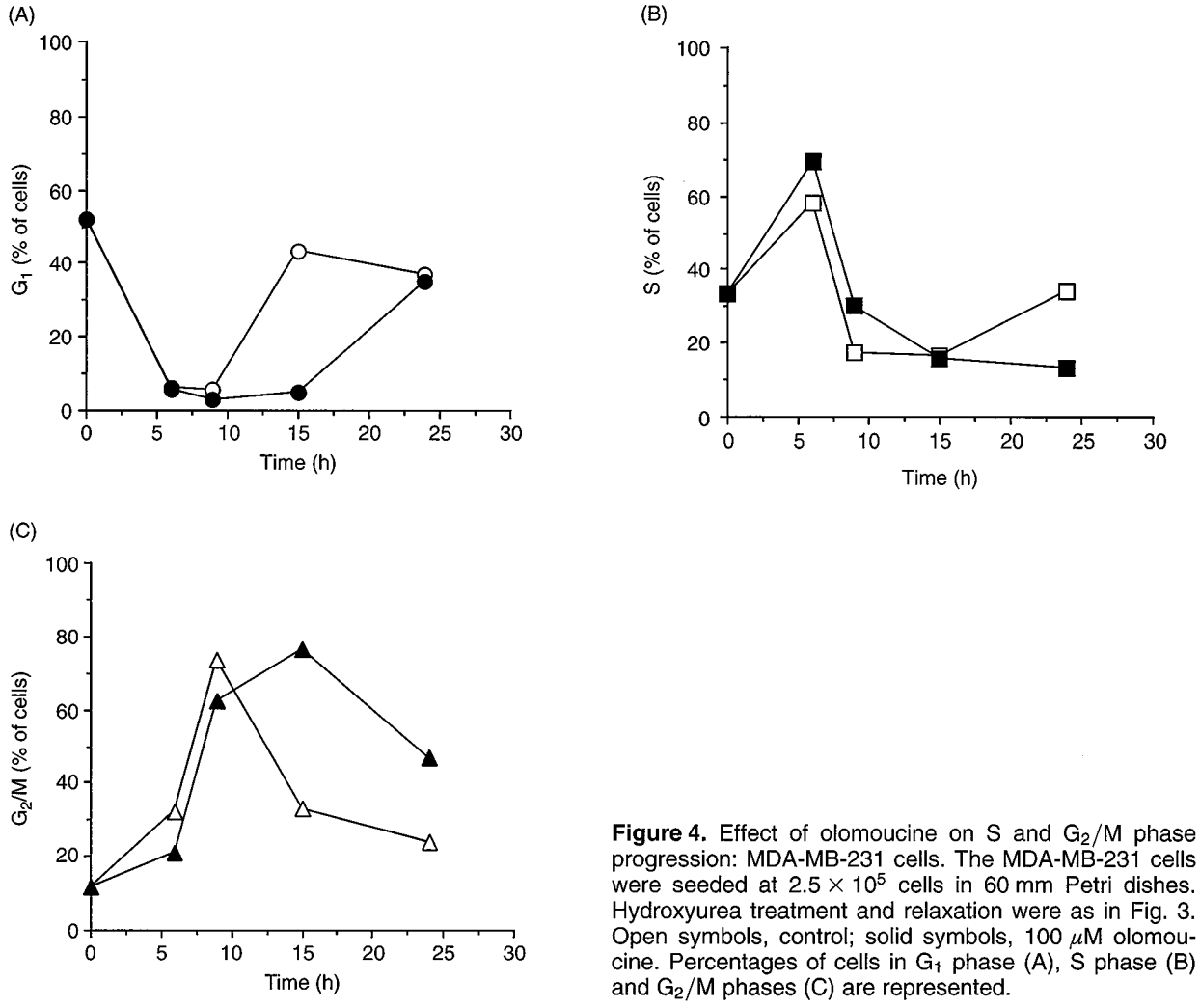

Figure 4. Effect of olomoucine on S and G_2/M phase progression: MDA-MB-231 cells. The MDA-MB-231 cells were seeded at 2.5×10^5 cells in 60 mm Petri dishes. Hydroxyurea treatment and relaxation were as in Fig. 3. Open symbols, control; solid symbols, 100 μM olomoucine. Percentages of cells in G_1 phase (A), S phase (B) and G_2/M phases (C) are represented.

exposure to olomoucine, both in the MDA-MB-231 cells and in the KB 3-1 cells (data not shown). The dephosphorylation of Rb under such conditions is probably an indirect consequence of a decreased cdk activity, possibly because of inhibition of early cell cycle progression due to the inhibition of MAPK by olomoucine.[1]

The discrepancy between the inhibition of [^{3}H]thymidine incorporation by olomoucine and absence of effect on S-phase progression was addressed in the following experiments.

Mechanism of the inhibition of [^{3}H]thymidine incorporation by olomoucine

We have first examined the time dependence of the effect of olomoucine on the incorporation of [^{3}H]thymidine into cellular DNA in the KB 3-1 cell line. The drug displays a biphasic action: there is a strong inhibition detected immediately upon addition of olomoucine, followed by a progressive additional decrease of [^{3}H]thymidine incorporation. The data shown in Figure 6 illustrate this observation; time zero corresponds to the incorporation of [^{3}H]thymidine during the first 30 min after the simultaneous addition of olomoucine and the radioactive precursor, whereas the '24 h' curve reflects the incorporation of [^{3}H]thymidine between 24 and 24.5 h after the addition of the drug to the culture medium. Apparently, olomoucine directly interferes with either the uptake or incorporation of [^{3}H]thymidine ('fast' inhibition), and causes an additional reduction in the incorporation of [^{3}H]thymidine as a consequence of inhibition of the cells' entry into S phase. The 'fast' inhibitory effect of olomoucine is readily reversible as revealed by the immediate

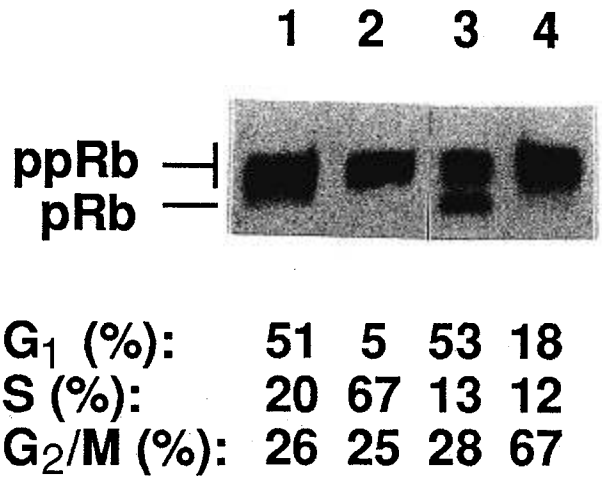

Figure 5. Rb protein phosphorylation: effects of olomoucine and lovastatin. The MDA-MB-231 cells were seeded at 10^6 cells in 60 mm Petri dishes. On the next day, exponentially growing cells (lane 1) were incubated in the presence of 2.5 mM hydroxyurea for the following 15 h. Relaxation was then carried out during 6 h in the absence of drugs (lane 2) or during 24 h in the presence of lovastatine (20 μM; lane 3) or olomoucine (100 μM; lane 4). Cells were either analyzed by flow cytometry or lysed and the extract analyzed by Western blotting (revelation by ECL) as described in Materials and methods (5 s exposure to a XAR5 Kodak film). Migration of the hypophosphorylated (pRb) and hyperphosphorylated (slower-migrating; ppRb) forms of the Rb protein are indicated. At each lane, the percentages of the cells in the different phases of the cell cycle are shown.

increase in the rate of [^{3}H]thymidine incorporation upon removal of olomoucine from the culture medium of cells incubated with the drug for 24 h (Figure 6b). After a delay of 8 h or more following removal of olomoucine, an additional increase in [^{3}H]thymidine incorporation is observed, probably due to the entry into S phase of the cells arrested in G_1 phase by olomoucine. This delay indicates that the arrest was situated in early G_1 rather than at the G_1/S interface.

There are at least three alternative mechanisms which could account for the 'fast' inhibitory action of olomoucine: inhibition of DNA replication, inhibition of thymidine kinase activity and inhibition of the entry of [^{3}H]thymidine into the cells. The inhibition of DNA replication can be excluded (see above). We have next studied the effect of olomoucine on the thymidine kinase activity *in vitro* (Table 1). The results show a weak inhibition detectable only at high concentrations (20% or less inhibition at 100 μM), insufficient in itself to explain the effects observed in living cells. In contrast, there was a significant inhibition of [^{3}H]thymidine entry into the KB 3-1 cells (Figure 7), as 100 μM olomoucine caused approximately 50% inhibition. These studies were carried out with cells synchronized by incubation with hydroxyurea and relaxed for 3 h in order to enter S phase; during the [^{3}H]thymidine entry experiment, aphidicolin was added to prevent the

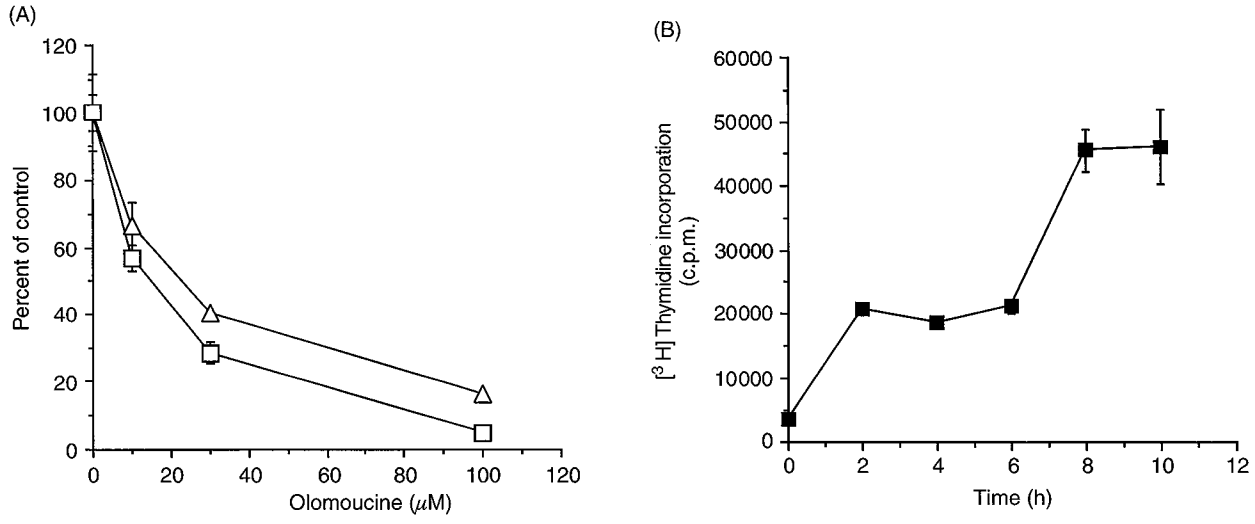

Figure 6. Effect of olomoucine on the [^{3}H]thymidine incorporation. KB 3-1 cells were seeded in 24-well boxes (5×10^4 cells/wells) and allowed to attach during 24 h (A). On the following day, the cells were exposed to olomoucine at the concentrations indicated either between 0 and 30 min ('time 30 min') or between 0 and 24 h ('time 24 h'). [^{3}H]Thymidine incorporation was evaluated during the 30 min pulses between 0 and 30 min (triangles) or between 24 and 24.5 h (squares). The data shown are means of triplicates ± SEM. (B) The cells were maintained in the presence of olomoucine at the concentrations indicated for 48 h. Then olomoucine was removed and the cells were incubated in drug-free medium. [^{3}H]Thymidine incorporation was measured during 30 min pulses every 2 h. The data shown are means of triplicates ± SEM.

Table 1. Effect of olomoucine on thymidine kinase activity *in vitro*

Olomoucine (μM)	Thymidine kinase activity (% of control)
0	100.0 ± 2.5
10	96.0 ± 12.3
30	102.0 ± 1.0
100	80.7 ± 4.9

Thymidine kinase activity was measured in the cytoplasmic fraction of the KB 3-1 cells, in the presence of increasing concentrations of olomoucine. The results (means ± SEM) are expressed as percent of control (value measured in the absence of olomoucine).

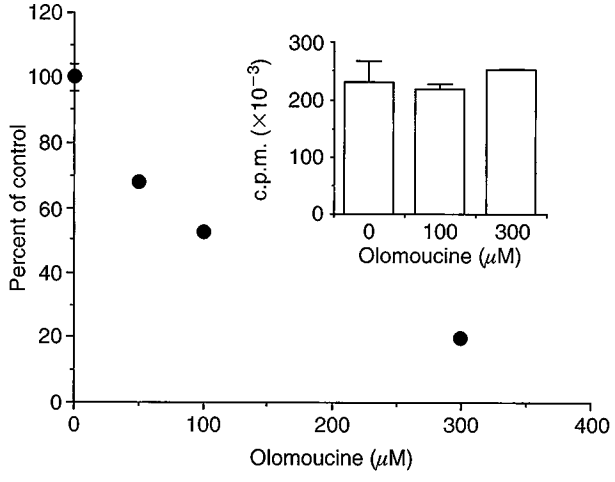

Figure 7. Effect of olomoucine on the entry of [^{3}H]thymidine into the cells. KB 3-1 cells were seeded in 35 mm Petri dishes (1.5×10^4 cells/dish). The cells were synchronized by incubation in the presence of hydroxyurea during 15 h. To allow progression into the S phase, the cells were relaxed from the hydroxyurea block and maintained in drug-free medium during the next 3 h. The cells were then placed in fresh medium containing aphidicoline (2 μM), olomoucine at the concentrations indicated and [^{3}H]thymidine (2 μCi/ml). Entry of thymidine was determined as described in Materials and methods. The data are expressed as percentages of the cells non-treated with olomoucine; means of triplicates ± SEM are shown. Insert: cells in S phase were precharged during 30 min with [^{3}H]thymidine in the presence of aphidicoline (2 μM). Aphidicoline was then removed by washing with olomoucine-containing medium and the cells were maintained for the next 30 min in the presence of the concentrations of olomoucine as indicated. [^{3}H]Thymidine incorporation into DNA was terminated by acidification and measured as described in Materials and methods. The data shown are means of triplicates ± SEM.

incorporation of the labeled precursors into DNA. If the cells were precharged with [^{3}H]thymidine in the absence of olomoucine (and in the presence of aphidicolin to block DNA synthesis), 100 μM olomoucine failed to affect the incorporation when the DNA polymerase inhibitor was removed, confirming that the 'fast' inhibitory action of olomoucine was indeed attributable to the steps preceding DNA synthesis itself.

Table 2. Effect of olomoucine on the uptake of [^{35}S]Met + [^{35}S]Cys by the KB 3-1 cells

Olomoucine (μM)	c.p.m. ($\times 10^{-3}$)
0	80.4 ± 0.3
50	90 ± 0.05
100	82.4 ± 0.6
300	76.9 ± 1.3

Exponentially growing cells were incubated at 37°C with the mixture of [^{35}S]Met + [^{35}S]Cys (1 μCi/ml) in the presence of increasing concentrations of olomoucine. After 30 min, the culture medium was acidified with ascorbic acid and the radioactivity incorporated into trichloroacetic acid-insoluble material was determined. The data shown are means of triplicates ± SEM.

Discussion

In this work, we have studied the effects of olomoucine, a selective inhibitor of cdk, on the cell cycle of human cancer cells. Since this drug is an effective inhibitor of two of the cdk needed for the replication of chromosomal DNA (cdk2) and for mitosis (cdc2), we anticipated that it would interfere with these two essential processes and thus block cell proliferation. While growth inhibition by olomoucine has been systematically observed, the cell cycle effects of olomoucine did not conform to our expectations. In particular, DNA replication was not prevented by olomoucine in any of the three cell lines analyzed. This observation may mean that cdk2 activity is not indispensable for DNA synthesis in S phase, a conclusion incompatible with earlier studies obtained in cell-free conditions[3] as well as in cells expressing a dominant negative mutant cdk2[15] which have demonstrated that DNA replication cannot proceed in the absence of cdk2 activity. Alternatively, olomoucine may not efficiently cross the cell membrane, so that its intracellular concentrations are insufficient to inhibit this enzyme in the living cell. The permeability of the cell membrane to olomoucine may differ according to the cell type as well as the phase of the cell cycle. A definitive answer to this question depends on the availability of a radioactively labeled form of the drug.

Another surprising observation was the relative inefficacy of olomoucine to prevent mitosis: the duration of the G_2/M phase was only extended in

the MDA-MB-231 and Evsa-T cells, and to an even lesser extent in the KB 3-1 cells. If olomoucine had blocked the activity of the cyclin B–cdc2 complex (as observed in cell-free studies),[1] one would expect a total arrest in G_2. Again, insufficient permeability of the cell membrane to olomoucine may be the reason for the low activity of the drug. In this context, however, it is of interest that olomoucine has been reported to enhance the DNA damage-induced apoptosis, by inhibiting cdc2.[16] A partial inhibition of cdc2 may be sufficient to produce this effect.

The fact that olomoucine failed to directly affect the state of phosphorylation of the Rb protein brings additional support in favor of the conclusion that the limited rate of entry of the drug does not allow rapid accumulation of a concentration sufficient to efficiently inhibit the appropriate cyclin-dependent kinases. The intracellular concentration of olomoucine may progressively increase with time; this would explain the increased proportion of cells in the G_1 phase (as well as the accumulation of hypophosphorylated Rb) seen after a long exposure to the drug. The kinetics of [^{3}H]thymidine incorporation after removal of olomoucine indicates that after a long exposure to olomoucine the cells end up arrested in early G_1 phase (see Figure 6B), probably as a consequence of the inhibition of MAPK.

An unexpected effect of olomoucine concerned the inhibition of [^{3}H]thymidine uptake by cells in the absence of DNA synthesis. This action of olomoucine was immediate. Although the inhibition is relatively weak (EC_{50} approximately 100 μM), it may explain the 'fast' component of olomoucine inhibition of [^{3}H]thymidine incorporation: the following step required for the utilization of thymidine, phosphorylation by thymidine kinase, is also (weakly) inhibited by olomoucine, and a reduced intracellular concentration of total thymidine phosphate (metabolic plus that produced from the [^{3}H]thymidine taken up from culture medium) would lead to a still greater reduction of the cellular content of the final DNA precursor, thymidine triphosphate. The mechanism by which olomoucine inhibits [^{3}H]thymidine entry into cells has not been further studied. A plausible hypothesis is the inhibition of the equilibrative nucleoside transporters,[17] either directly (competition) or indirectly.

Inhibition of the cell proliferation could be a consequence of totally non-specific actions of a drug. In the case of olomoucine, interference with the uptake of nutrients was a possibility to consider, in relation to the observed inhibition of the entry of thymidine. However, olomoucine had little or no effect on the entry of ^{35}S-labeled Met and Cys (Table 2), at a concentration (100 μM) sufficient to efficiently inhibit cell proliferation.

Conclusion

Our data show that olomoucine (at concentrations of the order of 10^{-5} M) slows down the proliferation of cultured cancer cells, but does not rapidly block the intracellular processes which depend on the activities of enzymes (cdk; MAPK) known to be efficiently inhibited by nanomolar concentrations of this compound under cell-free conditions. The most likely explanation of the relatively low efficiency of olomoucine *in vivo* is a limited rate of entry across the cell membrane. The inhibition of cell proliferation may be in part due to the action of olomoucine at the cell membrane level (reflected by the inhibition of uptake of [^{3}H]thymidine). Further development of compounds capable of entering the cell and inhibiting cdk activities *in vivo* is desirable.

Acknowledgments

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