"CELL DIFFERENTIATION, CASPASE INHIBITION AND MACROMOLECULAR SYNTHESIS BLOCKAGE, BUT NOT BCL-2 OR BCL-XL PROTEINS, PROTECT SH-SY5Y CELLS FROM APOPTOSIS TRIGGERED BY TWO CDK INHIBITORY DRUGS."

Judit Ribas and Jacint Boix#

Molecular Pharmacology Group, Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina, Universitat de Lleida. Av. Rovira Roure, 44. 25198-Lleida. Catalunya. SPAIN.

#To whom correspondence should be addressed:

Jacint Boix MD PhD
Molecular Pharmacology Group,
DCMB, Univ. of Lleida, School of Med.
Av. Rovira Roure, 44
25198-Lleida, SPAIN

Phone: +34-973702404
Fax: +34-973702426
E-mail: jacint.boix@cmb.udl.es

Abstract

Olomoucine and Roscovitine are two ATP-competing compounds described as specific inhibitors of cyclin-dependent kinases (CDK). Both drugs showed to induce apoptosis in SH-SY5Y, a neuroblastoma-derived cell line. In these cells, neither Bcl-2 nor Bcl-XL overexpression conferred any resistance to both drugs. However, a partial protective effect was detected when cells were treated with a general inhibitor of caspases (zVADfmk), cycloheximide (CHX) or actinomycin D (DAct). Interestingly, a synergism in cell protection was observed between zVADfmk and the macromolecular synthesis inhibitors, thus suggesting different apoptotic pathways in distinct subpopulations of the cell culture. On the other hand, no lethality was found when cells were treated with either PD98059 or UO126. This discarded Erk1/Erk2 inhibition as the cause of apoptosis. Furthermore, SH-SY5Y cells became resistant to either Olomoucine or Roscovitine upon the induction of differentiation. This resistance correlated with the extent of differentiation and, therefore, the number of cells entering a quiescent state. In conclusion, our results seem to support a role for CDK inhibition as the cause of the apoptotic process triggered by Olomoucine and Roscovitine. In addition, we contribute to define a promising profile as anticancer drugs for both compounds, at least in the treatment of neuroblastoma.

Key words: Olomoucine, Roscovitine, CDK, Apoptosis, Bcl-2, Bcl-XL, Caspase, Cycloheximide, Erk, SH-SY5Y.

Introduction

Protein kinases are pivotal elements in the regulation of cellular functions. The human genome is estimated to contain 518 protein kinases grouped in 7 major evolutionarily defined families [1]. Most human pathologies are the result of cellular dysfunction. Protein kinases are involved in either the genesis or the modulation of most of these dysfunctions. As a consequence, protein kinases become an obvious target for the design of new drugs. Cyclin-dependent kinases (CDK) are a family of protein kinases involved in the ordered succession of the cell cycle phases. CDK are controlled by several independent mechanisms such as the binding of positive and negative regulatory proteins, the state of phosphorylation and the location inside the cell [2]. Their pharmacological modulation is approached with increasing interest by the drug industry. Based on an ATP competing mode of action, several drugs are presently available to inhibit CDK. These drugs display a variable degree of specificity [3-5]. Staurosporine (STS), for instance, is an unspecific inhibitor of protein kinases with a spectrum that includes CDK. Other compounds, like UCN-01 or Flavopiridol, have reached clinical phases of development as anticancer drugs. Flavopiridol is the best example of a first generation of inhibitors, specific for CDK but with a low discriminating power among CDK subtypes. A second generation of compounds with increased specificity towards a precise set of CDK has been also synthesized. Olomoucine and Roscovitine belong to this second generation and show "in vitro" specificity for CDK1, CDK2 and CDK5 [6,7].

Neoplasia is characterized by an uncontrolled cell proliferation. In this context, the pharmacological inhibition of CDK implies the stop of the cell cycle and a brake to cell proliferation [8]. This can be defined as a cytostatic effect. In addition, CDK inhibitory drugs could induce cell differentiation. STS, for instance, stimulates cell differentiation of neuroblastoma [9]. Cell differentiation is a mechanism that opposes to cancer development. Therefore, a differentiation effect must be considered in these drugs. Finally, these drugs are effective in triggering apoptosis [4,8]. As a consequence, a cytocidal effect completes a promising profile as anticancer agents. Surprisingly, there are few studies approaching the cell and molecular mechanisms involved in the induction of apoptosis by this type of drugs.

Apoptosis is a specific type of cell death. Apoptosis was initially defined on a morphological basis, in brief cell shrinking and fragmentation, chromatin condensation and phagocytosis by neighboring or specialized cells. Apoptosis is an ordered process, an orchestrated succession of cell and molecular events shared by most experimental paradigms of the phenomenon. In the process we can distinguish three phases named induction, execution and resolution. The resolution phase is the latest one and comprises the cell fragmentation and phagocytosis phenomena. The execution phase is defined by caspase activation [10,11]. Caspases are a family of proteases characterized by having a cysteine in their catalytic center and cleaving proteins at aspartic residues. As a consequence the acronym caspase was coined [12]. The intracellular activation of caspases causes specific proteolysis in a specific set of proteins that translates into the

cell apoptotic phenotype [13]. The mechanisms that lead to caspase activation have been classified into two pathways, the intrinsic and the extrinsic pathway. The extrinsic pathway is initiated in membrane receptors, Fas or TNF-R1 for instance, named death receptors (DR). Upon binding of their specific ligands, these receptors transduce their death signal by means of a caspase, mainly caspase 8 [14]. Therefore, the pharmacological inhibition of Caspase 8 implies the blockage of the death signal and cells are able to survive facing killer ligands like Fas-L or TNF. The intrinsic pathway is defined on the basis of mitochondrial implication. Mitochondria seem to act as a cell damage sensor that releases a set of proteins, kept in their intermembrane space, upon stressing stimuli. Cytochrome c is one of such proteins and acts by promoting the apoptosome assembly and the specific activation of caspase 9 [15]. In this context, the pharmacological inhibition of caspases blocks most of the characteristic features of apoptosis, but does not avoid cell death [9]. These mitochondrial events are regulated by the Bcl-2 family of proteins [16]. These proteins contain homology domains that allow reciprocal interactions and specific differences of affinity can be found among them. Some members are Bcl-2 agonists and act by preventing apoptosis (Bcl-XL, Bcl-w, Mcl-1, etc.). The others (Bax, Bak, Bid, etc.) behave as antagonists and promote it. Bcl-2 and Bcl-XL are able to inhibit the translocation of cytochrome c to the cytoplasm. As a consequence, their overexpression in a cell implies an increased resistance to stimuli activating the intrinsic pathway.

The induction of apoptosis by means of the pharmacological inhibition of CDK is the focus of our research interests. Little is known about the molecular events that take place and connect cell detection of CDK dysfunction and apoptosis. In order to investigate these mechanisms, we decided to use Olomoucine and Roscovitine, two CDK inhibitory drugs. As a cell model, we chose SH-SY5Y, a cell line derived from a human neuroblastoma. One reason was our previous experience in characterizing the apoptotic phenotype and the molecular mechanisms triggered by STS in these cells [9,17,18]. Another reason was their remarkable capability to differentiate in cell culture conditions [9,19]. In these cells, Olomoucine and Roscovitine behaved as effective inductors of apoptosis. In order to elucidate the biochemical pathways involved, we examined the effects of Bcl-2 and Bcl-XL overexpression and were null. In addition, we tested caspase inhibition and the blockage of new protein expression. Either action reduced apoptosis and displayed synergism in doing so. Finally, the induction of differentiation in the cell cultures minimized the ratios of cell death and apoptosis. The implications of these results envisaging cell cycle phases, apoptosis and cancer therapy will be discussed further.

Materials and methods

Antibodies and chemical reagents

AntiBcl-2 (clone 124) monoclonal antibody was purchased from DAKO (Glostrup, Denmark). AntiBcl-XL (Catalog # B22630) monoclonal antibody was acquired from

Transduction Laboratories (Lexington, KY, USA). AntiRb (clone G3-245) was provided by BD Biosciences (Madrid, Spain). AntiβActin (clone AC-15) and secondary peroxidase conjugated antibodies were supplied by Sigma (St. Louis, MO, USA). Olomoucine, Iso-Olomoucine, Roscovitine, PD98059 and Geneticin (G418) were purchased from Calbiochem (San Diego, CA, USA). UO126 was purchased from Tocris (Bristol, UK). zVADfmk (Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) and AcDEVDafc (Acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin) were obtained from Enzyme Systems Products (Livermore, CA, USA). zVADfmk (N-1510) is presently being obtained from Bachem (Bubendorf, Switzerland). zVADfmk from Enzyme Systems Products was used at a concentration 50μM. zVADfmk from Bachem requires a concentration 100μM to yield equivalent results. Fetal calf serum was supplied by Life Technologies/Gibco BRL (Barcelona, Spain). CellTiter 96® and Cytotox 96® kits were provided by Promega (Madison, WI, USA). TUNEL reactions were performed with the *In situ* Cell Death Detection kit, Fluorescein, from Roche (Barcelona, Spain). Culture media, STS, bisBenzimide (Hoechst 33342), propidium iodide, CHX and DAct were purchased from Sigma (St. Louis, MO, USA). Unless otherwise stated, the non-listed reagents were also from Sigma.

Cell lines, specific culture conditions and drug treatments

SH-SY5Y cells were grown as reported before [9,17]. SH-SY5Y populations transfected with the pcDNA3/Bcl-2, pcDNA3/Bcl-XL and empty pcDNA3 vectors were

obtained and characterized in previous research projects [9]. We have observed a reduced proliferation in Bcl-2 overexpressing cells, this implied a growing advantage for Bcl-2 non-expressing cells. In order to keep overexpression in most cells, G-418 was permanently present in cultures unless the experiment was terminal. In addition, Bcl-2 levels were routinely checked by Western Blot. A similar strategy was applied to Bcl-XL overexpressing and pcDNA3/empty control populations. Along our experiments, cell cultures were periodically checked for mycoplasm contamination and were repeatedly negative by PCR assays. To induce differentiation in SH-SY5Y cells, we followed an established procedure [19]. In brief, cells were cultured on collagen coated plates and 10μM RA (*all-trans*-Retinoic Acid) was added for five days. RA was purchased from Tocris (Bristol, UK). Culture dishes and other plastic disposable tools were supplied by Corning (Corning, NY, USA), Sarstedt (Newton, NC, USA), Bibby Sterilin (Staffordshire, UK) and Becton Dickinson (Franklin Lakes, NJ, USA). Olomoucine and Iso-Olomoucine were dissolved in DMSO at a concentration 50mM. Roscovitine and zVADfmk were prepared with DMSO at a concentration 20mM. Finally, STS was also adjusted with DMSO to 1mM. From these working stock solutions, drugs were added to complete media. Then serial dilutions were performed in order to obtain the final concentrations reported in the text and figures. The resulting DMSO concentration never surpassed 1% in the medium. At this concentration we have determined DMSO does not influence SH-SY5Y cell viability, differentiation or neurite outgrowth. CHX and DAct were soluble in culture media and adjusted to the final concentrations reported also in text and figures.

Cell viability and cell death determinations

CellTiter 96® kit provides the MTS reagent, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt. MTS is a water-soluble tetrazolium salt, that permeates cell membranes and is chemically reduced to a colored formazan salt inside metabolically competent cells. This fact allows the determination of viability in cell cultures. MTS derived formazan salt is characterized by being water-soluble, thus circumventing cell lysis or DMSO dissolving steps required in the similar MTT procedure [17]. The assay is devised to be applied to multi-well plates, thus easing the performance of many independent determinations and further statistical analysis. We plated 40.000 SH-SY5Y cells/well in 96 multi-well plates (M96), routinely. Color changes were quantified by means of an ELISA plate reader after 24 hours of drug treatment. Final values were the result of subtracting 620nm from 490nm lectures. Lectures proved to be linear following 3-6 hours of incubation in MTS. Each experimental condition was systematically tested in several wells and each experiment repeated several times, as stated in the figure legends. Cell viability ratios were obtained by referring values from treated to those of untreated, control cultures.

Cytotox 96® kit provides the reagents and standards to determine lactate deshydrogenase activity (LDH) in culture media or cell lysates. LDH enzyme is released from cells only upon membrane rupture, as a consequence its measurement quantifies death ratios in cell cultures. The assay is also devised to be performed in multi-well

plates. In the LDH assays, we plated M96 with 30.000 SH-SY5Y cells/well. Following 48 hours of drug treatment, LDH release was determined according to the kit instructions. In apoptosis, the LDH release is a later event than the decrease of MTS reduction. As a consequence, only at 48 hours of drug treatment LDH determinations were consistent with MTS ones, which were performed at 24 hours. The increase in LDH was detected as a change in color, that was quantifiable by an ELISA plate reader. Final values were also the result of subtracting 620nm from 490nm lectures. Each experimental condition was systematically tested in several wells and each experiment repeated several times, as stated in the figure legends. Cell death ratios were obtained by referring the LDH values in the medium of a treated culture to the total LDH content of an equally treated control culture. The total LDH content is the result of cell lysis by addition of Triton X-100 to the culture medium, at a final concentration of 0.9%. The LDH value of complete medium and complete medium plus 0.9% Triton X-100 were subtracted from the corresponding values. The cell death percentage found in untreated cultures was averaged and subtracted from the final death ratios.

Apoptosis assessment

We have used five different criteria to asses apoptosis in cell cultures. First, nuclear morphology as evidenced by direct 0.05 μg/ml bisBenzimide (Hoechst 33342) staining and fluorescence microscopy. Frequently, bisBenzimide/propidium iodide double staining was performed. In this case, propidium iodide was added at a final

concentration of 25μg/ml. Second, the large DNA fragments of approximately 50 kbp (kilobase pairs). This DNA fragmentation was detected by means of a reported procedure [20]. However, we used a contour homogeneous electric field (CHEF) system (BioRad, Hercules, CA, USA) to perform the agarose gel electrophoresis of the DNA samples. The running conditions were 15 hours, at 6 V/cm, initial switching time of 50 seconds and final switching time of 90 seconds. Third, the internucleosomal DNA fragmentation. The ladder pattern of DNA degradation was easily detected by means of an standard electrophoresis in 1.5% agarose gels. Fourth, the surge and increase of effector caspase activity. We employed a procedure based on reading the fluorescence released from the AcDEVDafc synthetic substrate after its direct addition to the culture medium, detergent cell lysis and incubation at 37°C. This method allows measurements in a M96 format and has been validated in our previous works [9,18]. As a minor improvement, Nonidet P-40 detergent has been reduced to 1.6% in the 2X lysis buffer. Finally, TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP Nick End-Labelling) assays were performed according to the kit instruction manual. Flow cytometry readings were obtained by means of an EPICS® XL unit from Coulter Científica, SA (Madrid, Spain).

Cell cycle analysis

Cell cultures were kept on ice and incubated for 15 minutes in 25μg/ml propidium iodide, 10μg/ml RNase A and 0,1% Triton X-100. Then, cell suspensions were subjected to analysis in the flow cytometer referred before.

Protein extractions and Western Blotting

To study cytochrome c release from mitochondria, a 0.05% digitonin cytosolic extraction was performed according to a reported procedure [21]. The remaining mixture of cell constituents (nuclei, mitochondria and other organelles) was extracted as will be next described for whole cells. To obtain whole cell extracts, cells were washed with phosphate buffered saline (PBS), lysed in a lysis-buffer containing 100 mM Tris/ClH pH 6.8, 2% SDS, 1 mM EDTA, 1 mM PMSF, 2 μg/ml Aprotinin, 1 μg/ml Leupeptin and 1 μg/ml Pepstatin, sonicated, and boiled for 5 min. Following a centrifugation at 12000 g for 15 minutes, the protein concentration was determined in the supernatants by means of the DC Protein Assay from BioRad. These supernatants were subjected to electrophoresis in SDS 7-12% polyacrylamide gels. Based on the protein assay, equal amounts of protein were loaded per lane. Then, proteins were transferred to PVDF membranes (Amersham, Buckinghamshire, UK) by means of a MINI® trans-blot module from BioRad. Membranes were reacted with the primary antibodies described above. Immunoblots were finally developed with the appropriate peroxidase labeled secondary antibody and either the SuperSignal® West Pico or the SuperSignal® West Dura reagents from Pierce (Rockford, IL, USA).

Results

Characterization of the cell death process induced by Olomoucine and Roscovitine in SH-SY5Y cells

In order to characterize the lethal effects of Olomoucine and Roscovitine on the SH-SY5Y cell line, cells were cultured on M96 and subjected to serial dilutions of these drugs. After 24 hours of treatment, cell viability was determined by measuring the ability to reduce MTS (Fig. 1A). Olomoucine and Roscovitine induced cell death in a concentration-dependent manner. The drug concentration lethal for 50% of the cell population (LC_{50}) was 127,5μM and 25μM, respectively. This was consistent with the "in vitro" reports about Roscovitine being a more potent CDK inhibitor than Olomoucine [3,4]. Concentrations had to be raised to 200μM Olomoucine or 50μM Roscovitine to kill most of the cell population after one day of treatment. Therefore, these higher concentrations have become the most frequently employed in our studies. On the other hand, Iso-Olomoucine is an isomer of Olomoucine that causes “in vitro” CDK inhibition at concentrations far greater than Olomoucine. As it is shown in Fig. 1A, Iso-Olomoucine did not cause cell death in the same range of Olomoucine concentrations. As a consequence, Iso-Olomoucine has become the most appropriate negative control in our experiments.

As we have commented before, cell viability measurements were based on determinations of the cell ability to reduce MTS. Since cell reduction potential can decrease by other contingencies not related to cell death, all along our experiments we

decided to confirm our MTS data by an independent procedure. The chosen procedure was the quantification of the LDH activity released into the culture medium (Fig. 1B). This parameter indicates cell membrane rupture and, unequivocally, cell death. Though it is also adapted to M96 assays like the MTS method, there is a difference to be noted between both procedures. In the MTS procedure, the values from treated cultures can be only referred to those of control untreated ones in order to obtain the percentage of cell viability. In the LDH procedure, the values of LDH released in the treated cultures can be referred to the total LDH content of analogously treated cultures, where total LDH has been released by means of cell lysis. This limits the number of determinations to be performed per plate and reduces the feasibility to study the broad range of concentrations attainable with the MTS procedure (compare Fig. 1A and 1B, for example). However, LDH method allows the direct calculation of cell death percentages. A parameter that is complementary to the cell viability one obtained with the MTS procedure. Along our experiments, LDH and MTS procedures yielded complementary values in a reproducible and consistent manner.

Once we had standardized the concentration and time parameters for cell death induction, we proceeded to characterize the type of cell death triggered by Olomoucine and Roscovitine in SH-SY5Y cells. First, we characterized the nuclear morphology of cells by means of chromatin staining with bisBenzimide and fluorescence microscopy. As shown in Fig. 2A, Olomoucine and Roscovitine generated the reduced size and condensed nuclei, progressively more fragmented, typical of apoptosis. These images

were not found in cultures of SH-SY5Y cells treated with Iso-Olomoucine. Then, we analyzed the DNA of Olomoucine, Roscovitine and Iso-Olomoucine treated cells in agarose gel electrophoresis under a continuos or a contour-clamped homogeneous electric field (CHEF). As expected, Olomoucine and Roscovitine generated the internucleosomal DNA degradation and the 50 kbp fragments characteristic of apoptosis (Fig. 2B). In order to assess the mitochondrial involvement in this apoptotic process, we characterized the cytoplasmic translocation of cytochrome c. After nine hours of treatment, cells treated with Olomoucine (Fig. 2C) and Roscovitine (not shown) but not Iso-Olomoucine displayed cytochrome c in a cytosolic fraction. The subsequent activation of effector caspases was tested by evidencing the specific proteolysis of several substrates like poly-ADPribose polymerase (PARP), Fodrin and Lamin B1 in Western Blots (not shown). Alternatively, we followed a more quantitative approach by determining the activation kinetics of effector caspases 3 and 7. The surge and extent of DEVD cleaving activity (DEVDase activity) was measured by means of the fluorescence released, upon cleavage, from a synthetic substrate (AcDEVDafc). As shown in Fig. 2D, a linear increase in DEVDase activity was found up to 9 hours of drug treatment. Olomoucine and Roscovitine kinetics did not differ significantly. However, as expected, Iso-Olomoucine kept DEVDase activity at basal levels. In conclusion, all the apoptotic features, we had so far checked, were positive. Olomoucine and Roscovitine caused cell death by inducing apoptosis in SH-SY5Y cells.

MTS and LDH procedures are characterized to quantify all types of cell death and not only the apoptotic type. The characterization of apoptosis we have just described is basically qualitative. Therefore, a matter of concern was if a significant amount of cells were undergoing a non-apoptotic type of cell death. As we have mentioned before, we have routinely performed bisBenzimide/propidium iodide double staining and propidium iodide had never evidenced a significant population of dead cells without apoptotic features. In order to definitively discard this issue, we quantified apoptosis by counting individual cells by flow cytometry. The TUNEL procedure was chosen to label the apoptotic cells. As shown in Fig. 2E, barely all cells became TUNEL positive after 48 hours of treatment. In other words, they had undergone apoptotic cell death.

Effects of Bcl-2 and Bcl-XL overexpression on Olomoucine and Roscovitine induced apoptosis

As we have commented before, Bcl-2 and Bcl-XL overexpression is a very effective resource to prevent apoptosis in many experimental paradigms, particularly, in those characterized by following an intrinsic or mitochondrial pathway. Therefore, we were interested to know if any of these proteins could afford some protection to SH-SY5Y cells challenged with Olomoucine or Roscovitine. SH-SY5Y populations overexpressing either Bcl-2 or Bcl-XL protein were available from our previous studies. Both proteins conferred resistance to SH-SY5Y facing STS, being Bcl-XL overexpression far more protective than Bcl-2 one [9]. Along the following experiments,

the protection from STS treatments was routinely tested as a control of Bcl-2 or Bcl-XL overfunction.

The viability profiles of SH-SY5Y pcDNA3/Bcl-2 and pcDNA3/empty cell populations were determined by the MTS procedure after 24 hours of treatment with either Olomoucine or Roscovitine (Fig. 3A). No difference was observed between both populations. Cell death was also assessed by the LDH procedure and the null protective effect of Bcl-2 was also confirmed (Fig. 3B). Bcl-2 overexpressing cells continued to die displaying an apoptotic phenotype as revealed by bisBenzimide (not shown) and the ladder pattern of DNA degradation (Fig. 3C). Bcl-2 is a protein constitutively expressed by SH-SY5Y cells, however the Bcl-2 content resulting from overexpression was neatly greater (Fig. 3D). As shown also in this figure, Bcl-2 overexpression did not change the Bcl-XL content, which remained undetectable in our Western Blots.

Analogously, we determined the viability of SH-SY5Y pcDNA3/Bcl-XL cells subjected to Olomoucine or Roscovitine treatments (Fig. 4A). As shown in the figure, Bcl-XL overexpression did not protect from these drugs, either. The LDH release determinations confirmed this assertion (Fig. 4B). SH-SY5Y pcDNA3/Bcl-XL cells were dying by apoptosis as evidenced by the internucleosomal DNA degradation (Fig. 4C). Note the first lane of this image, where no ladder is observed in cells that have been treated with STS. As stated before, this is a control of an excess of Bcl-XL function in these cells [9]. On the other hand, Bcl-XL overexpression did not change the cell content of Bcl-2 (Fig. 4D). In summary, neither Bcl-2 nor Bcl-XL overfunction was able to modify

the death ratios caused by Olomoucine and Roscovitine treatments. A Bcl-2 or Bcl-XL regulated pathway to initiate apoptosis, in other words a conventional intrinsic pathway, seemed not to be activated by these drugs.

Effects of zVADfmk on SH-SY5Y cells facing Olomoucine and Roscovitine

It is broadly accepted that, in the extrinsic pathway, the signal from the death receptors is driven through caspase activation. As a consequence, the pharmacological inhibition of caspases translates into cell survival in these models. Conversely, in the intrinsic pathway, caspase inhibition translates into blockage of the apoptotic features but the ratios of cell death remain unchanged. These facts prompted us to study the effect of caspase inhibition in cells treated with Olomoucine and Roscovitine. We chose zVADfmk by its broad spectrum of caspase inhibition. The addition of 50μM zVADfmk to SH-SY5Y cells challenged with Olomoucine resulted in an increase of cell viability (Fig. 5A) and a corresponding decrease in the cell death ratios (Fig. 5B). Similar results were obtained if Roscovitine was used instead of Olomoucine (Fig. 5A and 5B). The protection afforded by zVADfmk was partial, since many cells continued to die. Dying cells did not show the typical features of apoptosis. For instance, bisBenzimide/propidium iodide double staining showed no nuclear fragmentation in the dead cells (not shown). Consistently, no apoptotic DNA ladder was seen despite cell death was taking place (Fig. 5C). In summary, zVADfmk was capable of blocking the apoptotic phenotype in all cells but prevented cell death in only a subpopulation.

We observed that Olomoucine and Roscovitine were very stable in culturing conditions, however zVADfmk experienced an important decay after 24 hours in these conditions. By carefully refreshing zVADfmk after 24 hours of culture, we found its protective effect could be maintained up to 48 hours of Olomoucine and Roscovitine treatment (Fig. 5B). This suggested that the aforementioned cell subpopulation could survive in the presence of either Olomoucine or Roscovitine, as far as zVADfmk was active and caspases were inhibited. This behavior is characteristic of the apoptotic models that follow an extrinsic pathway. Taken together with the previous Bcl-2 and Bcl-XL results, all the data were pointing to a type I extrinsic pathway or, alternatively, to a caspase initiating event in the apoptotic process of this cell subpopulation.

In a few experiments Olomoucine concentration was raised to 800μM, interestingly zVADfmk maintained the percentage of cell viability at 56,96 ± 0,91 in this condition. Note that this viability value did not differ significantly from that observed at 200μM. This indicated that greater concentrations of Olomoucine did not trigger any additional, new mechanism, of cell death or if Olomoucine did, it was also blocked by zVADfmk.

Effect of CHX and DAct on SH-SY5Y cells treated with Olomoucine or Roscovitine

In the characterization of apoptosis models, a frequently asked question has been the requirement of new protein synthesis for the process to be accomplished. To answer this question, CHX and DAct have been generally used. In our experiments, we

employed these reagents at concentrations far above those described to inhibit macromolecular synthesis. Initially, we tested the direct toxicity of these concentrations on SH-SY5Y cells (Fig. 6A). After 48 hours of treatment, neither CHX nor DAct caused significant amounts of LDH release. The cell death ratios were 10,3 ± 2,1 for CHX and 20,2 ± 1,6 for DAct. The lower toxicity of CHX justified its preferential use in further experiments. Moreover, these concentrations of CHX were not able to activate effector caspases in SH-SY5Y cells (See Fig. 6D and 6E). In conclusion, up to 48 hours of treatment, CHX was not efficiently inducing apoptosis in the SH-SY5Y cell line.

The effect of CHX and DAct on cultures of SH-SY5Y cells treated with either Olomoucine or Roscovitine was then assayed. As shown in Figure 6C, both compounds performed very well at preventing cell death. The ratios of cell protection surpassed those afforded by zVADfmk alone. The results obtained by the MTS procedure were also confirmed by the LDH method (Fig. 6B). Next we wondered about a possible synergism between macromolecular synthesis and caspase inhibition. As displayed in Figure 6B and 6C, this synergism was found. In conclusion, three SH-SY5Y cell subpopulations seemed to exist in our cultures. A population of cells protected by CHX but not by zVADfmk. An overlapping population protected by both compounds and, finally, a small population protected only by zVADfmk (this is schematized in Fig. 9). In order to demonstrate that a subpopulation was eluding CHX protection, DEVDase activation kinetics was determined in SH-SY5Y cell cultures. As expected, CHX was only partially inhibiting DEVDase activity in SH-SY5Y cells treated with 200μM Olomoucine (Fig. 6D)

or 50μM Roscovitine (Fig. 6E). This coincided with the apoptotic phenotype observed in the scarce cells not protected by CHX (bisBenzimide images, not shown).

On the other hand we explored the possible effects of protein synthesis inhibition on cell cycle regulation. We explored CDK activity by analyzing the state of phosphorylation of a known substrate, the Rb protein. As shown in Fig. 6F, the reduction in Rb phosphorylation was already detectable after 24 hours of treatment with CHX. The implications of this result will be discussed further.

Olomoucine, Roscovitine and cell differentiation in SH-SY5Y cells

Another interesting aspect concerning the mode of action of Olomoucine and Roscovitine is their putative ability to induce the differentiation of SH-SY5Y cells. We have tested low and high doses, short and long times of incubation, and looked for a perceptible neurite outgrowth. The results were repeatedly negative, particularly if compared with the prominent neuritogenesis caused by STS [9] or RA differentiating treatments (Fig. 7A).

Cell differentiation is a physiological process that implies a downregulation of CDK function. As a consequence, we thought of studying the effect of cell differentiation on cell sensitivity to Olomoucine and Roscovitine. SH-SY5Y cells were seeded on collagen coated plates and treated with 10μM RA for five days. The result was a cell population displaying a differentiated phenotype (Fig. 7A) and mostly arrested in G0/G1 phase of the cell cycle (Fig. 7B). The CDK inhibition associated with differentiation was

checked by analyzing the state of phosphorylation of Rb protein. As expected, Rb was found to be hypophosphorylated in the differentiated cells (Fig. 7C). In conclusion, a cell population mostly in a quiescent state was available to test the effects of Olomoucine and Roscovitine. Not surprisingly, differentiation implied an increase of cell viability in our experimental paradigm as measured by the MTS procedure (Fig. 7D). This result was also confirmed by the LDH method (not shown). To better characterize the resistance associated with the differentiation process, we performed an experiment in which we determined the protection afforded by shorter times of differentiation. As shown in Figure 7E, there was a strong correlation between cell resistance and the time required for an optimal differentiation. At time 0 no protection was found, thus ruling out the putative protective effects of 10μM RA and of culturing on collagen coated plates. A minimum of 24 hours, for differentiation to take place in the absence of Olomoucine or Roscovitine, was required to begin to detect some resistance.

Effect of PD98059 and UO126 compounds on proliferating SH-SY5Y cells.

Extracellular regulated kinases (Erk1 and Erk 2) have been described as targets of Olomoucine, Roscovitine and other closely related compounds [4, 22]. In order to explore the involvement of these kinases in our model, we proceeded to evaluate the lethal effects of PD98059 and UO126 on SH-SY5Y cells. PD98059 and UO126 are not chemically related, however they are both cell-permeable and selective inhibitors of MAP kinase kinase (MEK). This implies the inhibition of Erk1/Erk2 enzymatic activity. Both

compounds were employed at final concentrations known to inhibit Erk1/Erk2. We observed no reduction in SH-SY5Y cell viability, either measured by the MTS procedure at 24 hours (Fig. 8A) or determined by the release of LDH after 48 hours of treatment (Fig. 8B). Consistently, no increase in effector caspase activity was found up to 9 hours of treatment (Fig. 8C). In these experiments, STS was used as a positive control to induce cell death by apoptosis. Our PD98059 and UO126 compounds proved to be active when periodically checked in standardized measurements of Erk1/Erk2 activation in response to neurotrophins [23]. In conclusion, a putative inhibition of Erk1/Erk2 by Olomoucine and Roscovitine was ruled out as the cause of apoptosis in SH-SY5Y cells.

Discussion

In this work we have characterized some basic aspects of the cell death process triggered by two CDK inhibitory drugs, Olomoucine and Roscovitine, in a human cell line derived from neuroblastoma, SH-SY5Y. We have determined the cell death process to be dose-dependent and the range of concentrations that caused it. Care was taken not to rely on only one procedure to quantify cell death. Two different methods detecting independent cell death phenomena were used. In fact, a non-quantifiable third one, cell detachment and fragmentation, was routinely assessed and was always congruent with the numerical data. We have identified apoptosis as the type of cell death induced by Olomoucine and Roscovitine. In order to gain insight into the mechanisms activated to generate apoptosis, we used SH-SY5Y cells overexpressing functional Bcl-2 and Bcl-XL

proteins. Surprisingly, no protective effect was found. This result is in agreement with the one reported in HeLa cells overexpressing Bcl-2 and facing Flavopiridol [24]. It has also been reported that an increased Bcl-2 content does not afford any resistance to Flavopiridol in cells from a chronic B lymphocytic leukemia [25]. However, Bcl-2 overexpression proved capable of protecting MB-468 breast cancer cells from this drug [26]. One report is also found about Roscovitine induced apoptosis being prevented by Bcl-2 overexpression in a human leukemia cell line [27]. Finally, Bcl-2 and Bcl-XL prevented apoptosis triggered by a new CDK1 specific inhibitor, CGP74514A [28]. We have not detected any additional report addressing this specific issue. The drug and cell type contingency notwithstanding, we have not found any satisfactory explanation for the discrepancies.

In order to inhibit a broad spectrum of caspases, we used zVADfmk and found it was not only able to inhibit the apoptotic phenotype but cell death caused by Olomoucine and Roscovitine. The inhibition of cell death was partial and seemed to be maintained as long as zVADfmk was not degraded. This indicated a caspase initiating event in a specific subpopulation of the cell culture. Moreover, the null protective effect of Bcl-2 and Bcl-XL suggested this subpopulation was behaving like cells following a type I extrinsic pathway. Usually, this means the involvement of Fas type death receptors (DR). However, the initiation of a caspase cascade in the endoplasmic reticulum or Golgi apparatus can mimic a type I pathway [29]. Moreover, the cascade could be initiated in the nucleus, where CDK exert their function. Interestingly, caspase 9

has been described to be inhibited by forming a complex with survivin in mitotic cells. A complex which integrity is dependent upon CDK1 mediated phosphorylation of survivin [30]. Therefore, pharmacological inhibition of CDK1 should imply caspase 9 release and a type I apoptosis in a subpopulation of mitotic cells. Before exploring these alternative pathways, in our future experiments we will try to discard first the involvement of DR in the apoptotic process induced by Olomoucine and Roscovitine.

Protein synthesis inhibition by means of CHX and DAct reduced Olomoucine and Roscovitine induced apoptosis. A similar result has been reported in a lung carcinoma cell line treated with Flavopiridol [31]. The effect of CHX and DAct on apoptosis was an important point in the early times of apoptosis studies, that has not attracted much attention afterwards. In many models, both agents prevented apoptosis from occurring. In these models, the synthesis of new proteins was hypothesized to be required for apoptosis to take place. In other models, both agents triggered cell death by themselves and defined the release type of apoptosis [32]. In the release models, the existence of a short living, apoptosis blocking protein, that is continuously synthesized, was hypothesized. In both types of models, there has been a very limited success in identifying the hypothesized gene products. The molecular machinery of apoptosis has revealed to be constitutive and not to require new protein synthesis to be activated [10,14,15]. An increase of reduced glutathione, caused by CHX, was proposed to prevent oxidative stress and to be the explanation of the CHX antiapoptotic properties [33]. We propose an alternative hypothesis, CHX and DAct are going to block the

synthesis of cyclins [34,35], then inhibit CDK and stop the cell cycle. If cells are able to withstand this insult, as SH-SY5Y cells seem to, they will no longer offer a target for CDK inhibitory drugs. Indeed, we have evidence that CHX and DAct markedly stop cell growth in SH-SY5Y cells (estimations from the total LDH content). This is consistent with the Rb hypophosphorylation we have found in CHX-treated SH-SY5Y cells (Fig. 6F). Moreover, our hypothesis is reinforced by the study of thymocytes [36,37] and neuronal cell [38,39] models in which CDK activity is required for apoptosis to be accomplished. In these experimental paradigms, Olomoucine and Roscovitine have been useful tools to block apoptosis and to confirm the CDK involvement. Based on our hypothesis, CHX should prevent apoptosis in these paradigms. This seems to be the case either in thymocytes [40,41] or in the neuronal models [42, 43].

Another important point is the synergy observed between protein synthesis inhibition and zVADfmk treatments. Based on this combination of treatments, we can distinguish three different subpopulations in a culture of SH-SY5Y cells. Each subpopulation following a specific pathway towards cell death (schematized in Fig. 9). In two pathways there is an apical event blocked by CHX. In the third one the apical event seems to be the initiation of a caspase cascade. In the CHX protected cells, some are also protected by caspase inhibition while the other are not. In conclusion, Olomoucine and Roscovitine seem to trigger different molecular mechanisms that lead to apoptosis in SH-SY5Y cells. We believe the cell cycle is the cause of this heterogeneity. We favor the hypothesis that the referred subpopulations reflect the phase in which the cell was

cycling at the moment of drug treatment. Fig. 9 is a working hypothesis that we expect to test in the future. We will study the apoptotic process triggered by Olomoucine and Roscovitine in synchronized populations of SH-SY5Y. Following a similar approach, Flavopiridol has demonstrated to increase the induction of apoptosis in transformed cells synchronized in S phase [44].

The specificity of drugs is a general matter of concern in pharmacological studies. Presently, research efforts are being devoted to characterize as much "in vivo" targets of these CDK inhibitory drugs as possible. These new targets could help to explain the biological actions and the putative lack of specificity of these compounds [45]. However, in our experiments elevated Olomoucine concentrations seem not to engage additional mechanisms leading to cell death. In addition, we have discarded the involvement of Erk-1/Erk-2 inhibition as the mechanism causing apoptosis. We do not know if Olomoucine and Roscovitine can inhibit other CDK subtypes in our cells, for example CDK8 and CDK9. These CDK subtypes are mainly involved in transcriptional activation. Consistently, Roscovitine has demonstrated the ability to inhibit transcription in herpesvirus [46] and human cells [47]. Therefore, it could be proposed that Olomoucine and Roscovitine could be apoptotic agents through their inhibition of transcription. Our results do not support this hypothesis. Let us remember that very high concentrations of CHX or DAct do not induce apoptosis in SH-SY5Y cells (Fig. 6A). In other words, SH-SY5Y cells do not display a release model of apoptosis. Another advantage of the SH-SY5Y cell line is the capability to be differentiated in cell culture

conditions. Differentiation implies cell quiescence and a physiological downregulation of CDK activity. If apoptosis is the result of CDK inhibition by Olomoucine and Roscovitine, cell resistance would be expected in differentiated SH-SY5Y cells. Though partial, as partial is probably the extent of differentiation in the culture, the resistance has been evidenced in our work. Obviously, we can not exclude other differentiation-related events capable of explaining the resistance to both compounds. However, taken together our results, we believe the role of CDK1 and CDK2 as targets for Olomoucine and Roscovitine in apoptosis induction has been reinforced.

In the introduction section, we commented the putative cytostatic, differentiation-inducing and cytocidal properties of Olomoucine and Roscovitine to treat cancer. No differentiation-inducing properties have been observed in SH-SY5Y cells, however both compounds have induced apoptosis efficiently. Apoptosis induction was found in growing cells and markedly neutralized in quiescent, differentiated cells. As a consequence, a preference for proliferating and undifferentiated tumor cells can be inferred. This is a common property found in most anticancer drugs. However, Olomoucine and Roscovitine were able to circumvent the antiapoptotic effects of Bcl-2 and Bcl-XL proteins, two proven oncogenic factors that cause resistance to chemotherapy. In conclusion, Olomoucine, Roscovitine and, probably, other related compounds display a promising profile as anticancer drugs. Particularly, as we have just reported, in the treatment of neuroblastoma.

Acknowledgements

This work has been funded by "Paeria de Lleida" ("Ajuts a la recerca", 1998) and University of Lleida ("Programa de Grups Emergents", 2001). J. Ribas is a predoctoral fellow from DGR ("Generalitat de Catalunya"). We thank V.J. Yuste and C. Casas for critical reading of the manuscript. We thank J.X. Comella for making easier the start of the Molecular Pharmacology Group. We thank J. Esquerda and E. Herrero for continued support. We are greatly indebted to N. Agell for scientific discussion, encouragement and support.

References

[1] G. Manning, D.B. Whyte, R. Martinez, T. Hunter, S. Sudarsanam, The protein kinase complement of the human genome, Science 298 (2002) 1912-1934.

[2] C. Norbury, P. Nurse, Animal cell cycles and their control, Annu. Rev. Biochem. 61 (1992) 441-470.

[3] I.R. Hardcastle, B.T. Golding, R.J. Griffin, Designing inhibitors of cyclin-dependent kinases, Annu. Rev. Pharmacol. Toxicol. 42 (2002) 325-348.

[4] M. Knockaert, P. Greengard, L. Meijer, Pharmacological inhibitors of cyclin-dependent kinases, Trends Pharmacol. Sci. 23 (2002) 417-425.

[5] E.A. Sausville, Complexities in the development of cyclin-dependent kinase inhibitor drugs, Trends Mol. Med. 8 (2002) S32-S37.

[6] J. Vesely, L. Havlicek, M. Strnad, J.J. Blow, A. Donella-Deana, L. Pinna, D.S. Letham, J. Kato, L. Detivaud, S. Leclerc, L. Meijer, Inhibition of cyclin-dependent kinases by purine analogues, Eur. J. Biochem. 224 (1994) 771-786.

[7] L. Meijer, A. Borgne, O. Mulner, J.P.J. Chong, J.J. Blow, N. Inagaki, M. Inagaki, J.G. Delcros, J.P. Molinoux, Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5, Eur. J. Biochem. 243 (1997) 527-536.

[8] M.D. Garrett, A. Fattaey, CDK inhibition and cancer therapy, Curr. Opin. Genet. Dev. (1999) 104-111.

[9] V.J. Yuste, I. Sanchez-Lopez, C. Sole, M. Encinas, J.R. Bayascas, J. Boix, J.X. Comella, The prevention of the staurosporine-induced apoptosis by Bcl-X(L), but not by Bcl-2 or caspase inhibitors, allows the extensive differentiation of human neuroblastoma cells, J. Neurochem. 80 (2002) 126-139.

[10] V. Cryns, J. Yuan, Proteases to die for. Genes Dev. 12 (1998) 1551-1570.

[11] N.A. Thornberry, Y. Lazebnik, Caspases: enemies within, Science 281 (1998) 1312-1316.

[12] E.S. Alnemri, D.J. Livingston, D.W. Nicholson, G. Salvesen, N.A. Thornberry, W.W. Wong, J. Yuan, Human ICE/CED-3 protease nomenclature, Cell 87 (1996) 171.

[13] U. Fischer, R.U. Jänicke, K. Schulze-Osthoff, Many cuts to ruin: a comprehensive update of caspase substrates, Cell Death Differ. 10 (2003) 76-100.

[14] A. Ashkenazi, V.M. Dixit, Death receptors: signaling and modulation, Science 281 (1998) 1305-1308.

[15] J.M. Adams, S.Cory, Apoptosomes: engines for caspase activation, Curr. Opin. Cell. Biol. 14 (1998) 715-720.

[16] J.M. Adams, S. Cory, The Bcl-2 protein family: arbiters of cell survival. Science 281 (1998) 1322-1326.

[17] J. Boix, N. Llecha, V.J. Yuste, J.X. Comella, Characterization of the cell death process induced by staurosporine in human neuroblastoma cell lines, Neuropharmacology 36 (1997) 811-821.

[18] V.J. Yuste, J.R. Bayascas, N. Llecha, I. Sanchez-Lopez, J. Boix, J.X. Comella, The absence of oligonucleosomal DNA fragmentation during apoptosis of IMR-5 neuroblastoma cells: disappearance of the caspase-activated DNase, J. Biol. Chem. 276 (2001) 22323-22331.

[19] M. Encinas, M. Iglesias, Y. Liu, H. Wang, A. Muhaisen, V. Ceña, C. Gallego, J.X. Comella, Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells, J. Neurochem. 75 (2002) 991-1003.

[20] J.T. Wolfe, J.H. Pringle, G.M. Cohen, Assays for the measurement of DNA fragmentation during apoptosis, in: T.G. Cotter, S.J. Martin (Eds.), Techniques in Apoptosis, Portland Press Ltd, London, 1996, pp. 51-69.

[21] M. Piqué, M. Barragán, M. Dalmau, B. Bellosillo, G. Pons, J. Gil, Aspirin induces apoptosis through mitochondrial cytochrome c release, FEBS Lett. 480 (2000) 193-196.

[22] M. Knockaert, P. Lenormand, N. Gray, P. Schultz, J. Pouyssegur, L. Meijer, p42/p44 MAPKs are intracellular targets of the CDK inhibitor purvalanol, Oncogene 21 (2002) 6413-6424.

[23] J. Egea, C. Espinet, R.M. Soler, S. Peiró, N. Rocamora, JX Comella, Nerve Growth Factor activation of the extracellular signal-regulated kinase pathway is modulated by Ca^{2+} and Calmodulin, Mol. Cell. Biol. 20 (2000) 1931-1946.

[24] T.V. Achenbach, R. Muller, E.P. Slater, Bcl-2 independence of flavopiridol-induced apoptosis. Mitochondrial depolarization in the absence of cytochrome c release, J. Biol. Chem. 275 (2002) 32089-32097.

[25] C. Pepper, A. Thomas, T. Hoy, C. Fegan, P. Bentley, Flavopiridol circumvents Bcl-2 family mediated inhibition of apoptosis and drug resistance in B-cell chronic lymphocytic leukaemia, Br. J. Haematol. 114 (2001) 70-77.

[26] S. Wittmann, P. Bali, S. Donapaty, R. Nimmanapalli, F. Guo, H. Yamaguchi, M. Huang, R. Jove, H.G. Wang, K. Bhalla, Flavopiridol down-regulates antiapoptotic proteins and sensitizes human breast cancer cells to epothilone B-induced apoptosis, Cancer Res. 63 (2003) 93-99.

[27] C. Yu, M. Rahmani, Y. Dai, D. Conrad, G. Krystal, P. Dent, S. Grant, The lethal effects of pharmacological cyclin-dependent kinase inhibitors in human leukemia

cells proceed through a phosphatidylinositol 3-kinase/Akt-dependent process, Cancer Res. 63 (2003) 1822-1833.

[28] Y. Dai, P. Dent, S. Grant, Induction of apoptosis in human leukemia cells by the CDK1 inhibitor CGP74514A, Cell Cycle 1 (2002) 143-142.

[29] K.F. Ferri, G. Kroemer, Organelle-specific initiation of cell death pathways, Nature Cell Biol. 3 (2001) E255-E263.

[30] D.S. O'Connor, D. Grossman, J. Plescia, F. Li, H. Zhang, A. Villa, S. Tognin, C. Marchisio, D.C. Altieri, Regulation of apoptosis at cell division by $p34^{cdc2}$ phosphorylation of survivin, Proc. Natl. Acad. Sci. USA 97 (2000) 13103-13107.

[31] K.C. Bible, S.H. Kaufmann, Flavopiridol: a cytotoxic flavone that induces cell death in noncycling A549 human lung carcinoma cells, Cancer Res. 56 (1996) 4856-4861.

[32] J. Boix, J. Fibla, V.J. Yuste, J.M. Piulats, N. Llecha, J.X. Comella, Serum deprivation and protein synthesis inhibition induce two different apoptotic processes in N18 neuroblastoma cells, Exp. Cell Res. 238 (1998) 422-429.

[33] R.R. Ratan, T.H. Murphy J.M. Baraban, Macromolecular synthesis inhibitors prevent oxidative stress-induced apoptosis in embryonic cortical neurons by shunting cysteine from protein synthesis to glutathione, J. Neurosci. 14 (1994) 4385-4392.

[34] M. Polymenis, E.V. Schmidt, Coupling cell division to cell growth by translational control of the G1 cyclin CLN3 in yeast, Genes Dev. 11 (1997) 2522-2531.

[35] D.D. Hall, D.D. Markwardt, F. Parviz, W. Heideman, Regulation of the Cln3-Cdc28 kinase by cAMP in Saccharomyces cerevisiae, EMBO J. 17 (1998) 4370-4378.

[36] G. Gil-Gomez, A. Berns, H.J. Brady, A link between cell cycle and cell death: Bax and Bcl-2 modulate Cdk2 activation during thymocyte apoptosis, EMBO J. 17 (1998) 7209-7218.

[37] A. Hakem, T. Sasaki, I. Kozieradzki, J.M. Penninger, The cyclin-dependent kinase Cdk2 regulates thymocyte apoptosis, J. Exp. Med. 189 (1999) 957-968.

[38] D.S. Park, S.E. Farinelli L.A. Greene, Inhibitors of cyclin-dependent kinases promote survival of post-mitotic neuronally differentiated PC12 cells and sympathetic neurons, J. Biol. Chem. 271 (1996) 8161-8169.

[39] J. Padmanabhan, D.S. Park, L.A. Greene, M.L Shelanski, Role of cell cycle regulatory proteins in cerebellar granule neuron apoptosis, J. Neurosci. 19 (1999) 8747-8756.

[40] P. Golstein, D.M. Ojcius, D.-E. Young, Cell death mechanisms and the immune system, Immunol. Rev. 121 (1991) 29-63.

[41] B. Dallaporta, M. de Pablo, C. Maisse, E. Daugas, M. Loeffler, N. Zamzani, G. Kroemer, Proteasome activation as a critical event of thymocyte apoptosis, Cell Death Diff. 7 (2000) 368-373.

[42] R.N. Pittman, S. Wang, A.J. DiBenedetto, J.C. Mills, A system for characterizing cellular and molecular events in programmed neuronal cell death, J. Neurosci. 13 (1993) 3669-3680.

[43] J.B. Schulz, M. Weller, T. Klockgether, Potassium deprivation-induced apoptosis of cerebellar granule neurons: a sequential requirement for new mRNA and protein

synthesis, ICE-like protease activity, and reactive oxygen species, J. Neurosci. 16 (1996) 4696-4706.

[44] C.B. Matranga, G.I. Shapiro, Selective sensitization of transformed cells to Flavopiridol-induced apoptosis following recruitment to S-Phase, Cancer Res. 62 (2002), 1707-1717.

[45] M. Knockaert, L. Meijer, Identifying in vivo targets of cyclin-dependent kinase inhibitors by affinity chromatography, Biochem. Pharmacol. 64 (2002) 819-825.

[46] D.J. Davido, D.A. Leib, P.A. Schaffer, The cyclin-dependent kinase inhibitor roscovitine inhibits the transactivating activity and alters the posttranslational modification of herpes simplex virus type 1 ICP0, J. Virol. 76 (2002) 1077-1088.

[47] M. Ljungman, M.T. Paulsen, 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 (2001) 785-789.

FIG. 1. Cell death induced by Olomoucine and Roscovitine was concentration-dependent. **A)** SH-SY5Y cells in culture were treated for 24 hours with Roscovitine (❑) in a range of concentrations 6.7 to 50μM. Cells were also treated with Olomoucine (■) and Iso-Olomoucine (●) in a range of concentrations 26.7 to 200μM. Cell viability was quantified by means of the MTS reduction assay. The survival percentage was obtained by referring the obtained values to those of control untreated cells. Every point is the mean ± SEM (Standard Error of Media) of at least five independent experiments with six independent determinations per experiment. **B)** SH-SY5Y cells were treated for 48 hours with either Olomoucine or Roscovitine at the concentrations specified. Cell death was measured by determining the LDH released to the culture medium. The percentage was calculated by referring this value to that of the total LDH content of a control culture subjected to the same treatment. In addition, the cell death percentage found in untreated cultures was averaged and subtracted from the displayed final death ratios. Every bar indicates the mean ± SEM of at least four independent experiments with at least three independent measurements per experiment.

FIG. 2. Olomoucine and Roscovitine induced apoptosis in SH-SY5Y cells. **A)** SH-SY5Y cells were treated for 24 hours with 200μM Iso-Olomoucine, 200μM Olomoucine and 50μM Roscovitine. Following bisBenzimide staining of DNA, cells were photographed with an inverted fluorescence microscope. Arrows point to chromatin condensations typical of different stages of the apoptotic process. Scale bar = 50μM. **B)** DNA was

extracted from cells untreated (1) or treated for 12 hours with 200μM Olomoucine (2), 200μM Iso-Olomoucine (3) or 50μM Roscovitine (4). The ladder pattern of DNA degradation that characterizes apoptosis was detected in 1.5% agarose gels. The large DNA fragmentation that is characteristic of apoptosis was resolved by means of a CHEF electrophoresis. Std is a commercial standard of DNA. The position of the 50 kbp band is indicated in the figure. **C)** SH-SY5Y cells were treated with 200μM Olomoucine and 200μM Iso-Olomoucine. Then cytosolic extracts were performed at the time periods indicated in the figure. These extracts were analyzed by Western Blot to show the release of cytochrome c from mitochondria. The identity of the upper band is unknown but is an useful control of the amount of protein loaded per lane. **D)** Time-course of effector caspase activation (DEVDase activity measured in arbitrary fluorescence units). SH-SY5Y cells were treated with 200μM Olomoucine (■), 200μM Iso-Olomoucine (●) and 50μM Roscovitine (❑) for the time periods indicated in the graph and the fluorescence released from a AcDEVDafc substrate was determined. Every point is the mean ± SEM of two independent experiments with two independent measurements per experiment. **E)** Cells were treated as indicated in the graph, subjected to the TUNEL procedure and analyzed by flow cytometry. The percentage of apoptotic cells is indicated after 24 (full bars) and 48 hours of treatment (open bars). The bar is the mean ± SEM of at least two independent experiments.

FIG. 3. Bcl-2 overexpression did not protect cells from Olomoucine or Roscovitine

challenge. **A)** SH-SY5Y pcDNA3/Bcl-2 (❑) and pcDNA3/empty (■) cell populations were compared following a 24 hour treatment with either Olomoucine or Roscovitine, as indicated in the graphs. Cell survival was assessed by the MTS procedure as in figure 1. Every point is the mean ± SEM of three independent experiments with six independent measurements per experiment. **B)** SH-SY5Y pcDNA3/Bcl-2 (open bars) and pcDNA3/empty cell populations (full bars) were also treated with Olomoucine and Roscovitine for 48 hours. Cell death was determined by the LDH procedure. Every bar is the mean ± SEM of two independent experiments with three independent determinations per experiment. **C)** DNA ladders in 1.5% agarose gels. Cells were treated for 12 hours with 1μM STS (1), 200μM Iso-Olomoucine (2), 200μM Olomoucine (3) and 50μM Roscovitine (4). **D)** The Bcl-XL and Bcl-2 content of pcDNA3/Bcl-2 and pcDNA3/empty cell populations. Western Blots were performed and reacted with antiBcl-XL and antiBcl-2 antibodies. AntiActin antibody was used to demonstrate equal protein load.

FIG. 4. Bcl-XL overexpression did not protect cells from Olomoucine or Roscovitine challenge. **A)** SH-SY5Y pcDNA3/Bcl-XL (❑) and pcDNA3/empty (■) cell populations were compared following a 24 hour treatment with either Olomoucine or Roscovitine, as indicated in the graphs. Cell survival was assessed by the MTS procedure as in previous figures. Every point is the mean ± SEM of three independent experiments with six independent measurements per experiment. **B)** SH-SY5Y pcDNA3/Bcl-XL (open bars) and pcDNA3/empty cell populations (full bars) were also treated with Olomoucine and

Roscovitine for 48 hours. Cell death was determined by the LDH procedure. Every bar is the mean ± SEM of two independent experiments with three independent determinations per experiment. **C)** DNA ladders in 1.5% agarose gels. Cells were treated for 12 hours with 1μM STS (1), 200μM Iso-Olomoucine (2), 200μM Olomoucine (3) and 50μM Roscovitine (4). **D)** The Bcl-XL and Bcl-2 content of pcDNA3/Bcl-XL and pcDNA3/empty cell populations. Western Blots were performed and reacted with antiBcl-XL and antiBcl-2 antibodies. AntiActin antibody was used to demonstrate equal protein load.

FIG. 5. SH-SY5Y cells were partially protected from Olomoucine and Roscovitine challenge by zVADfmk. **A)** SH-SY5Y cells were treated with Olomoucine or Roscovitine for 24 hours in the presence (❑) or absence (■) of 50μM zVADfmk in the culture medium. Cell survival was assessed by the MTS procedure. Every point is the mean ± SEM of five independent experiments with six independent measurements per experiment. **B)** SH-SY5Y cells were treated for 48 hours as indicated in the figure. The culture medium contained (open bars) or did not contain (full bars) 50μM zVADfmk. To circumvent zVADfmk decay, it was refreshed at 24 hours of treatment. Every bar indicates mean ± SEM of at least three independent experiments with at least three independent measurements per experiment. **C)** SH-SY5Y cells were treated for 12 hours with 1μM STS (1), 200μM Iso-Olomoucine (2), 200μM Olomoucine (3) and 50μM Roscovitine (4). As shown in the picture, some cells were cultured in the presence of

50μM zVADfmk. DNA was extracted and internucleosomal DNA degradation observed in agarose gels.

FIG. 6. Inhibitors of new protein synthesis prevented apoptosis to a greater extent than zVADfmk in SH-SY5Y treated with either Olomoucine or Roscovitine. **A)** SH-SY5Y cells were treated for 48 hours with CHX, DAct and STS at the concentrations reported in the graph. LDH release was determined to quantify cell death. As evidenced, the toxicity derived from the inhibition of new protein synthesis was low. **B)** Cells were also challenged with 200μM Olomoucine (full bars) and 50μM Roscovitine (open bars), in the absence or presence of CHX, zVADfmk or both. Cell death was quantified following the LDH procedure. Bar value is the mean ± SEM that results from at least two independent experiments with at least 3 independent measurements per experiment. **C)** Cells treated with 200μM Olomoucine (full bars) and 50μM Roscovitine (open bars) were also treated with DAct, CHX, and zVADfmk as indicated in the graph. Cell viability was obtained by measuring the reduction of MTS. Bar value is the mean ± SEM of three experiments with 6 independent measurements per experiment. **D)** Time course of effector caspase activation was determined in SH-SY5Y cells treated with 200μM Olomoucine (▲), 12μg/ml CHX (■) or both (▼). Every point is the mean ± SEM of two independent experiments with two independent measurements per experiment. **E)** Time course of effector caspase activation in SH-SY5Y cells challenged with 50μM Roscovitine (▲), 12μg/ml CHX (■) or both (▼). Every point is the mean ± SEM of two independent

experiments with two independent measurements per experiment. DEVDase activity was measured in arbitrary fluorescent units. **F)** CHX induced hypophosphorylation of Rb protein. Rb from proliferating (control), 12μg/ml CHX for 24 hours (+CHX) and 10μM RA for five days (+RA) treated SH-SY5Y cells was analyzed by Western Blot.

Fig 7. Differentiated SH-SY5Y cells displayed an increased resistance to Olomoucine and Roscovitine. **A)** Phase contrast photographs of control and RA-differentiated SH-SY5Y cells (+RA). The arrow points to the prominent neurite projections characteristic of neuronal differentiation. Scale bar = 100μM. **B)** Cell cycle profile of control and differentiated SH-SY5Y cells. Differentiation implied S phase reduction and cell accumulation in G0/G1 phase. The profile is representative of several recorded profiles, obtained along the differentiation experiments. **C)** The state of phosphorylation of Rb protein was determined by Western Blot. The hyperphosphorylation characteristic of a growing control population decreases in a non-cycling differentiated one (+RA). **D)** SH-SY5Y cells differentiated with RA for five days (▲) and proliferating untreated controls (▼) were treated for 24 hours with Olomoucine and Roscovitine in the range of concentrations shown. Cell viability was quantified by the MTS assay. Every point is the mean ± SEM of at least five independent experiments with six independent determinations per experiment. **E)** SH-SY5Y cells were differentiated with RA for the days indicated in the graph. Then 200μM Olomoucine (●) or 50μM Roscovitine (▲) were added to the culture and cell viability determined after 24 hours. Cell resistance to

both drugs increased proportionally to the days of RA treatment. Cell viability was quantified by the MTS procedure. Every point is the mean ± SEM of six independent determinations.

FIG. 8. PD98059 and UO126 did not induce cell death in SH-SY5Y cells. **A)** Cells were treated as indicated in the figure. STS was used as a positive control of cell death. Cell viability was measured by the MTS procedure. The mean ± SEM of three independent experiments with at least six independent measurements per experiment is shown. **B)** Cells were treated as before and cell death was determined by the LDH procedure. Every bar is the mean ± SEM of two independent experiments with at least three independent determinations per experiment. **C)** Time-course of effector caspase activation. SH-SY5Y cells were treated with 50μM PD98059 (❍), 15μM UO126 (●) and 1μM STS (▲) for the time periods indicated. The value of control untreated cells is placed at time 0 (■). DEVDase activity was measured in arbitrary fluorescence units. Every point is the mean ± SEM of at least five independent determinations.

FIG. 9. Scheme of the putative pathways leading to cell death in SH-SY5Y cells facing Olomoucine or Roscovitine treatment. The three pathways are defined on the basis of the synergism detected between CHX and zVADfmk (Fig. 6C). Each pathway defines a specific subpopulation in the culture of SH-SY5Y cells. We believe a specific cycle phase is underlying each subpopulation. This is a working hypothesis. Each pathway is initiated

by the pharmacological inhibition of CDK. CDK ? means that the specific CDK number (either 1 or 2) is not known. $CASP_i$ means an initiating caspase, not an effector one. X is the unknown number identifying this specific caspase.

FIG. 1.

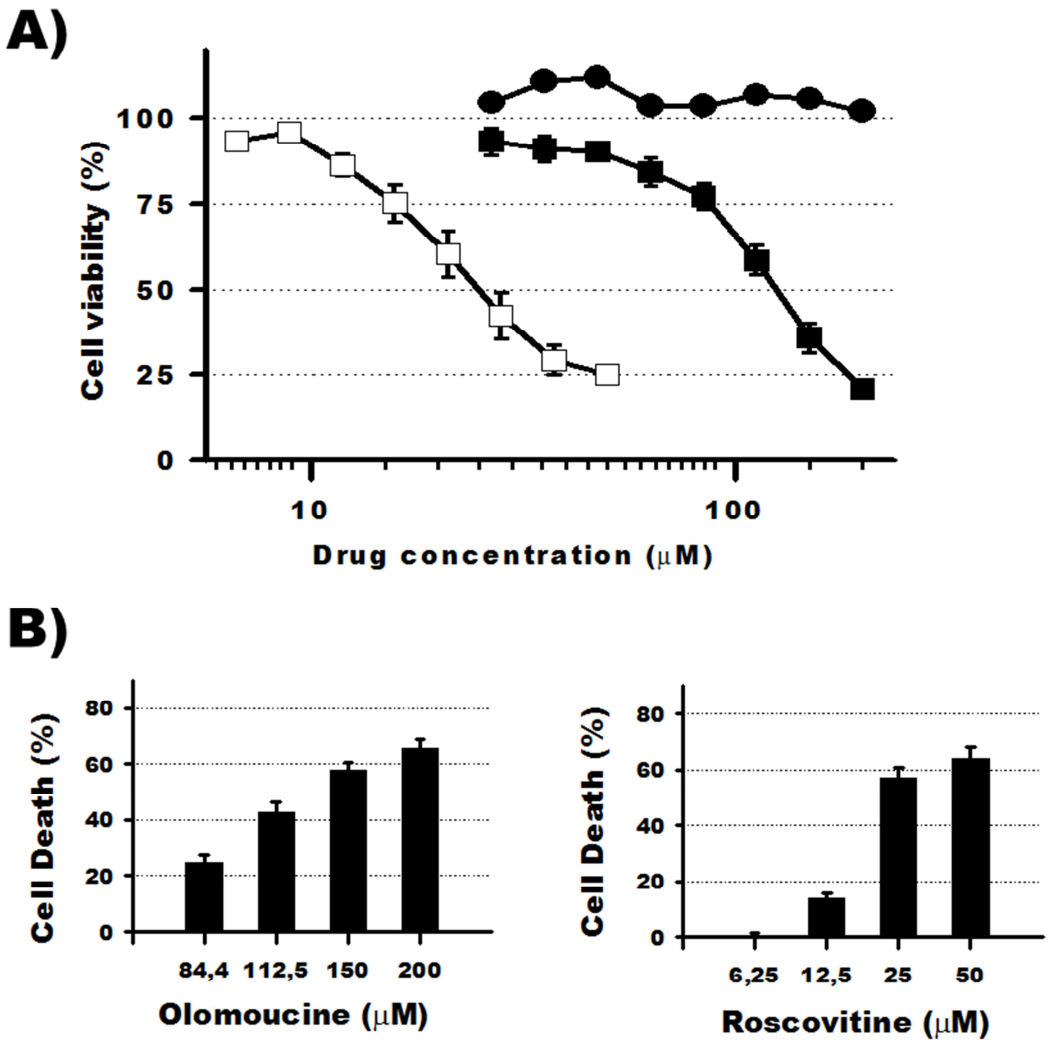

A)
Cell viability (%)
100
75
50
25
0
10
100
Drug concentration (μM)
B)
Cell Death (%)
80
60
40
20
0
84,4
112,5
150
200
Olomoucine (μM)
Cell Death (%)
80
60
40
20
0
6,25
12,5
25
50
Roscovitine (μM)

FIG. 2.

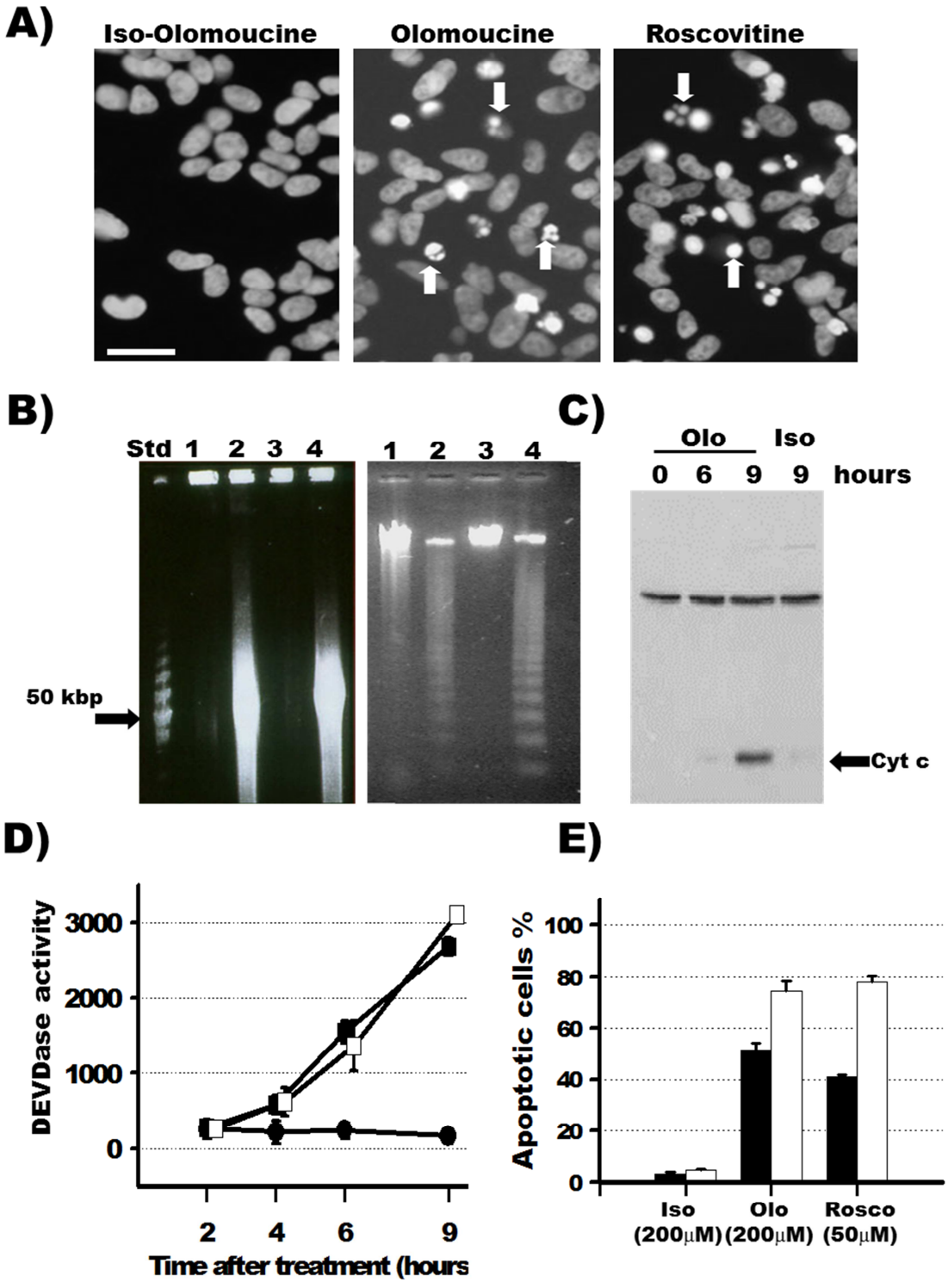

A)
Iso-Olomoucine
Olomoucine
Roscovitine
B)
Std 1 2 3 4
1 2 3 4
50 kbp
C)
Olo
Iso
0 6 9 9 hours
Cyt c
D)
DEVDase activity
3000
2000
1000
0
2 4 6 9
Time after treatment (hours
E)
Apoptotic cells %
100
80
60
40
20
0
Iso (200μM)
Olo (200μM)
Rosco (50μM)

FIG. 3.

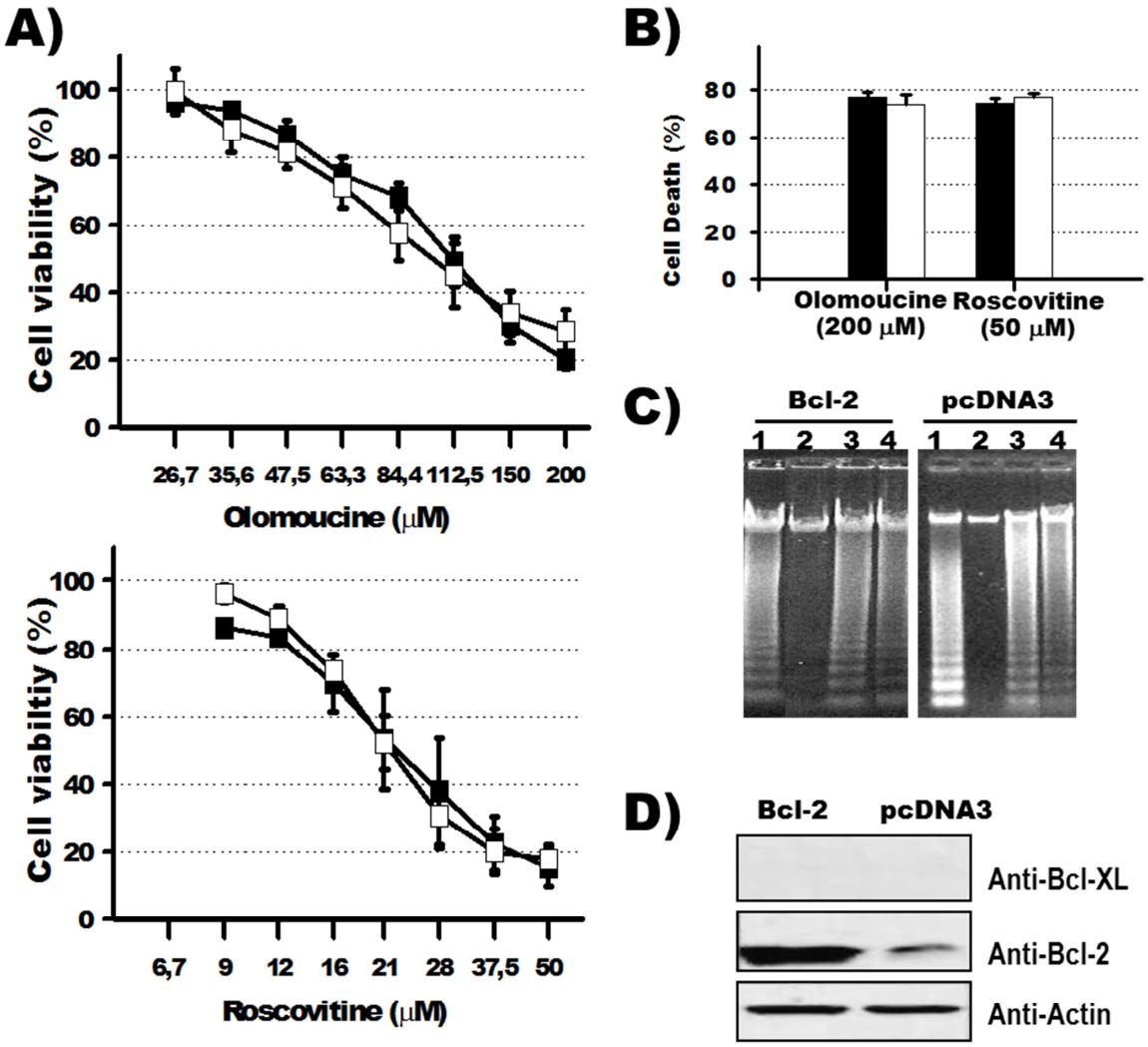

A)
Cell viability (%)
100
80
60
40
20
0
26,7 35,6 47,5 63,3 84,4 112,5 150 200
Olomoucine (μM)
Cell viabiltiy (%)
100
80
60
40
20
0
6,7 9 12 16 21 28 37,5 50
Roscovitine (μM)
B)
Cell Death (%)
80
60
40
20
0
Olomoucine (200 μM)
Roscovitine (50 μM)
C)
Bcl-2
pcDNA3
1 2 3 4 1 2 3 4
D)
Bcl-2
pcDNA3
Anti-Bcl-XL
Anti-Bcl-2
Anti-Actin

FIG. 4.

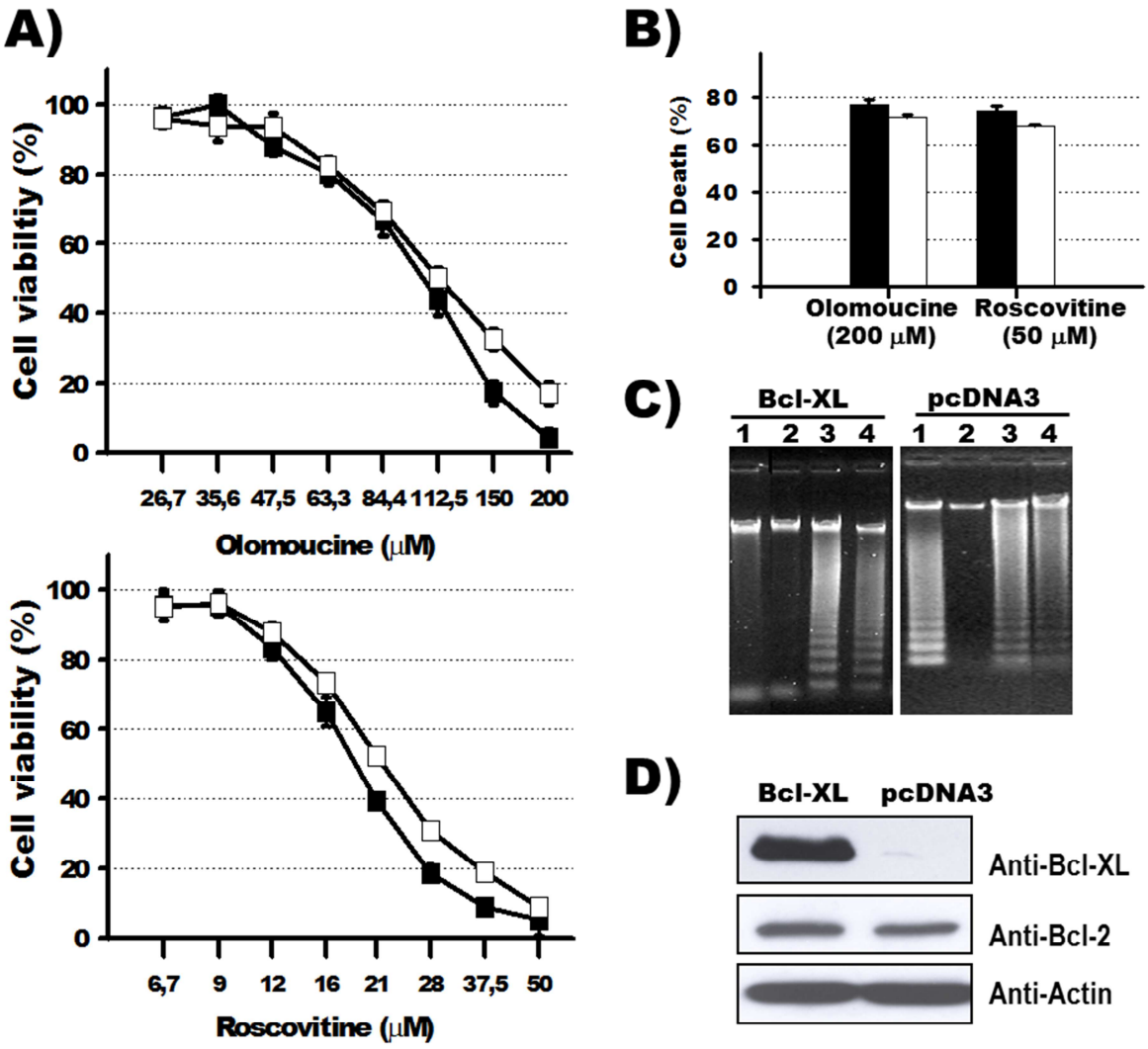

A)
Cell viabiltiy (%)
100
80
60
40
20
0
26,7 35,6 47,5 63,3 84,4 112,5 150 200
Olomoucine (μM)
Cell viability (%)
100
80
60
40
20
0
6,7 9 12 16 21 28 37,5 50
Roscovitine (μM)
B)
Cell Death (%)
80
60
40
20
0
Olomoucine (200 μM)
Roscovitine (50 μM)
C)
Bcl-XL
pcDNA3
1 2 3 4 1 2 3 4
D)
Bcl-XL
pcDNA3
Anti-Bcl-XL
Anti-Bcl-2
Anti-Actin

FIG. 5.

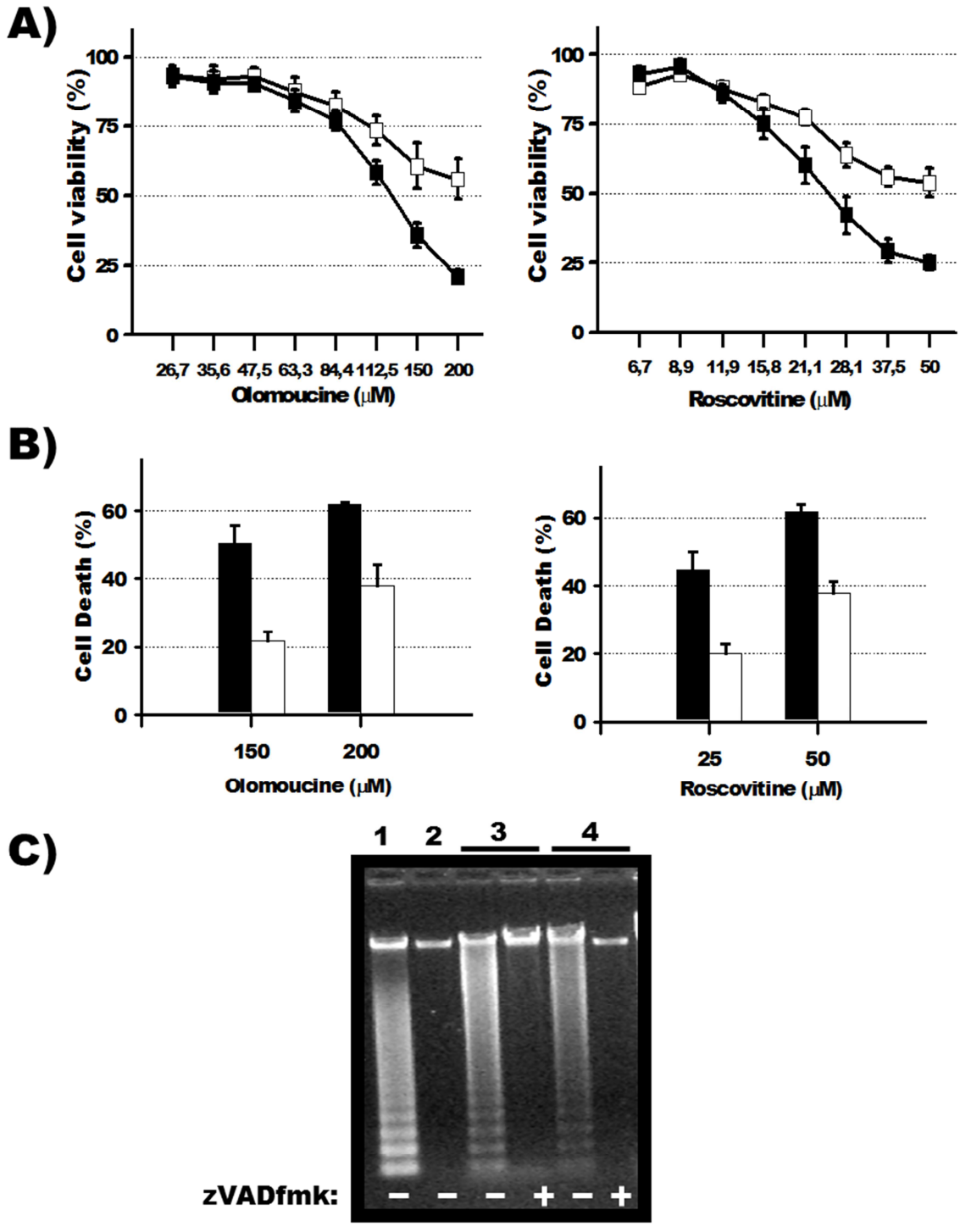

A)
Cell viability (%)
100
75
50
25
0
26,7 35,6 47,5 63,3 84,4 112,5 150 200
Olomoucine (μM)
Cell viability (%)
100
75
50
25
0
6,7 8,9 11,9 15,8 21,1 28,1 37,5 50
Roscovitine (μM)
B)
Cell Death (%)
60
40
20
0
150 200
Olomoucine (μM)
Cell Death (%)
60
40
20
0
25 50
Roscovitine (μM)
C)
1 2 3 4
zVADfmk: − − − + − +

FIG. 6.

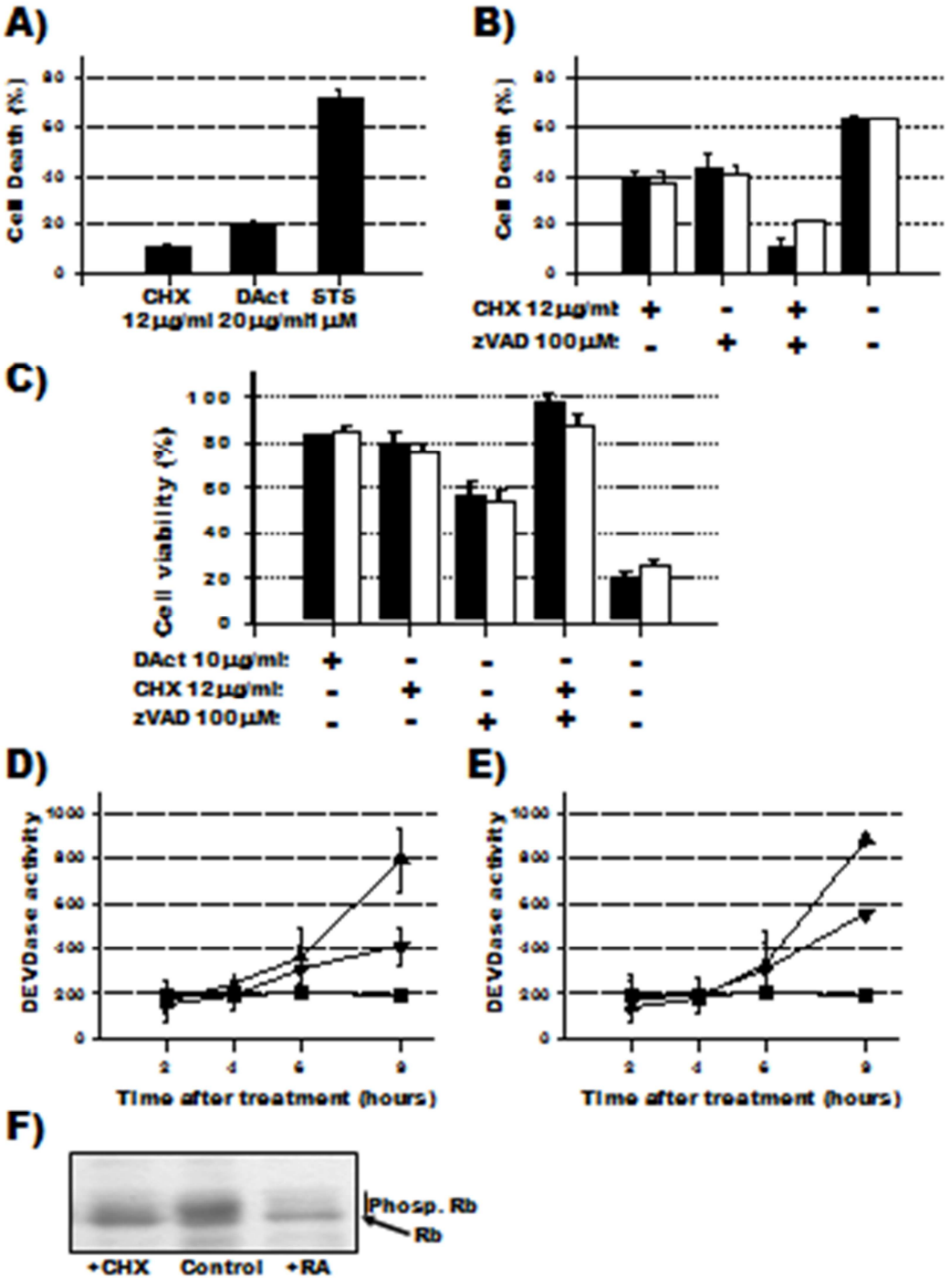

A)
Cell Death (%)
CHX 12µg/ml
DAct 20µg/ml
STS 1µM
B)
Cell Death (%)
CHX 12µg/ml
zVAD 100µM
C)
Cell viability (%)
DAct 10µg/ml:
CHX 12µg/ml:
zVAD 100µM:
D)
DEVDase activity
Time after treatment (hours)
E)
DEVDase activity
Time after treatment (hours)
F)
Phosp. Rb
Rb
+CHX
Control
+RA

FIG. 7.

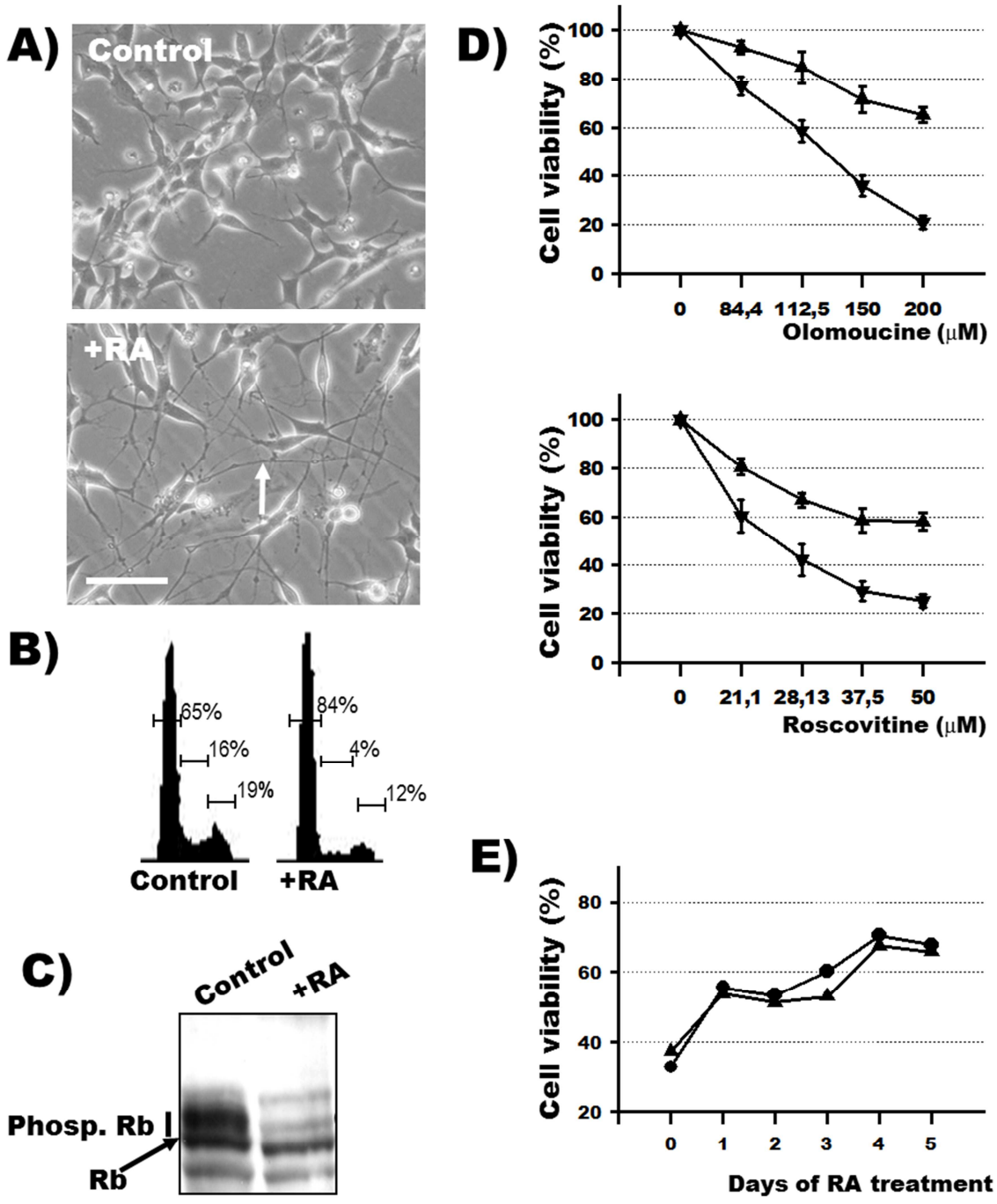

A)
Control
+RA
B)
65%
16%
19%
84%
4%
12%
Control
+RA
C)
Control
+RA
Phosp. Rb
Rb
D)
Cell viability (%)
100
80
60
40
20
0
0
84,4
112,5
150
200
Olomoucine (μM)
Cell viability (%)
100
80
60
40
20
0
0
21,1
28,13
37,5
50
Roscovitine (μM)
E)
Cell viability (%)
80
60
40
20
0
1
2
3
4
5
Days of RA treatment

FIG. 8.

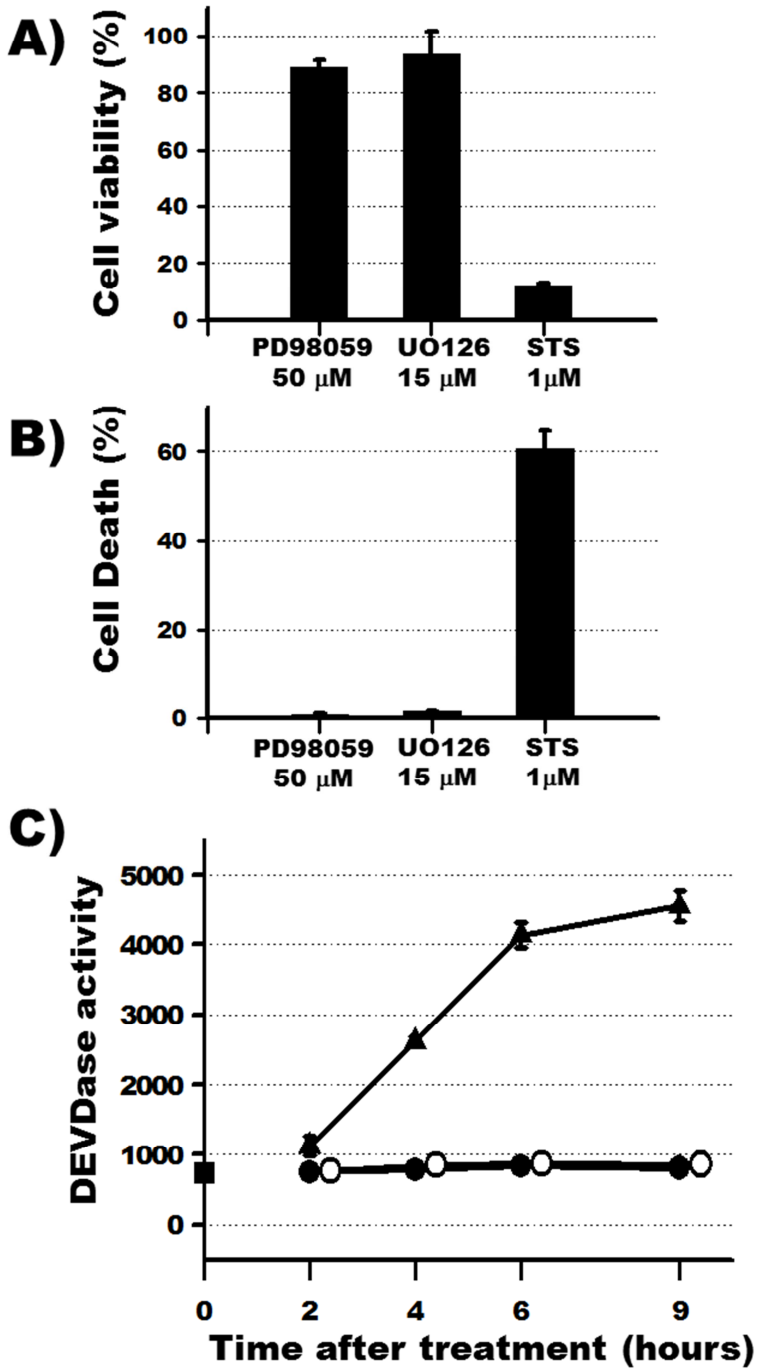
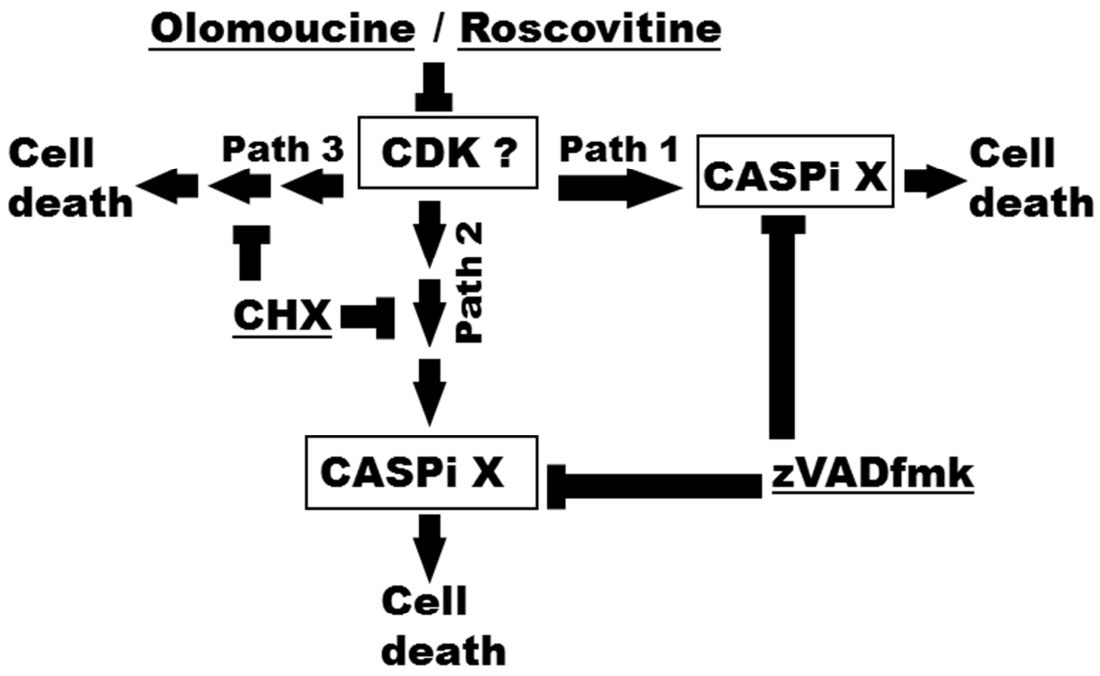

A)
Cell viability (%)
100
80
60
40
20
0
PD98059
50 μM
UO126
15 μM
STS
1μM
B)
Cell Death (%)
60
40
20
0
PD98059
50 μM
UO126
15 μM
STS
1μM
C)
DEVDase activity
5000
4000
3000
2000
1000
0
0
2
4
6
9
Time after treatment (hours)

FIG. 9.


Olomoucine / Roscovitine
CDK ?
Path 3
Cell death
Path 1
CASPi X
Cell death
CHX
Path 2
CASPi X
zVADfmk
Cell death