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FAST TRACK

A New, Unexpected Action of Olomoucine, a CDK Inhibitor, on Normal Human Cells: Up-Regulation of CLIMP-63, a Cytoskeleton-Linking Membrane Protein

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Abstract Inhibition of cyclin-dependent kinases (CDKs) is a novel strategy in the therapy of human malignancies. The pharmacological CDK inhibitors representing a few distinct classes of compounds exert different target specificity. Considering the fact that dividing and quiescent cells differ in their CDK activity and in the pattern of their expression, one might expect that anti-proliferative efficiency of the pharmacological CDK inhibitors would depend on the mitotic index of treated cells. The present article shows that olomoucine (OLO), a weak CDK2 inhibitor has new, unexpected activity. At concentrations up to 100 μM OLO did not inhibit proliferation of normal human cells, but arrested growth of human HL-60 leukemia cells. The anti-proliferative effect of OLO was clearly weaker than that of roscovitine (ROSC). Surprisingly, OLO at low doses strongly up-regulated a cellular protein with approximately 65 kDa in normal, but not in immortalized and cancer cells. By mass spectrometric analysis CLIMP-63, a cytoskeleton-linking membrane protein was identified as the major component of the up-regulated protein band. These results were subsequently confirmed by immunoblotting. Further experiments revealed that OLO, but not ROSC, strongly up-regulates CLIMP-63 in a dose- and time-dependent manner solely in senescent cells. J. Cell. Biochem. 102: 1405–1419, 2007. © 2007 Wiley-Liss, Inc.

Key words: CDK inhibitors; cell cycle arrest; quiescence; senescence; life-span; population doubling

Cyclin-dependent kinases (CDKs) that regulate sequential steps of the cell cycle in higher eukaryotes, received much attention of oncologists during the past two decades [for reviews, see van den Heuvel and Harlow, 1993; Hunter and Pines, 1994; Vermeulen et al., 2003; Senderowicz, 2004]. CDKs, which are serine/threonine kinases, form complexes with cyclins, their specific regulatory components. The activation of CDKs is mediated by binding to their proper cyclin(s) and by their subsequent phosphorylation at specific residues catalyzed by cyclin activating kinase (CAK). Most cancer cells harbor mutations in genes controlling the cell cycle resulting in an aberrant cell cycle progression [Kozar et al., 2004; Malumbres et al., 2004]. According to the current opinion CDK4/6 and CDK2 play a key role in the G_1/S transition. Interestingly, abnormally elevated CDK4 activity is implicated in cancer primarily by mutations or alterations of its partner, cyclin D as well as by inactivation of its cellular inhibitor $p16^{INK4A}$ [Rane et al., 1999; Senderowicz, 2004]. On the other hand, CDK2 seems to be not directly affected by the enhancement of the activity or by mutations. Considering the increased activity of CDKs in malignant

Abbreviations used: APF, anti-proliferative factor; CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; CKAP4/p63, cytoskeleton-associated protein 4/p63; CLIMP-63, cytoskeleton-linking membrane protein-63; ER, endoplasmic reticulum; FACS, fluorescence-activated cell sorting; HIPK2, homeodomain protein kinase-2; LPC-1, late PDL cDNA-1; MTs, microtubules; OLO, olomoucine; PDL, population doubling level; PI, propidium iodide; PVDF, polyvinylidene difluoride; ROSC, roscovitine; WCL, whole cell lysate; MS/MS, tandem mass spectrometry.

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cells, their inhibition by specific drugs offers a new and promising therapeutic strategy in the defense against cancer [Senderowicz, 2004; Fischer and Gianella-Borradori, 2005; Wesierska-Gadek and Schmid, 2006]. Different pharmacological CDK inhibitors were developed [Fischer and Gianella-Borradori, 2005; Wesierska-Gadek and Schmid, 2006] that include broad spectrum agents, inhibiting a number of CDKs, as well as more specific inhibitors exhibiting high selectivity. The broad range CDKI seem to be cytotoxic due to the inhibition of a number of kinases in the regulation of basal cellular functions [Sedlacek, 2001; Fischer and Gianella-Borradori, 2005]. The relatively selective inhibitors of CDKs belonging to the group of substituted purines encompass among others roscovitine (ROSC), olomoucine (OLO), and purvalanol [De Azevedo et al., 1997; Havlicek et al., 1997; Meijer et al., 1997]. According to predictions, pharmacological inhibitors of CDKs compete with ATP for the binding to the active CDK site and thereby display increased anti-proliferative effects on cycling cells, especially malignant cells [Gray et al., 1999]. Depending on the selectivity profiles of these novel drugs and intrinsic features of the cancer cells, growth inhibition in different phases of the cell cycle is observed [Schutte et al., 1997; Alessi et al., 1998; Wesierska-Gadek et al., 2003]. Compounds targeting the activity of CDK4/6 block cells in early G_1 [Marzec et al., 2006], whereas selective inhibitors of CDK1/2 arrest the cell cycle at the G_1/S [Alessi et al., 1998] or G_2/M transition [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a]. Interestingly, some inhibitors, especially those targeting the activity of CDK2, are able to selectively induce apoptosis in cancer cells [Schutte et al., 1997; Wojciechowski et al., 2003; Wesierska-Gadek et al., 2004a, 2005a]. ROSC and OLO were found to exert a strong inhibitory effect on the CDK2/cyclin A and CDK1/cyclin B kinase complexes [Yuan et al., 2004]. Noteworthy, recent reports demonstrating that some cancer cells can proliferate despite CDK2 inhibition and that CDK2 is dispensable for cell cycle progression of somatic cells question the central role of CDK2 in the cell cycle supervision [Berthet et al., 2003]. Therefore, the suitability of CDK2 as a therapeutic target became questionable.

Indeed, although selective CDK2 inhibitors seem unsuitable for the therapy of CDK2-independent tumors, it appears that they could be successfully used for another purpose, namely to protect the normal cells from undesired consequences of chemotherapy. One might speculate that pretreatment with CDK2 inhibitors would arrest at least a substantial number of normal cells in G_1/G_0 phase rendering these cells transiently resistant to chemotherapy.

In the present article we tested the effect of OLO, a weak CDK2 inhibitor, on normal and transformed human cells. According to the expectations, OLO showed low, if any, cytotoxicity against non-transformed human MRC-5 cells and up to a final concentration of 100 μM it did not affect their proliferation. This was attributable primarily to the high ratio of G_1-arrested cells. Surprisingly, exposure of human MRC-5 cells to OLO resulted in an induction of a protein with 65 kDa in a dose- and time-dependent manner. Mass spectrometric analysis of the Coomassie-Blue-stained up-regulated protein band revealed that the cytoskeleton-linking membrane protein-63 (CLIMP-63) alternatively also termed cytoskeleton-associated protein 4/p63 (CKAP4/p63), is its major component [Schweizer et al., 1993a; Banham et al., 1997; Gupta et al., 2006]. Immunoblotting experiments with anti-CLIMP-63 antibodies confirmed these data. In untreated MRC-5 cells CLIMP-63 protein was barely detectable. After exposure of the cells to increasing OLO concentrations the intensity of the CLIMP-63 protein band strongly increased. OLO induced CLIMP-63 protein in normal quiescent/senescent fibroblasts but not in immortalized or transformed cells. Thus, our results indicate that even less potent pharmacological CDK inhibitors exert strong biological effects on normal tissues. The functional consequences of the OLO-mediated up-regulation of CLIMP-63 protein in normal healthy cells and their potential exploitation for, for example, protection of normal cells from the undesired side effects of chemotherapy have to be systematically elucidated.

MATERIALS AND METHODS

Cell Culture

Normal human MRC-5 skin and F2000 lung fibroblasts, immortalized mouse A-19 embryo fibroblasts, as well as human cancer cells (MCF-7 breast cancer cells and HL-60 leukemia cells) were used in this study. MRC-5, F2000,

A-19, and MCF-7 cells were grown up to 60–70% confluence as a monolayer in Dulbecco's medium supplemented with 10% fetal calf serum in an atmosphere of 8% CO_2. The latter were cultivated in phenol red-deprived medium. HL-60 cells were grown as suspension culture in RPMI medium with 10% fetal calf serum in an atmosphere of 5% CO_2. Cells were treated with ROSC and OLO at a final concentration ranging from 1 to 150 μM for indicated periods of time.

Antibodies

We used the following antibodies: monoclonal anti-CLIMP-63 (G1/296) (Alexis Biochemicals) and polyclonal antibody (a kind gift from Dr. J. Rohrer) [Schweizer et al., 1994, 1995], monoclonal anti-PCNA (clone PC-10) antibody (Oncogene Research Products (Cambridge, MA)), monoclonal anti-MCM-7 (clone DCS141.2) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), p53 DO-1 antibody which was a kind gift from Dr. B. Vojtesek (Masaryk Memorial Cancer Institute, Brno), and monoclonal anti-actin antibody (Clone C4) from ICN Biochemicals (Aurora, OH).

Immunostaining

For microscopic investigations, cells were plated on slides in chambers and appropriately cultivated, as described previously in detail [Wesierska-Gadek et al., 2002]. After treatment for the indicated time, cells were washed three times in PBS and immediately fixed in ice-cold methanol/acetone (3:2) mixture for 20 min and washed in PBS. The fixed cells were permeabilized in 0.2% Triton X-100 in PBS for 20 min and then saturated in 5% BSA in PBS for 1 h and thereafter incubated with primary antibodies in 3% BSA in PBS overnight at +4°C in humidity chambers at a dilution of 1:2,000. Immune complexes were detected after incubation with appropriate secondary antibodies coupled with fluorochrome Cy2. Finally, cells were stained with propodium iodide and examined using a Leica TSC 4D confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). Negative control for the method included cells incubated without a primary antibody.

Determination of the Number of Viable Cells

Proliferation of human cells and their sensitivity to increasing concentrations of drugs were determined by the CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corporation, Madison, WI). As described recently in more detail [Wesierska-Gadek et al., 2004b], the CellTiter-Glo® Luminescent Cell Viability Assay, generating luminescent signals, is based on quantification of the cellular ATP levels. Tests were performed at least in quadruplicates. Luminescence was measured in the Wallac 1420 Victor, a multilabel, multitask plate counter. Each point represents the mean ± SD (bars) of replicates from at least three experiments.

To discriminate between initial cell killing and inhibition of cell proliferation, we measured additionally the direct cytotoxicity of the studied drugs by the dye exclusion test [Wesierska-Gadek et al., 2004b]. Cells were collected, adherent cells were detached by trypsin or by accutase treatment (PAA Laboratories, GmbH, Coelbe, Germany), and all cells were washed with PBS. The vital dye 7-amino-actinomycin D (7-AAD; BD Biosciences, San Diego, CA) appropriately diluted with PBS was added and after 10 min the accumulation of the 7-AAD fluorescent dye was quantified by flow cytometry using a fluorescence-activated cell sorter FACScan cytometer (Becton Dickinson).

Measurement of DNA Content in Single Cells by Flow Cytometry

The measurement of DNA content was performed by flow cytometric analysis based on a slightly modified method [Wesierska-Gadek and Schmid, 2000] described previously by Vindelov et al. [1983]. Propidium iodide-stained cells were measured by flow cytometry using the Becton Dickinson fluorescence activated cell sorter (FACScan). Distribution of cells in distinct cell cycle phases was determined using ModFIT cell cycle analysis software. DNA histograms were obtained by the CellQuest evaluation program.

Electrophoretic Separation of Proteins and Immunoblotting

Total cellular proteins dissolved in SDS sample buffer were separated on 10% SDS slab gels and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (Amersham International, Little Chalfont, Buckinghamshire, England). Equal protein loading and protein transfer was confirmed by Ponceau S staining. Blots were incubated with

specific primary antibodies at the appropriate final dilution and the immune complexes were detected using appropriate peroxidase-conjugated secondary antibodies and the enhanced chemiluminescent detection reagent ECL+ (Amersham International). In some cases, blots were used for several sequential incubations. Incubation with anti-actin antibodies additionally confirmed equal protein loading [Wesierska-Gadek et al., 1999, 2003].

Mass-Spectrometric Analysis

Unless otherwise noted, the various steps of the procedure were performed at room temperature and all incubation steps were performed under shaking. The protein band of interest in the SDS-gel was excised, cut into small pieces, transferred into a 0.25 ml polyethylene sample vial and washed twice with 150 μl 10 mM NH_4HCO_3, pH = 8.9 (15 min at 30°C). The supernatant was discarded and the gel pieces were shrunk by dehydration in 150 μl of 50% v/v acetonitrile/10 mM NH_4HCO_3 (15 min at 30°C). This step was performed twice and, after removing all liquid, the gel pieces were dried in a vacuum centrifuge. The gel pieces were swollen in a digestion buffer containing 10 mM NH_4HCO_3 and 0.05 μg/μl of trypsin (Roche, sequencing grade) at 4°C for 20 min. The supernatant was removed and replaced with 20 μl of the same buffer without trypsin. Digestion took place overnight at 37°C. Peptides were extracted by adding 50 μl 10 mM NH_4HCO_3 (37°C, 15 min), and 50 μl acetonitrile (37°C, 15 min). After collecting the supernatant 100 μl 10% formic acid/20% acetonitrile/20% 2-propanol was added to the gel pieces (37°C, 10 min). The supernatants were combined, concentrated by drying to approximately 5 μl, and stored at −20°C. Tryptic protein digestions were analyzed using capillary HPLC connected online to a LCQ ion trap instrument (ThermoFinnigan, San Jose, CA) equipped with a nanospray interface. The nanospray voltage was set at 1.6 kV and the heated capillary was held at 170°C. MS/MS spectra were searched against a human protein database using SEQUEST (LCQ BioWorks; ThermoFinnigan).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism and significance levels were evaluated using Bonferroni's Multiple Comparison Test.

RESULTS

OLO Affects the Viability and Number of Human Cancer But Not of Normal Cells

We examined the direct cytotoxic effect of two CDK inhibitors, OLO and ROSC, on normal human (MRC-5 and F2000) as well as cancer (HL-60 and MCF-7) cells. To asses the direct cytotoxicity, we determined the number of viable cells after continuous exposure of cells to the drugs for 24 h at concentrations ranging from 0 to 150 μM by two independent methods: by the flow cytometric dye exclusion test using 7-AAD as a staining agent and measurement of cellular ATP content using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corporation). The cellular ATP level reflects the physiological status of the cells and exactly correlates with the number of living cells. Both CDK inhibitors differentially affected the viability and the proliferation of tested human cells. Exposure of normal human cells (MRC-5 and F2000) to increasing concentrations of OLO for 24 h reduced only negligibly the number of living cells. At a final OLO concentration of 150 μM the number of MRC-5 cells decreased by approximately 10% (Fig. 1A). Unlike OLO, ROSC at low doses markedly affected the viability of normal human cells. At a final concentration of 40 μM ROSC the number of living MRC-5 cells was reduced by 70% (Fig. 1A). The reduction of the cell number after treatment with 40 μM ROSC was statistically very highly significant ($P < 0.001$; Fig. 1B). The determination of the viability of cells by dye exclusion tests revealed that both drugs were not directly cytotoxic for normal human cells. The quantification of the 7-AAD uptake by flow cytometry revealed that only a low number of MRC-5 cells accumulated the dye after exposure to OLO (Fig. 1C). However, OLO strongly affected the viability of exponentially growing human HL-60 leukemia cells. After exposure of HL-60 cells to OLO at a final concentration of 150 μM for 24 h, the number of living cells was reduced by approximately 50% (Fig. 2A). This correlated with a marked increase of 7-AAD uptake (Fig. 1C).

Effect of Both CDK Inhibitors in the Cell Cycle Progression of Normal Human Cells

The cell cycle status of untreated control human MRC-5 fibroblasts largely differed from

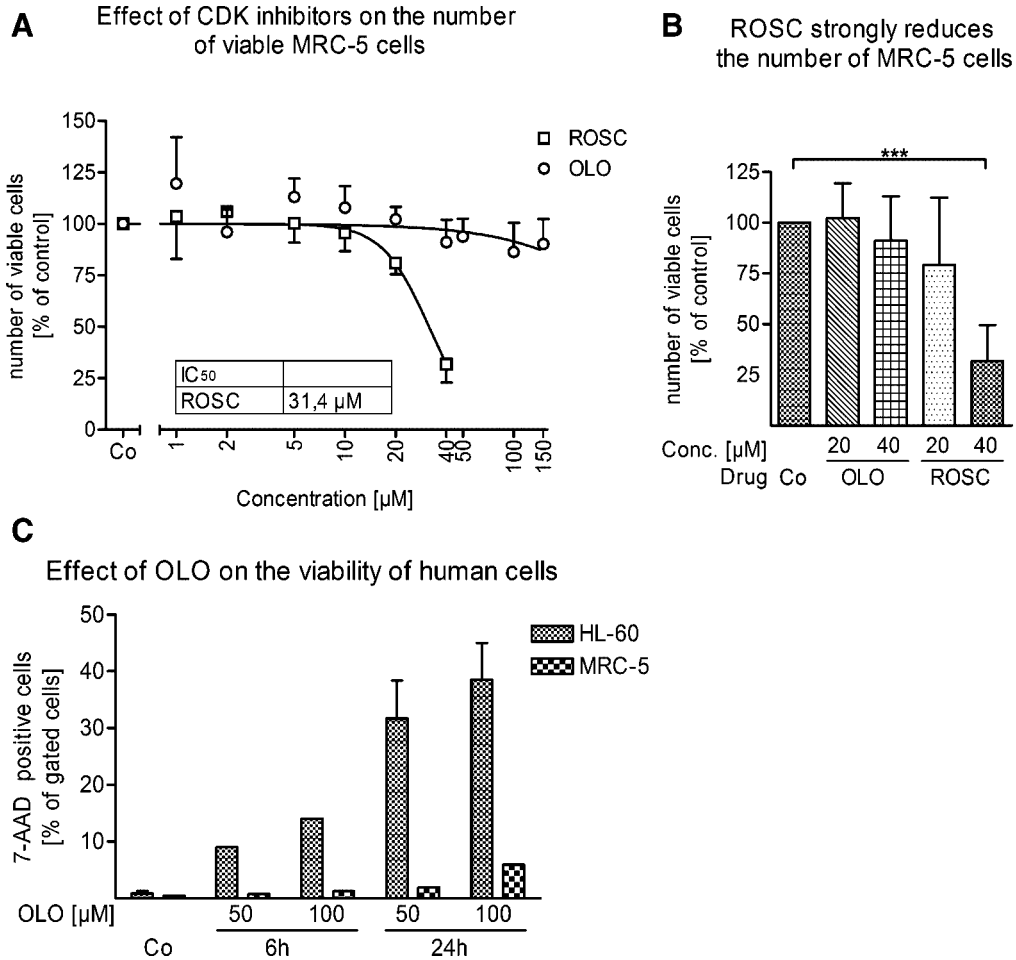

Fig. 1. Reduction of the number of viable cells after treatment with CDKIs. **A**: Unlike ROSC, a higher dose of OLO is necessary to inhibit proliferation of normal human MRC-5 cells. MRC-5 cells plated in 96-well microtiter plates were treated for 24 h with increasing concentrations of OLO, or ROSC. The cellular ATP concentration reflecting the number of viable cells was determined directly after treatment using the CellTiter-Glo Assay. The graph represents mean values from four independent experiments, each performed at least in quadruplicates. $IC_{50} = 31.4$ µM ROSC after 24 h treatment; the IC_{50} for OLO was beyond the tested drug doses. OLO at a final concentration of 300 µM reduced the number of living MRC-5 cells by approximately 20% (data not shown). **B**: Statistical relevance of the ROSC-mediated reduction of the number of living MRC-5 cells. Statistical relevance was determined using Bonferroni's Multiple Comparison Test. The difference of cell number after treatment with 40 µM ROSC compared to the untreated control is statistically very highly significant ($P < 0.001$). **C**: Comparison of 7-AAD uptake in MRC-5 and HL-60 cells exposed to OLO. Control and CDK inhibitors treated MRC-5 and HL-60 cells were collected, PBS-washed and suspended in 500 µl PBS. Thereafter, 5 µl of 7-AAD solution were added. Cells were incubated in the dark for 10 min and the cellular accumulation of fluorescent 7-AAD was measured by flow cytometry.

that of HL-60 cells. Whereas approximately 75% of MRC-5 cells were in the G_1 phase, HL-60 cells were predominantly accumulated in the S-phase. The flow cytometric determination of DNA concentration in single cells revealed that unlike normal human cells (Fig. 3A), HL-60 leukemia cells were strongly affected by OLO at higher concentrations. After exposure to OLO for 8 h the G_1 cell population increased and the ratio of the S-phase cell population decreased. After treatment of HL-60 cells with 150 µM OLO for 24 h a slight increase of cells arrested at the G_2/M transition was observed. Moreover, OLO induced apoptosis. The ratio of the sub-G_1 population increased in a time- and concentration-dependent manner (Fig. 3B). Thus, the flow cytometric analyses not only confirmed the results of the determination of the cell viability but also explained the reasons of the strong diminution of the number of living cells after treatment of HL-60 cells with 150 µM OLO for 24 h.

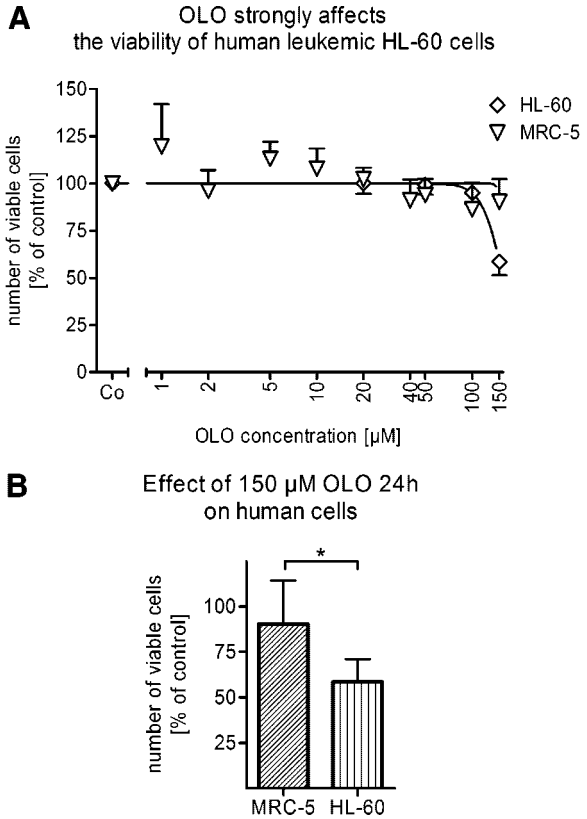

Fig. 2. Exposure of human leukemia HL-60 cells to high dose of OLO negatively affects their viability. MRC-5 and HL-60 cells plated in 96-well microtiter plates were treated for 24 h with increasing concentrations of OLO. The cellular ATP concentration reflecting the number of viable cells and statistical significance was determined as described in detail in Figure 1.

OLO But Not ROSC Strongly Increases Cellular Levels of an Unknown Protein

Surprisingly, the exposure of normal human fibroblasts (MRC-5 and F2000 cells) to 20 μM OLO for 6 h resulted in a strong increase of an unknown protein at approximately 65 kDa. As depicted in Figure 4, administration of OLO but not of ROSC, obviously enhanced in human MRC-5 cells the intensity of the Coomassie-Blue-stained protein band of approximately 65 kDa. After longer OLO treatment this protein further accumulated and at 15 h became the most prominent band as visualized by Coomassie-Blue staining (Fig. 4).

Identification of CLIMP-63 Protein by Mass Spectrometry

To identify the protein very strongly up-regulated after treatment of MRC-5 cells with 20 μM OLO for 15 h, the Coomassie-Blue-stained protein band was excised from the gel, submitted to limited proteolysis by trypsin and the trypsin-generated peptides were analyzed by mass spectrometry. The analysis revealed the CLIMP-63 protein as a major component of the excised, most prominent protein band. The identified protein with an estimated molecular weight of 68 kDa, possessing the ID number Q07065, was the major component of the spot. Moreover, two other minor proteins were detected in the excised band: pro-lamin A at 74 kDa (ID number P02545) and dolichyl-diphospho-oligosaccharide-protein glycosyltransferase at 68 kDa (ID number PO4843). These experiments were repeated three times: the recovery of CLIMP-63 protein in the major band in the samples obtained from MRC-5 cells treated with 20 μM OLO for 15 h varied between 26.9% and 27.9%. Furthermore, the recovery of CLIMP-63 protein in the excised gel slices from samples obtained after exposure to OLO for 6 h was reduced approximately to a quarter. Neither in samples obtained from untreated MRC-5 control cells nor in the lane with the protein marker (as a background control) was the CLIMP-63 protein detectable. Thus, these results show that out of two tested CDK inhibitors only OLO up-regulated CLIMP-63 protein in a time-dependent manner. The highest accumulation of CLIMP-63 protein in this experimental series was observed after exposure to OLO for 15 h (Fig. 4).

A Low Dose of OLO Strongly Enhances Cellular Levels of CLIMP-63 Protein in Untransformed Human Cells

Finally, to substantiate the results of the analysis of the most prominent protein band by mass spectrometry, immunoblotting experiments were performed using whole cell lysates (WCLs) obtained from untreated and OLO-treated human MRC-5 (Fig. 5) and F2000 diploid fibroblasts (not shown). CLIMP-63 protein was detected solely in samples prepared from cells treated with OLO and the strongest signals were found in cells treated for 24 h (Fig. 5). Moreover, the position of the strongly immunoreactive signals generated by anti-CLIMP-63 antibody coincided with the position of the most prominent band visualized on the membrane by Ponceau S staining. Interestingly, anti-CLIMP-63 antibody stained an additional immunoreactive protein band at approximately 120 kDa. This

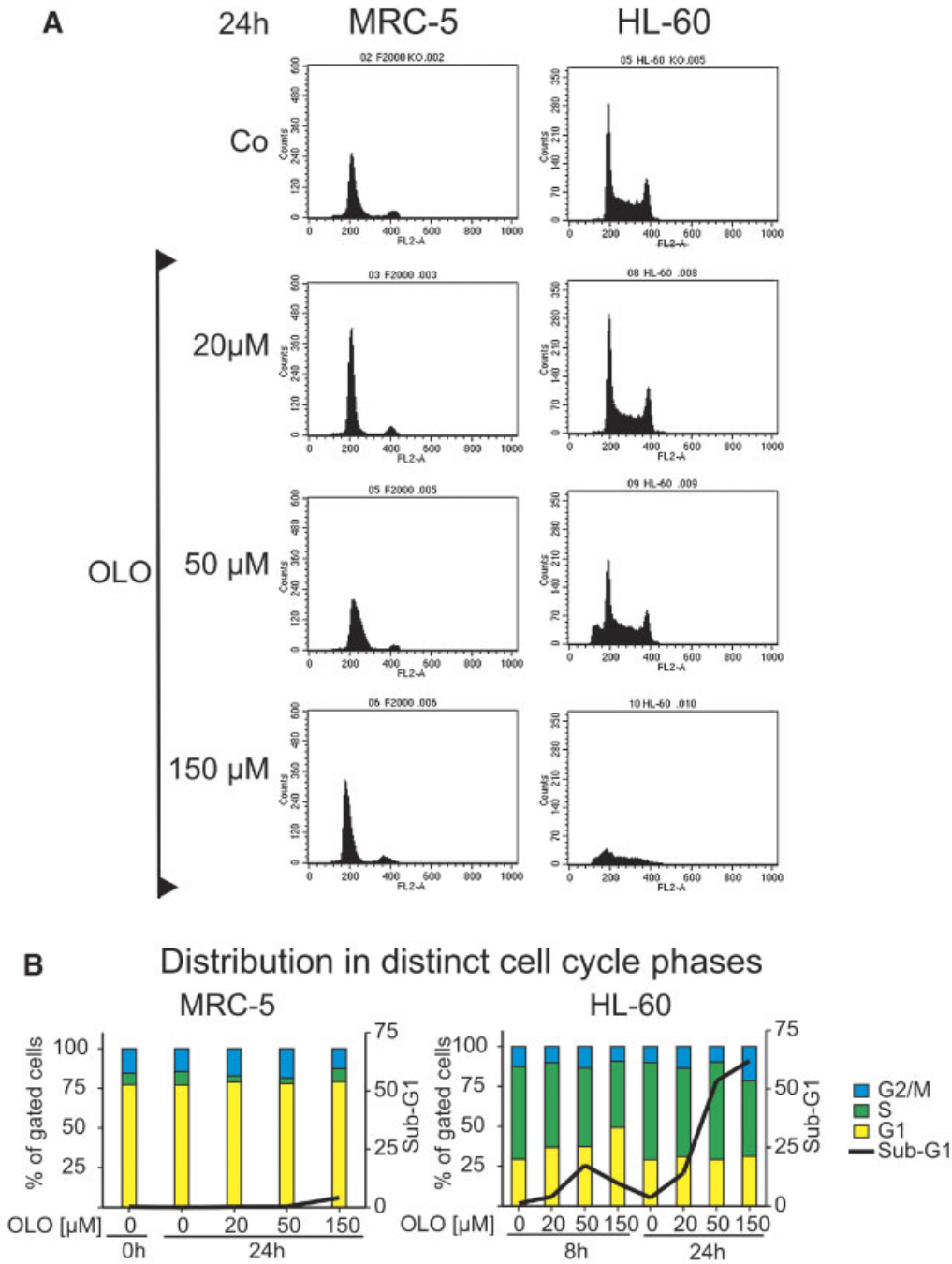

Fig. 3. OLO differentially affects cell cycle progression of human MRC-5 and HL-60 cells. Human MRC-5 and HL-60 cells were treated with OLO as indicated. Thereafter, cells were harvested and stained with propidium iodide. Conditions of staining and measurement as described in the Materials and Methods Section. **A**: DNA histograms depicting a representative experiment performed in duplicate were prepared using the CellQuest evaluation program. **B**: Diagram showing the changed distribution of cells in distinct cell cycle phases and the frequency of sub-G_1 cell population. The distribution was determined using ModFIT cell cycle analysis software. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

band appeared solely in samples accumulating CLIMP-63 protein and considering the capability of CLIMP-63 protein to undergo complex formation it could represent its dimers.

Taken together, the results of the determination of the CLIMP-63 expression by immunoblotting fully confirmed those obtained by protein analysis using mass spectrometry. To

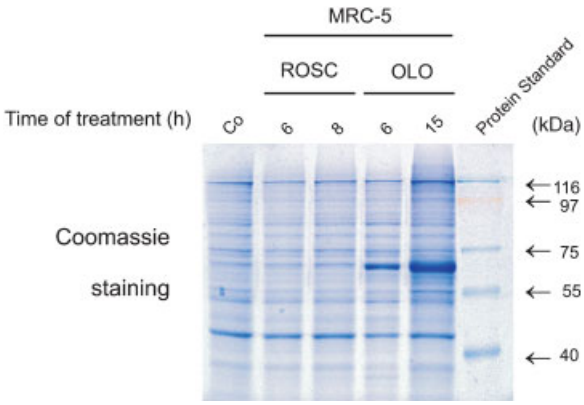

Fig. 4. Strong induction of a 65 kDa protein after OLO treatment. WCLs obtained from MRC-5 control cells and cells treated with ROSC or OLO at a final concentration of 20 μM for indicated periods of time were separated on 10% SDS-slab gels. Gels were fixed and proteins were visualized by Coomassie-Blue staining.

substantiate our observation, we proved the expression and subcellular distribution of CLIMP-63 in MRC-5 cells upon treatment with OLO. As illustrated in Figure 6 (upper panel), a very weak CLIMP-63 signal was detected in cytoplasma of untreated controls. However, upon exposure of MRC-5 cells to OLO a strong cytoplasma staining was detected (Fig. 6, lower panel). The sequential images from confocal microscopy (Fig. 6, lower panel) depict the spatial distribution of the CLIMP-63 protein

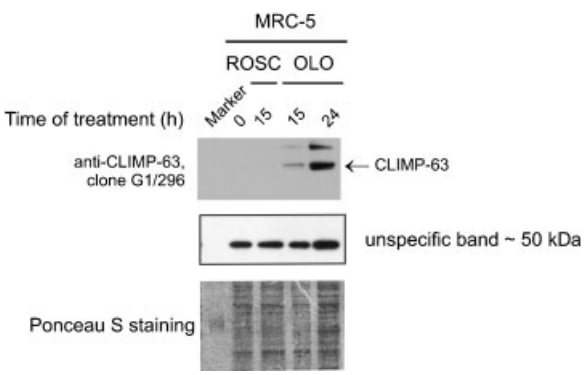

Fig. 5. OLO up-regulates CLIMP-63 protein in MRC-5 cells in a time-dependent manner. WCLs obtained from MRC-5 control cells and cells treated with ROSC or OLO at a final concentration of 20 μM for indicated periods of time were separated on 10% SDS gels and electroblotted onto the PVDF membrane. Protein transmission and loading was checked by Ponceau S staining. Blots were incubated with monoclonal anti-CLIMP-63 antibodies (clone G1/296) at a final dilution of 1:2,000. The immune complexes were detected after incubation with anti-mouse-HRP secondary antibody diluted to a final concentration of 1:20,000 using ECL+ reagent.

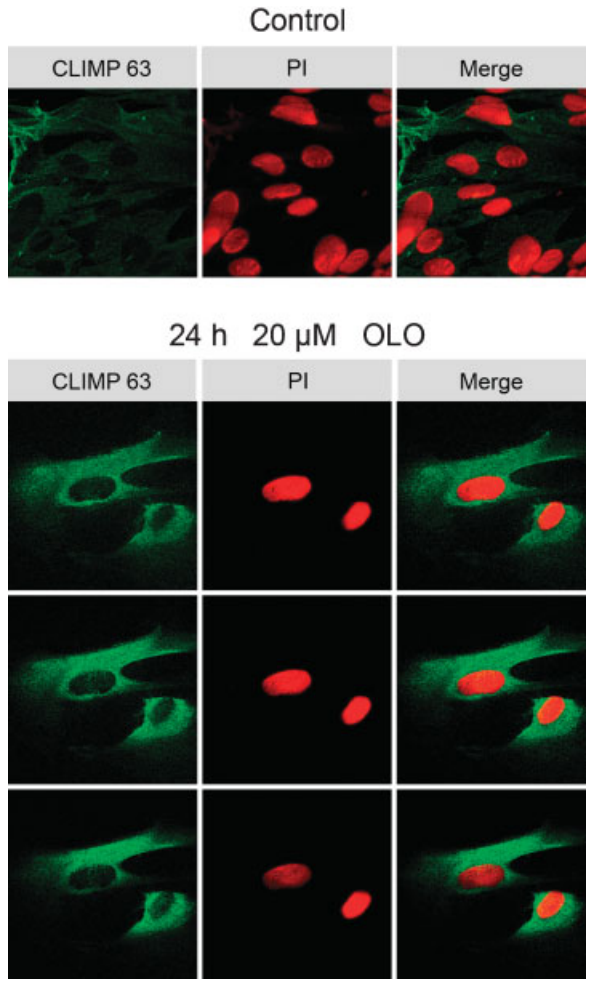

Fig. 6. Strong expression of CLIMP-63 protein in MRC-5 cells after exposure to OLO. Control MRC-5 cells and cells treated with OLO were fixed and stained with anti-CLIMP-63 antibody. The immune complexes were detected using secondary antibodies coupled to fluorochrome Cy2. The chromatin was visualized by propidium iodide staining. Images using filter for FITC and rhodamine were sequentially prepared under confocal microscopy.

up-regulated in human MRC-5 cells after exposure to OLO.

OLO Fails to Induce the Accumulation of CLIMP-63 Protein in Immortalized and Cancer Cells

Exposure of immortalized mouse embryo fibroblasts A-19 (MEFs) as well as human cancer cells (MCF-7 breast cancer or HL-60 leukemia cells) to OLO does not enhance the cellular level of CLIMP-63. Analysis of Coomassie-Blue-stained gels on which WCLs obtained from OLO-treated mouse fibroblasts (Fig. 7A) and human MCF-7 cells (Fig. 8) were resolved, revealed a lack of a detectable accumulation of

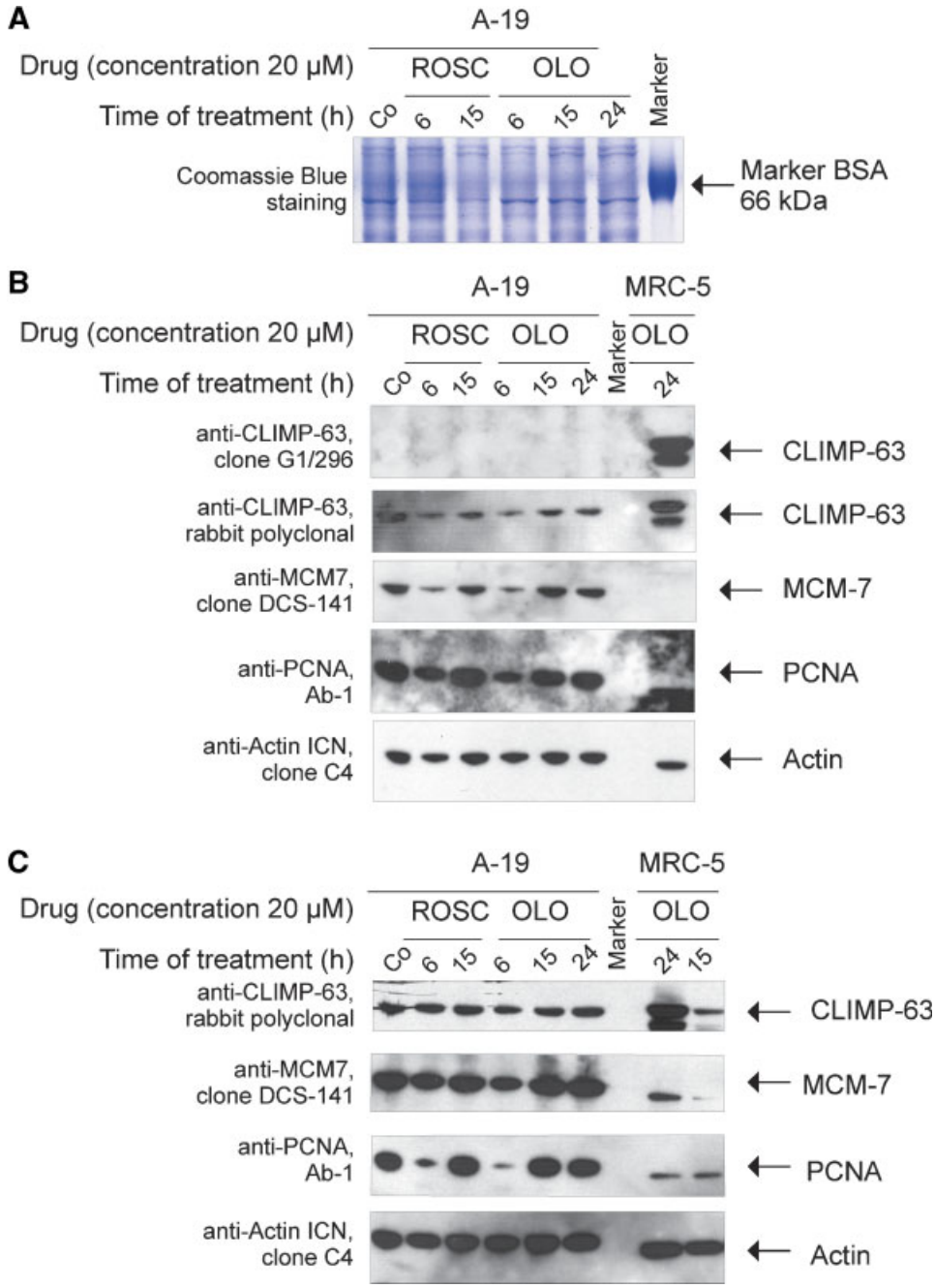

Fig. 7. OLO enhances expression of CLIMP-63 protein in quiescent but not in proliferating cells. WCLs were obtained from immortalized mouse (A-19) and quiescent human MRC-5 fibroblasts. Samples prepared from control cells and cells treated with ROSC or OLO at a final concentration of 20 μM for indicated periods of time were separated on 10% SDS gels, Coomassie-Blue-stained or electroblotted onto the PVDF membrane. Protein electrotransmission and loading was checked by Ponceau S staining. Blots were incubated with monoclonal anti-CLIMP-63 antibodies (clone G1/296) at a final dilution of 1:2,000 or rabbit polyclonal anti-CLIMP-63 antibodies at a final dilution of 1:1,000 [Schweizer et al., 1994, 1995]. The immune complexes were detected after incubation with anti-mouse-HRP or anti-rabbit-HRP secondary antibody diluted to a final concentration of 1:20,000 or 1:10,000, respectively, using ECL+ reagent. **A**: Coomassie-Blue staining. **B**: Membrane was incubated with monoclonal anti-CLIMP-63 antibodies (clone G_1/296) and sequentially with rabbit polyclonal anti-CLIMP-63 antibodies. **C**: A twin blot was directly incubated with rabbit polyclonal anti-CLIMP-63 antibodies. Blots were sequentially probed with other antibodies as indicated. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

a protein band at approximately 65 kDa. Unlike in samples of normal human cells, in samples of immortalized A-19 MEFs neither accumulation of a prominent protein band at p65 kDa after analysis of Coomassie-Blue-stained gels (Fig. 7A), nor up-regulation of CLIMP-63 protein could be detected by immunoblotting (Fig. 7B,C). To avoid false negative results, a sample of OLO-treated human MRC-5 cells was loaded as a positive control. The monoclonal anti-CLIMP-63 antibody G_1/296 heavily stained a protein band at approximately 65 kDa in the sample of MRC-5 cells, but not in samples obtained from mouse A-19 fibroblasts (Fig. 7B). This antibody raised against an antigen of human origin probably fails to recognize its murine counterpart. Therefore, we performed further immunoblots with the rabbit polyclonal anti-CLIMP-63 antibody reacting with the mouse antigen [Schweizer et al., 1994, 1995]. As shown in Figure 7B, sequential incubation of the blot with the polyclonal anti-CLIMP-63 antibodies (lower panel), revealed positive signals in samples of mouse fibroblasts. The intensity of CLIMP-63 protein band in WCLs obtained from untreated control as well as in CDKI-treated A-19 cells was comparable thereby evidencing that OLO did not affect the cellular level of CLIMP-63 protein in mouse A-19 fibroblasts. Direct incubation of the twin blot with the polyclonal CLIMP-63 antibodies (Fig. 7C) substantiated the observation that exposure of mouse A-19 cells to OLO did not change the cellular level of CLIMP-63 protein.

Sequential incubations of the blots showed a marked down-regulation of cellular levels of MCM-7 and PCNA proteins in MRC-5 cells as compared with mouse A-19 fibroblasts. MCM-7, a marker of cycling cells, is absent in cells resting in G_0 phase of the cell cycle. Thus, these results revealed a strong indication that the action of OLO may depend on the cell cycle status of treated cells.

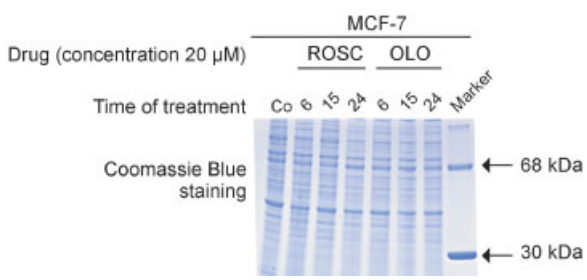

Fig. 8. No induction of CLIMP-63 band in human MCF-7 breast cancer cells. WCLs obtained from untreated human MCF-7 cells and cells treated with ROSC or OLO at a final concentration of 20 μM for indicated periods of time were separated on 10% SDS-slab gels. Gels were fixed and proteins were visualized by Coomassie-Blue staining (for comparison, see Figure 4). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

OLO Induces the Accumulation of CLIMP-63 Protein in Non-Cycling Cells

Unlike mouse A-19 fibroblasts [Wesierska-Gadek et al., 1999], human diploid MRC-5 cells, exhibit a limited proliferative life span when continuously cultivated [Hayflick, 1965]. Therefore, in our further experiments we compared the effect of OLO on human MRC-5 cells differing in their mitotic potential. Cells with increasing population doubling level (PDL) were treated with the drug. As shown in Figure 9, exposure of cycling human MRC-5 fibroblasts (passage no. 37 and 39) to OLO did not induce increase of CLIMP-63 protein. Unlike in dividing cells, in quiescent/senescent cells (passage no. 43) that were characterized by a remarkable down-regulation of cellular levels of MCM-7 protein and PCNA, OLO markedly elevated levels of CLIMP-63 protein. Human MRC-5 fibroblasts at higher number of population doublings cessate to divide and become senescent. It seems that they after approximately 40 population doublings reach terminal and irreversible senescence. After addition of fresh FCS even to a final concentration of 15% or after replating in a fresh medium they were not able to re-enter the active cell cycle (not shown).

DISCUSSION

Substituted purines such as OLO and ROSC are well-characterized CDK inhibitors exhibiting increased selectivity towards CDK2 and CDK7/9 [De Azevedo et al., 1997; Havlicek et al., 1997; Meijer et al., 1997; Gray et al., 1999]. The biological action of OLO is much weaker than that exerted by the structurally related compound ROSC. Whereas ROSC is able to efficiently inhibit proliferation of malignant cells in concentrations ranging from 15 to 40 μM, OLO displays no inhibitory action at comparable concentrations [Schutte et al., 1997; Wesierska-Gadek et al., 2004a]. The comparison of the susceptibility of human untransformed and malignant cells to the action of increasing concentrations of OLO revealed that the drug

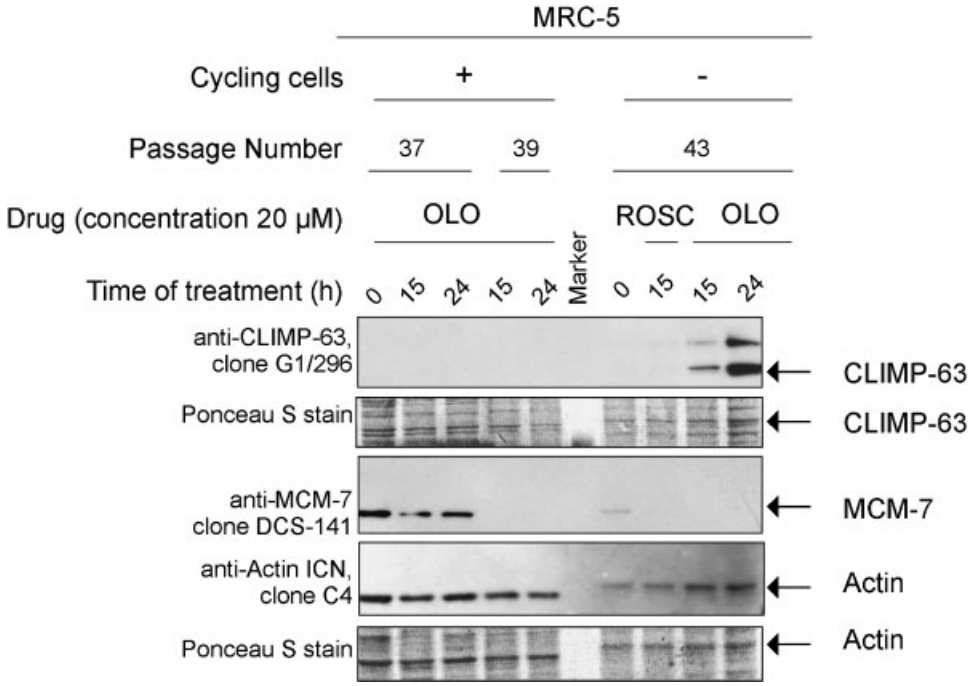

Fig. 9. OLO-mediated accumulation of CLIMP-63 protein in senescent human fibroblast. MRC-5 cells after different number of population doublings were exposed to the drugs. WCLs obtained from untreated human MRC-5 fibroblasts and cells treated with ROSC or OLO at a final concentration of 20 μM for indicated periods of time were separated on 10% SDS-slab gels and electroblotted. Blots were incubated with monoclonal anti-CLIMP-63 antibodies (clone G1/296) and sequentially with anti-MCM-7 antibodies. Conditions of immunoblotting as described in detail in Figure 5. Proper protein electrotransfer and equal protein loading was proved by Ponceau S staining. Region of the blot representing the range of CLIMP-63 was additionally shown. Cycling (in passage no. 37 and 39) as well senescent MRC-5 cells (in passage no. 43) were treated with CDK inhibitors. Addition of fresh FCS or re-plating of cells in a fresh medium containing FCS did not induce cell cycle re-entry of senescent MRC-5 fibroblasts.

administered at higher doses affected solely the proliferation of the latter. The flow cytometric analyses of OLO-treated cells revealed that normal human MRC-5 and F2000 cells were almost completely arrested in G_0/G_1 phase whereas leukemia HL-60 cells responded with an increase of the ratio of G_2/M arrested cells and the induction of apoptosis that became obvious by monitoring of the sub-G_1 population. These results are in concordance with previously published data [Schutte et al., 1997; Alessi et al., 1998; Wesierska-Gadek et al., 2003, 2004a] and explain, at least partially, the reduced susceptibility of normal human fibroblasts to the action of OLO. Moreover, OLO at low doses did not activate wt p53 [Schutte et al., 1997; Wesierska-Gadek et al., 2003].

Surprisingly, OLO at low doses that did not affect cell cycle progression, markedly up-regulated a protein of approximately 65 kDa. The increase of the protein level was so strong that the accumulation of this protein became obvious even by Coomassie-Blue staining that facilitated the identification of the induced protein by mass spectrometry. The assessed sequence revealed that CLIMP-63, a cytoskeleton-linking membrane protein [Schweizer et al., 1993a], was induced by OLO. Interestingly, the semi-quantitative analysis of the protein band corresponding to CLIMP-63 protein in MRC-5 cells by mass spectrometry showed its highest expression at 15–24 h after onset of OLO treatment. These data exactly correlate with the results of immunoblotting.

These unexpected observations raised a number of issues. First, by which mechanism does OLO up-regulate the cellular expression of CLIMP-63 protein in normal human cells? It has been previously reported that distinct CDK inhibitors, for example, 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) [te Poele et al., 1999] or ROSC [Ljungman and Paulsen, 2001; Lu et al., 2001] increase expression of some proteins, for example, of wt p53 tumor suppressor protein through inhibition of RNA synthesis. It has been proposed that the blockage of global transcription leads to down-regulation of MDM-2, a negative p53 regulator,

at both mRNA and protein levels [Lu et al., 2001]. However, considering more recent data [O'Hagan and Ljungman, 2004] and the fact that unlike OLO, ROSC did not affect the levels of CLIMP-63 protein, this explanation seems to be not accurate. Moreover, we reported recently that the inhibition of global transcription is not a general mechanism responsible for up-regulation of short-living proteins after treatment with CDK inhibitors [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a]. We found that ROSC enhanced approximately 40-fold the stability of wt p53 protein in human MCF-7 cells [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a] due to its phosphorylation on Ser46. It has been observed previously that the extent of p53 phosphorylation on Ser46 closely correlates with the extent of its elevation (MCF-7 parental cells vs. MCF-7 cells reconstituted with human caspase-3) and with the extension of the p53 half-life [Wojciechowski et al., 2003; Wesierska-Gadek et al., 2005a]. More recently, the activation of cellular homeodomain protein kinase-2 (HIPK2) in cells exposed to ROSC was reported [Wesierska-Gadek et al., 2007].

In this context it is worth to mention that CLIMP-63 protein, a non-glycosylated type II integral endoplasmic reticulum (ER) membrane component [Schweizer et al., 1995] generating disulfide-linked dimers, is a phosphoprotein and its modification state is regulated in a cell cycle-dependent manner [Vedrenne et al., 2005]. The human CLIMP-63 protein, originally identified and cloned by Schweizer et al. [1993a] consists of 602 amino acids. It contains three main domains: a short NH_2-terminal cytoplasmic segment of 106 amino acids, a single transmembrane domain, and a large extracytoplasmic luminal fragment of 474 amino acids [Schweizer et al., 1993a; Klopfenstein et al., 2001]. The cytoplasmic segment binds to microtubules (MTs) [Klopfenstein et al., 2001]. Interestingly, CLIMP-63 protein is reversibly palmitoylated during mitosis [Schweizer et al., 1993b]. Deletion of the luminal but not cytoplasmic fragment of CLIMP-63 protein abrogated subcompartment-specific localization suggesting that the luminal segment of CLIMP-63 protein is involved in the oligomerization into alpha–helical complexes that excludes it from the nuclear envelope [Schweizer et al., 1993b]. It has been reported that all three domains and the ability to oligomerize are required for the retention of CLIMP-63 protein in an ER-Golgi intermediate compartment [Schweizer et al., 1994]. Although CLIMP-63 protein was first localized to the rough ER of fibroblast-like, epithelial and plasma cells [Schweizer et al., 1995], it has been recently shown to also localize at the cell membrane and to be a functional vascular smooth muscle cell membrane receptor for tissue plasminogen activator [Razzaq et al., 2003] and a receptor for the frizzled-8 protein-related anti-proliferative factor (AIF) [Conrads et al., 2006]. AIF, a low molecular weight sialoglycopeptide that is secreted by bladder cells in patients suffering from chronic bladder disorder termed interstitial cystis markedly inhibits proliferation of both normal bladder epithelial cells as well as bladder carcinoma. The binding of AIF to CLIMP-63 receptor seems to be required for its growth inhibitory action [Conrads et al., 2006].

More recently, it has been reported that CLIMP-63 protein is phosphorylated and that the extent of its phosphorylation increases during mitosis [Vedrenne et al., 2005]. The cytosolic segment of CLIMP-63 protein contains four typical consensus sites for phosphorylation, three of which (Ser3, Ser19, and Ser101) are characteristic protein kinase C sites, and one (Ser17) is a casein kinase II site. Phosphorylation of CLIMP-63 protein seems to control CLIMP-63-mediated anchoring of the ER to MTs [Vedrenne et al., 2005]. In the light of these observations one might speculate that OLO could reduce the phosphorylation state of CLIMP-63 protein or of MTs and thereby contribute to its redistribution [Vedrenne et al., 2005; Nikonov et al., 2007]. It could be a result of a changed balance between kinase and phosphatase activities. Since CLIMP-63 protein during interphase links ER membranes to MTs, it is conceivable that changes of the phosphorylation status of MTs could affect their binding to CLIMP-63. It has been recently shown that the overexpression of a mutated CLIMP-63 protein lacking the MT-binding domain resulted in a marked increase of the mobility of the translocon complexes [Nikonov et al., 2007]. Given the possibility that OLO affects the activity of a distinct kinase or phosphatase that directly or indirectly regulates the phosphorylation status of CLIMP-63 protein or MTs, this effect seems to be specific for OLO, because ROSC did not change expression of CLIMP-63 protein. However, this does not explain how the prevention of the phosphorylation of CLIMP-63

protein might affect its stability. One may speculate that site-specific phosphorylation could determine the half-life of CLIMP-63 protein as in the case of survivin [O'Connor et al., 2000; Wall et al., 2003]. It is also conceivable that prevention of phosphorylation would affect the functional organization of CLIMP-63 protein and in this way increase the efficiency of protein synthesis [Nikonov and Kreibich, 2003].

Furthermore, CLIMP-63 expression is regulated in cell cycle-dependent mode and protein becomes phosphorylated during mitosis [Vedrenne et al., 2005]. Therefore, changing of the phosphorylation status of CLIMP-63 protein could play a quite distinct role in different rapidly proliferating cells but not in non-transformed human fibroblasts that are characterized by low mitotic potential [Wesierska-Gadek et al., 2005b]. Indeed, its cell cycle-dependent regulation is lost in senescent fibroblasts [Pignolo et al., 1998a,b].

One might speculate that prevention of the phosphorylation of CLIMP-63 protein could support the induction of apoptosis in exponentially growing cancer cells. Moreover, the disassembly of the Golgi apparatus occurs during mitosis [Misteli and Warren, 1995; Vedrenne and Hauri, 2006]. Considering the fact that unlike human HL-60 cells, normal human fibroblasts (MRC-5 and F2000) stop to divide after approximately 40 doublings, the status of the Golgi apparatus differs from that in exponentially dividing cells with high mitotic index. One might also speculate that OLO would differentially affect cells with a disassembled Golgi apparatus.

In the context of our results it is noteworthy to mention a previously published paper reporting an increase of the overexpression of LPC-1 (late PDL cDNA-1), a putative transmembrane shock factor, in senescent WI-38 fibroblast-like cells [Pignolo et al., 1998b]. Pignolo and his colleagues found a number of genes differentially expressed between non-proliferating early (quiescent) and late (senescent) cells. Among others they observed a two- to fivefold increase of the mRNA level for LPC-1 [Pignolo et al., 1998b] in serum-starved senescent versus similarly treated young WI-38 cells. Sequence analysis revealed the identity of LPC-1 with CLIMP-63 [Pignolo et al., 1998b]. On the other hand the senescent WI-38 cells fail to express EPC-1, a gene that is induced in young counterparts after serum starvation [Pignolo et al., 1993]. Upon entry into the G_0 state young cells expressed an approximately 100-fold higher level of EPC-1 mRNA than senescent cells under the same experimental conditions [Pignolo et al., 1993]. These observations evidencing that non-proliferating young and senescent cells exhibit differential gene expression program support our observations and implicate that the differential action of OLO on cycling and senescent MRC-5 may be attributable to the changed gene expression patterns resulting in an alteration of stress response, for example, inhibition of cellular CDKs.

Thus, our results evidence for the first time that even weaker pharmacological CDK inhibitors exert strong biological effects on normal quiescent/senescent tissues. The functional consequences of the OLO-mediated increase of cellular level of CLIMP-63 protein in normal healthy cells and their potential exploitation for, for example, protection of normal cells from the undesired side effects of chemotherapy have to be systematically explored.

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REFERENCES

Alessi F, Quarta S, Savio M, Riva F, Rossi L, Stivala LA, Scovassi AI, Meijer L, Prosperi E. 1998. The cyclin-dependent kinase inhibitors olomoucine and roscovitine arrest human fibroblasts in G1 phase by specific inhibition of CDK2 kinase activity. Exp Cell Res 245:8–18.

Banham AH, Turley H, Pulford K, Gatter K, Mason DY. 1997. The plasma cell associated antigen detectable by antibody VS38 is the p63 rough endoplasmic reticulum protein. J Clin Pathol 50:485–489.

Berthet C, Aleem E, Coppola V, Tessarollo L, Kaldis P. 2003. Cdk2 knockout mice are viable. Curr Biol 13:1775–1785.

Conrads TP, Tocci GM, Hood BL, Zhang CO, Guo L, Koch KR, Michejda CJ, Veenstra TD, Keay SK. 2006. CKAP4/

p63 is a receptor for the frizzled-8 protein-related antiproliferative factor from interstitial cystitis patients. J Biol Chem 281:37836–37843.

De Azevedo WF, Leclerc S, Meijer L, Havlicek L, Strnad M, Kim SH. 1997. Inhibition of cyclin-dependent kinases by purine analogues: Crystal structure of human cdk2 complexed with roscovitine. Eur J Biochem 243:518–526.

Fischer PM, Gianella-Borradori A. 2005. Recent progress in the discovery and development of cyclin-dependent kinase inhibitors. Expert Opin Investig Drugs 14:457–477.

Gray N, Detivaud L, Doerig C, Meijer L. 1999. ATP-site directed inhibitors of cyclin-dependent kinases. Curr Med Chem 6:859–875.

Gupta N, Manevich Y, Kazi AS, Tao JQ, Fisher AB, Bates SR. 2006. Identification and characterization of p63 (CKAP4/ERGIC-63/CLIMP-63), a surfactant protein A binding protein, on type II pneumocytes. Am J Physiol Lung Cell Mol Physiol 291:L436–L446.

Havlicek L, Hanus J, Vesely J, Leclerc S, Meijer L, Shaw G, Strnad M. 1997. Cytokinin-derived cyclin-dependent kinase inhibitors: Synthesis and cdc2 inhibitory activity of olomoucine and related compounds. J Med Chem 40:408–412.

Hayflick L. 1965. The limited in vitro lifetime of human diploid cell strains. Exp Cell Res 37:614–636.

Hunter T, Pines J. 1994. Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age. Cell 79:573–582.

Klopfenstein DR, Klumperman J, Lustig A, Kammerer RA, Oorschot V, Haur HP. 2001. Subdomain-specific localization of CLIMP-63 (p63) in the endoplasmic reticulum is mediated by its luminal alpha-helical segment. J Cell Biol 153:1287–1300.

Kozar K, Ciemerych MA, Rebel VI, Shigematsu H, Zagozdzon A, Sicinska E, Geng Y, Yu Q, Bhattacharya S, Bronson RT. et al. 2004. Mouse development and cell proliferation in the absence of D-cyclins. Cell 118:477–491.

Ljungman M, Paulsen MT. 2001. The cyclin-dependent kinase inhibitor roscovitine inhibits RNA synthesis and triggers nuclear accumulation of p53 that is unmodified at Ser15 and Lys382. Mol Pharmacol 60:785–789.

Lu W, Chen L, Peng Y, Chen J. 2001. Activation of p53 by roscovitine-mediated suppression of MDM2 expression. Oncogene 20:3206–3216.

Malumbres M, Sotillo R, Santamaria D, Galan J, Cerezo A, Ortega S, Dubus P, Barbacid M. 2004. Mammalian cells cycle without the D-type cyclin-dependent kinases Cdk4 and Cdk6. Cell 118:493–504.

Marzec M, Kasprzycka M, Lai R, Gladden AB, Wlodarski P, Tomczak E, Nowell P, Deprimo SE, Sadis S, Eck S, et al. 2006. Mantle cell lymphoma cells express predominantly cyclin D1a isoform and are highly sensitive to selective inhibition of CDK4 kinase activity. Blood 108:1744–1750.

Meijer L, Borgne A, Mulner O, Chong JP, Blow JJ, Inagaki N, Inagaki M, Delcros JG, Moulinoux JP. 1997. Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. Eur J Biochem 243:527–536.

Misteli T, Warren G. 1995. Mitotic disassembly of the Golgi apparatus in vivo. J Cell Sci 108:2715–2727.

Nikonov AV, Kreibich G. 2003. Organization of translocon complexes. Biochem Soc Trans 31:1254–1256.

Nikonov AV, Hauri H-P, Lauring B, Kreibich G. 2007. Climp-63-mediated binding of microtubules to the ER effects the lateral mobility of translocon complexes. J Cell Sci 129:2248–2258.

O'Connor DS, Grossman D, Plescia J, Li F, Zhang H, Villa A, Tognin S, Marchisio PC, Altieri DC. 2000. Regulation of apoptosis at cell division by p34cdc2 phosphorylation of survivin. Proc Natl Acad Sci USA 97:13103–13107.

O'Hagan HM, Ljungman M. 2004. Nuclear accumulation of p53 following inhibition of transcription is not due to diminished levels of M DM2. Oncogene 23:5505–5512.

Pignolo RJ, Cristofalo VJ, Rotenberg MO. 1993. Senescent WI-38 cells fail to express EPC-1, a gene induced in young cells upon entry into the G0 state. J Biol Chem 268:8949–8957.

Pignolo RJ, Martin BG, Horton JH, Kalbach AN, Cristofalo VJ. 1998a. The pathway of cell senescence: WI-38 cells arrest in late G1 and are unable to traverse the cell cycle from a true G0 state. Exp Gerontol 33:67–80.

Pignolo RJ, Rotenberg MO, Horton JH, Cristofalo VJ. 1998b. Senescent WI-38 fibroblasts overexpress LPC-1, a putative transmembrane shock protein. Exp Cell Res 240:305–311.

Rane SG, Dubus P, Mettus RV, Galbreath EJ, Boden G, Reddy EP, Barbacid M. 1999. Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in beta-islet cell hyperplasia. Nat Genet 22:44–52.

Razzaq TM, Bass R, Vines DJ, Werner F, Whawell SA, Ellis V. 2003. Functional regulation of tissue plasminogen activator on the surface of vascular smooth muscle cells by the type-II transmembrane protein p63 (CKAP4). J Biol Chem 278:42679–42685.

Schutte B, Nieland L, van Engeland M, Henfling ME, Meijer L, Ramaekers FC. 1997. The effect of the cyclin-dependent kinase inhibitor olomoucine on cell cycle kinetics. Exp Cell Res 236:4–15.

Schweizer A, Ericsson M, Bachi T, Griffiths G, Hauri HP. 1993a. Characterization of a novel 63 kDa membrane protein. Implications for the organization of the ER-to-Golgi pathway. J Cell Sci 104:671–683.

Schweizer A, Rohrer J, Jeno P, DeMaio A, Buchman TG, Hauri HP. 1993b. A reversibly palmitoylated resident protein (p63) of an ER-Golgi intermediate compartment is related to a circulatory shock resuscitation protein. J Cell Sci 104:685–694.

Schweizer A, Rohrer J, Hauri HP, Kornfeld S. 1994. Retention of p63 in an ER-Golgi intermediate compartment depends on the presence of all three of its domains and on its ability to form oligomers. J Cell Biol 126:25–39.

Schweizer A, Rohrer J, Slot JW, Geuze HJ, Kornfeld S. 1995. Reassessment of the subcellular localization of p63. J Cell Sci 108:2477–2485.

Sedlacek HH. 2001. Mechanisms of action of flavopiridol. Crit Rev Oncol Hematol 38:139–170.

Senderowicz AM. 2003. Small-molecule cyclin-dependent kinase modulators. Oncogene 22:6609–6620.

Senderowicz AM. 2004. Targeting cell cycle and apoptosis for the treatment of human malignancies. Curr Opin Cell Biol 16:670–678.

te Poele RH, Okorokov AL, Joel SP. 1999. RNA synthesis block by 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) triggers p53-dependent apoptosis in human colon carcinoma cells. Oncogene 18:5765–5772.

van den Heuvel S, Harlow E. 1993. Distinct roles for cyclin-dependent kinases in cell cycle control. Science 262: 2050–2054.

Vedrenne C, Hauri HP. 2006. Morphogenesis of the endoplasmic reticulum: Beyond active membrane expansion. Traffic 7:639–646.

Vedrenne C, Klopfenstein DR, Hauri HP. 2005. Phosphorylation controls CLIMP-63-mediated anchoring of the endoplasmic reticulum to microtubules. Mol Biol Cell 16: 1928–1937.

Vermeulen K, Van Bockstaele DR, Berneman ZN. 2003. The cell cycle: A review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif 36:131–149.

Vindelov LL, Christensen IJ, Nissen NI. 1983. A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry 3:323–327.

Wall NR, O'Connor DS, Plescia J, Pommier Y, Altieri DC. 2003. Suppression of survivin phosphorylation on Thr34 by flavopiridol enhances tumor cell apoptosis. Cancer Res 63:230–235.

Wesierska-Gadek J, Schmid G. 2000. Overexpressed poly (ADP-ribose) polymerase delays the release of rat cells from p53-mediated G(1) checkpoint. J Cell Biochem 80: 85–103.

Wesierska-Gadek J, Schmid G. 2006. Dual action of the inhibitors of cyclin-dependent kinases: Targeting of the cell-cycle progression and activation of wild-type p53 protein. Expert Opin Investig Drugs 15:23–38.

Wesierska-Gadek J, Wang Z-Q, Schmid G. 1999. Reduced stability of regularly spliced but not alternatively spliced p53 protein in PARP-deficient mouse fibroblasts. Cancer Res 59:28–34.

Wesierska-Gadek J, Schloffer D, Kotala V, Horky M. 2002. Escape of p53 protein from E6-mediated degradation in HeLa cells after cisplatin therapy. Int J Cancer 101:128–136.

Wesierska-Gadek J, Gueorguieva M, Horky M. 2003. Dual action of cyclin-dependent kinase inhibitors: Induction of cell cycle arrest and apoptosis. A comparison of the effects exerted by roscovitine and cisplatin. Pol J Pharmacol 55:895–902.

Wesierska-Gadek J, Gueorguieva M, Wojciechowski J, Horky M. 2004a. Cell cycle arrest induced in human breast cancer cells by cyclin-dependent kinase inhibitors: A comparison of the effects exerted by roscovitine and olomoucine. Pol J Pharmacol 56:635–641.

Wesierska-Gadek J, Schloffer D, Gueorguieva M, Uhl M, Skladanowski A. 2004b. Increased susceptibility of poly(ADP-ribose) polymerase-1 knockout cells to anti-tumor triazoloacridone C-1305 is associated with permanent G2 cell cycle arrest. Cancer Res 64:4487–4497.

Wesierska-Gadek J, Gueorguieva M, Horky M. 2005a. Roscovitine-induced up-regulation of p53AIP1 protein precedes the onset of apoptosis in human MCF-7 breast cancer cells. Mol Cancer Ther 4:113–124.

Wesierska-Gadek J, Wojciechowski J, Ranftler C, Schmid G. 2005b. Role of p53 tumor suppressor in ageing: Regulation of transient cell cycle arrest and terminal senescence. J Physiol Pharmacol 56:15–28.

Wesierska-Gadek J, Schmitz ML, Ranftler C. 2007. Roscovitine-activated HIP2 kinase induces phosphorylation of wt p53 at Ser-46 in human MCF-7 breast cancer cells. J Cell Biochem 100:865–874.

Wojciechowski J, Horky M, Gueorguieva M, Wesierska-Gadek J. 2003. Rapid onset of nucleolar disintegration preceding cell cycle arrest in roscovitine-induced apoptosis of human MCF-7 breast cancer cells. Int J Cancer 106:486–495.

Yuan J, Yan R, Kramer A, Eckerdt F, Roller M, Kaufmann M, Strebhardt K. 2004. Cyclin B1 depletion inhibits proliferation and induces apoptosis in human tumor cells. Oncogene 23:5843–5852.