

BAX and BAK proteins are required for cyclin-dependent kinase inhibitory drugs to cause apoptosis

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Abstract

In previous reports, we have shown in SH-SY5 cells that olomoucine and roscovitine, two inhibitory drugs of cyclin-dependent kinases, caused apoptosis independent of the extrinsic pathway. In this experimental paradigm, apoptosis was refractory to the protective effects of either Bcl-2 or Bcl-XL overexpression. We are now reporting that the failure of Bcl-XL to prevent cell death was consistent with no effect on the kinetics of caspase activation and cytochrome *c* release. To further characterize this issue, we have discarded a direct effect of either olomoucine or roscovitine on mitochondrial permeability transition. Moreover, we have evidence that an intrinsic pathway took place in SH-SY5Y cells by showing the mitochondrial translocation of a GFP-Bax construct on transfection and treatment with cyclin-dependent kinase inhibitory drugs. Finally, we tested the effect of olomoucine and roscovitine on wild-type, *bax*^{-/-}, *bak*^{-/-}, and double *bax*^{-/-}*bak*^{-/-} mouse embryonic fibroblasts (MEF). In wild-type MEFs, both drugs induced cell death by apoptosis in a dose-dependent manner. In *bax*^{-/-}, *bak*^{-/-}, and, particularly, double *bax*^{-/-}*bak*^{-/-} MEFs, we observed the inhibition of apoptosis. In conclusion, olomoucine and roscovitine

caused apoptosis through an intrinsic pathway, with Bax and Bak proteins being involved. [Mol Cancer Ther 2008;7(12):3800–6]

Introduction

Cyclin-dependent kinases (CDK) have been an obvious target for inhibitory drug research because of their pivotal role in cell cycle regulation (1). Two CDK inhibitory drugs, flavopiridol (alvociclib) and *R*-roscovitine (selecciclib), have been the first to be used in humans and reached advanced phases of clinical development in cancer. Both interact at the ATP-binding site of CDKs but differ in chemical structure and selectivity. Both are pan-CDK inhibitors, but roscovitine does not include CDK4 and CDK6 in its spectrum of inhibition (2). Olomoucine, a 2,6,9-trisubstituted purine-like roscovitine, shares the same spectrum of inhibition. Olomoucine displays less pharmacologic potency and possesses one inactive isomer, iso-olomoucine.

The induction of apoptosis was a frequently reported action for these types of drugs, yet the mode of induction was not approached until a few years ago. For instance, roscovitine proved capable to activate p53 and up-regulate the p53AIP1 protein, an apoptosis inducer acting on mitochondria (3). However, p53 was not necessary for *R*-roscovitine to cause apoptosis in B-cell chronic lymphocytic leukemia (4). Consistently, we have found no indication of roscovitine resistance in cell lines deficient in p53, such as HL-60 or Jurkat.³ Several reports revealed a marked down-regulation of the Mcl-1 protein in the apoptotic processes triggered by *R*-roscovitine (5–7). Mcl-1 is an antiapoptotic protein analogous to Bcl-2 and Bcl-XL proteins, characterized by being a proteasomal substrate and having an elevated turnover. This fact and the inhibitory effect imposed on transcription by CDK inhibitory drugs explained the down-regulation of Mcl-1 (4, 6, 7). These reports led to two additional conclusions: (a) the interest of CDK9 as a target for drug development (8) and (b) the suggestion of an intrinsic or mitochondrial pathway was underlying the induction of apoptosis by CDK inhibitory drugs.

The cell line SH-SY5Y is derived from the metastasis of a human neuroblastoma and has become our model in the investigation of new therapies for this type of pediatric cancer. In this cell line, we have characterized the apoptotic process triggered by staurosporine (9), olomoucine, and roscovitine (10). In these cells, we have discarded the extrinsic pathway as the mode of apoptosis triggered by olomoucine and roscovitine (11). The purpose of this work was to provide conclusive evidence that these drugs caused

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³ J. Ribas and J. Boix, unpublished results.

apoptosis by the intrinsic pathway. In SH-SY5Y cells, an intrinsic pathway was confirmed by showing the translocation of Bax protein. Finally, we showed that Bax and Bak proteins were necessary for olomoucine and roscovitine to generate apoptosis in mouse embryonic fibroblasts (MEF).

Materials and Methods

Chemical Reagents

Cytotox 96 kit was supplied by Promega Biotech Ibérica. The AcDEVDafc (acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin) was purchased from MP Biomedicals Europe. Roscovitine and iso-olomoucine were acquired from Calbiochem. Olomoucine was purchased from Tocris. Staurosporine, bisbenzimidazole (Hoechst 33342), and propidium iodide were obtained from Sigma. Unless otherwise stated, the nonlisted reagents were also from Sigma.

Cell Culture and Drug Treatments

The immortalized MEFs derived from double *bax*^{-/-} *bak*^{-/-} mice and their wild-type counterparts were obtained from Dr. S.J. Korsmeyer (12, 13). SH-SY5Y is a cell line derived from the metastasis of a human neuroblastoma and was obtained from the American Type Culture Collection. The SH-SY5Y cell lines stably transfected with the pcDNA3/Bcl-XL and empty pcDNA3 vectors have been characterized in previous reports (9, 10, 14). Both, MEFs and SH-SY5Y cell lines were grown in 2 mmol/L L-glutamine supplemented DMEM plus antibiotics and a 10% volume of FCS (Invitrogen). General culturing conditions were 37°C and a water-saturated, 5% CO₂ atmosphere. Culture dishes and other plastic disposable tools were supplied by Iwaki, Sarstedt, Bibby Sterilin, and Becton Dickinson. Stock

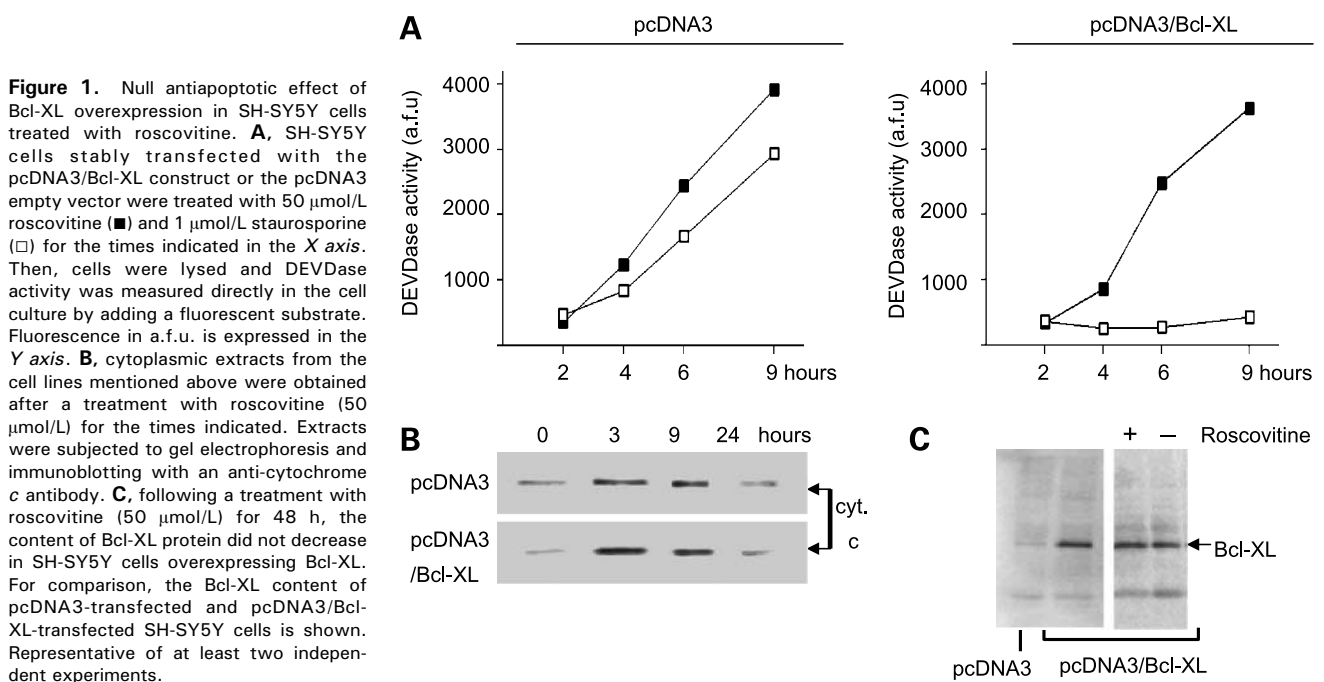
solutions of olomoucine, roscovitine, iso-olomoucine, and the other compounds used as drugs were prepared in DMSO. From these stock solutions, drugs were delivered into the culture medium and adjusted to the final concentrations reported in the text and figures. Drugs were serially diluted to determine the concentration-dependent toxicity. DMSO percentage never surpassed 1% in the culture medium, a concentration determined previously to be innocuous for these cells in culture.

Cell Death and Apoptosis Assessment

To quantify the ratios of cell death in the culture plates, the Cytotox 96 kit was used. The kit allows LDH quantification in the culture medium and has been routinely used in our experiments (10). To assess apoptosis in the cell cultures, two independent procedures were employed. First, nuclear morphology was evidenced by direct 0.05 µg/mL bisbenzimidazole (Hoechst 33342) plus 25 µg/mL propidium iodide double staining and fluorescence microscopy. Second, effector caspase activation was measured as DEVDase activity. This procedure quantified the fluorescence released from an AcDEVDafc substrate after its direct addition to the culture medium, cell lysis, and incubation at 37°C (11).

Detection of Mitochondrial Cytochrome *c* Release and Bcl-XL Content

To detect cytochrome *c* release, cytosolic extracts were obtained as standardized previously (15). These extracts were subjected to Western blotting with a rabbit polyclonal anti-cytochrome *c* antibody (Santa Cruz Biotechnology) at a dilution of 1:1,000 (16). To ascertain the Bcl-XL content, whole-cell extracts were obtained in buffer containing 100 mmol/L Tris-HCl (pH 6.8), 1 mmol/L EDTA, and 2% SDS. Following heat denaturation for 3 min, equal amounts



of total protein were separated in 12% SDS-PAGE and transferred to 0.45 μm nitrocellulose filters (Schleicher & Schuell) and filters were processed by the One-Step Complete Western kit (GenScript) according to the protocol provided with the kit. Anti-Bcl-XL monoclonal antibody was used at a dilution of 1:2,000 and acquired from Transduction Laboratories. Chemiluminescence was recorded by a Chemidoc XRS system (Bio-Rad).

In vitro Determination of Mitochondrial Permeability Transition

Mitochondria were isolated from livers of adult Sprague-Dawley rats by means of the procedure we have described for brain tissue (16). The permeability transition implies the swelling of mitochondria, a physical phenomenon detectable by spectrophotometry (17). Mitochondria were suspended to reach a protein concentration of 1 mg/mL in 200 mL of a solution containing 125 mmol/L KCl, 20 mmol/L HEPES, 2 mmol/L KH_2PO_4 , 1 mmol/L EGTA, 1 mmol/L MgCl_2 , 5 mmol/L malate, and 5 mmol/L glutamate with the pH adjusted to 7.08 with KOH. The decay of A_{540} , indicating mitochondrial swelling, was determined following the addition of the different compounds to be tested. Initial A_{540} values neared 0.8. Minor differences in the loading of the microplate wells were normalized by representing the data as the fraction of the initial absorbance determination remaining at a given time.

Evaluation of the Mitochondrial Translocation of GFP-Bax

The procedure was based on a transient transfection of SH-SY5Y cell with a fluorescent GFP-Bax construct and confocal microscopy as reported previously (18). In brief, SH-SY5Y cells were plated for 24 h before transfection at a density of 5.3×10^4 cells/cm² on poly(D-lysine)-coated glass slides. Transfection was done with a plasmid containing GFP-Bax (19) and the Lipofectamine reagent (Invitrogen). After 4 h coinubation, the transfection mixture was removed and replaced with fresh complete medium. Then, cells were incubated for 12 h to allow for sufficient GFP-Bax expression and then treated for 24 h with the different compounds to be tested. Cells were fixed in 4% paraformaldehyde and confocal microscopy was done. Mitochondrial translocation of the construct implied the shift from a cytoplasmic diffuse green fluorescence to a brighter spotted one localized to the mitochondria. The total number of transfected cells and those displaying the spotted pattern were quantified. The percentage of spotted cells over transfected cells was calculated, mean \pm SD of several independent experiments was obtained, and statistical signification was assessed by a Student's two tailed, unpaired *t* test. *P* values < 0.05 are considered significant.

Results

Analysis of the Intrinsic Pathway in Apoptotic SH-SY5Y Cells Treated with CDK Inhibitory Drugs

In a previous study, we were surprised that SH-SY5Y cells overexpressing either Bcl-2 or Bcl-XL proteins were not protected from apoptosis triggered by olomoucine and

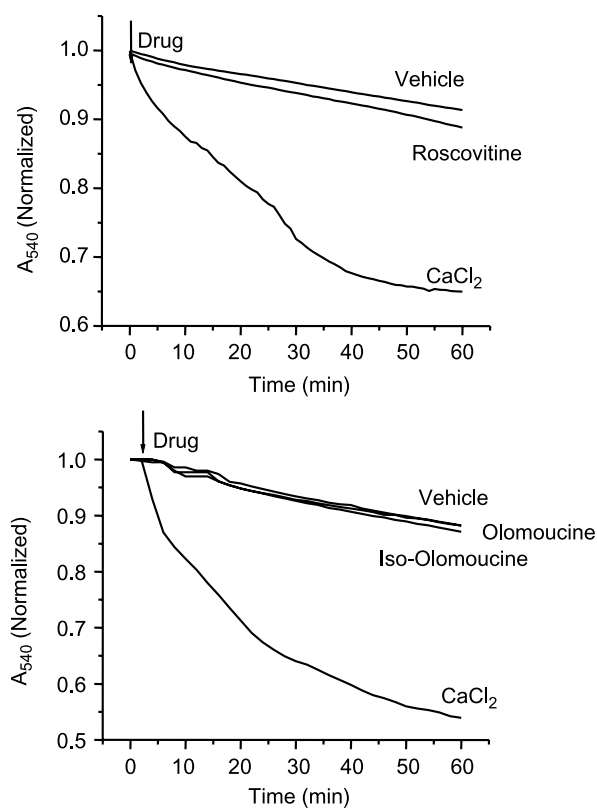
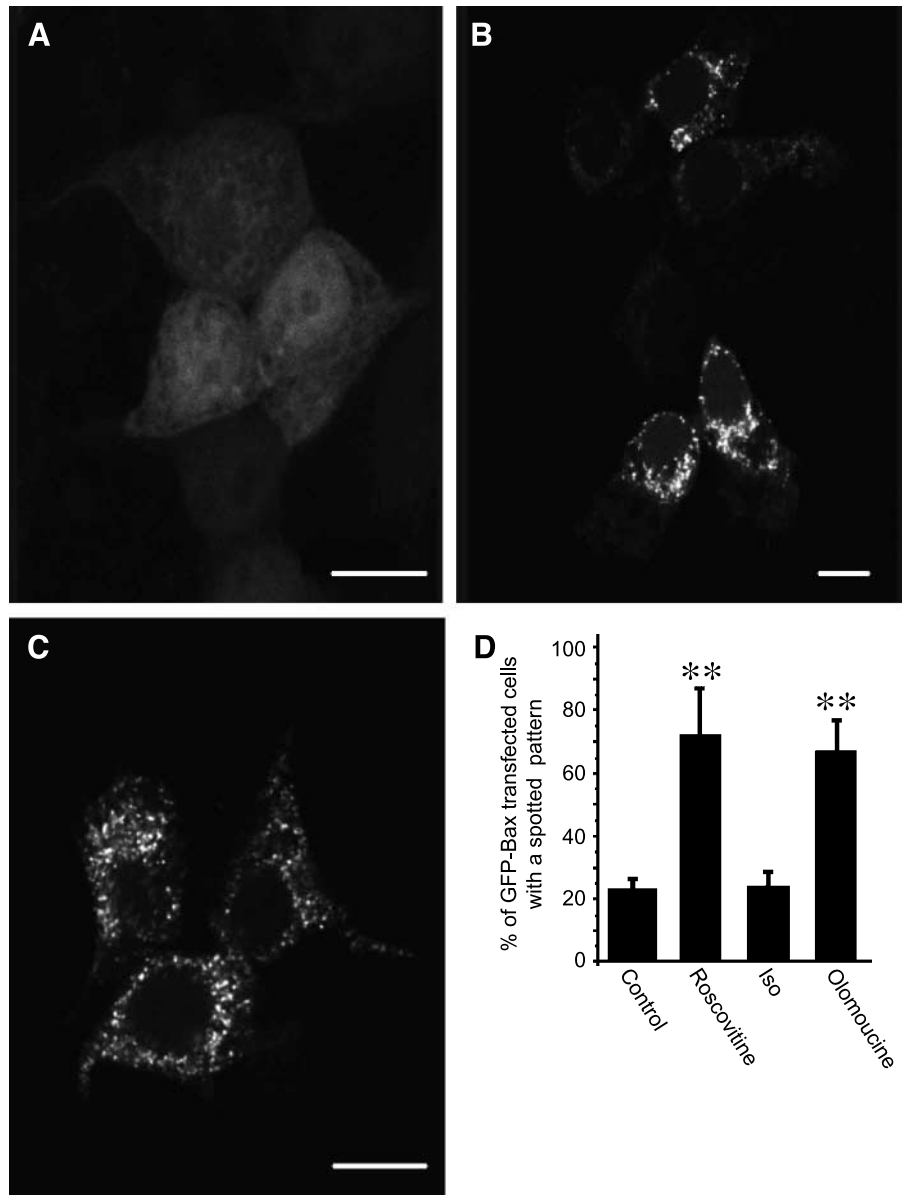


Figure 2. CDK inhibitory drugs do not promote swelling of isolated mitochondria. A_{540} was recorded after addition of roscovitine (50 $\mu\text{mol/L}$), olomoucine (200 $\mu\text{mol/L}$), and iso-olomoucine (200 $\mu\text{mol/L}$) to isolated liver mitochondria. CaCl_2 (75 $\mu\text{mol/L}$) was used as a positive control for liver mitochondrial swelling. Mean obtained from one experiment done by triplicate. Similar data were found in at least five different mitochondria preparations.

roscovitine (10). Bcl-2 and Bcl-XL proteins were active because these cells displayed significant protection from staurosporine (9). Remarkably, the extent of protection from Bcl-XL to SH-SY5Y cells surpassed that of Bcl-2 (9). These conclusions were further supported by assessing the kinetics of effector caspase activation (DEVDase activity). In a population of SH-SY5Y cells stably transfected with the empty pcDNA3 vector, DEVDase activity increased in a time-dependent manner on a treatment with either roscovitine (50 $\mu\text{mol/L}$) or staurosporine (1 $\mu\text{mol/L}$). An analogous population expressing the pcDNA3/Bcl-XL construct became refractory to caspase activation by staurosporine (1 $\mu\text{mol/L}$) but not to by roscovitine (50 $\mu\text{mol/L}$). Moreover, the kinetics of caspase activation by roscovitine was the same in the pcDNA3/Bcl-XL-transfected cells and the pcDNA3 control (Fig. 1A).

In this context, we speculated whether the failure of Bcl-XL to prevent caspase activation and apoptosis correlated with an inability to prevent cytochrome *c* release from mitochondria. We characterized the kinetics of cytochrome *c* release in control SH-SY5Y cells as well as SH-SY5Y cells overexpressing Bcl-XL. We observed that Bcl-XL was

Figure 3. Roscovitine and olomoucine induce GFP-Bax translocation to mitochondria. **A**, SH-SY5Y cells transfected with GFP-Bax as reported in Materials and Methods and left untreated. **B**, the same transfected cells after a treatment with 50 $\mu\text{mol/L}$ roscovitine for 24 h. **C**, the same transfected cells after a treatment with 200 $\mu\text{mol/L}$ olomoucine for 24 h. **D**, quantification of SH-SY5Y cells with GFP-Bax translocated to mitochondria after 24 h treatment with 50 $\mu\text{mol/L}$ roscovitine, 200 $\mu\text{mol/L}$ iso-olomoucine, 200 $\mu\text{mol/L}$ olomoucine, and untreated controls (*X axis*). Percentages are mean \pm SD of four separate experiments, each done in triplicate (*Y axis*). **, $P < 0.01$, versus control. Bar, 12 μm .



neither preventing nor delaying the release of cytochrome *c* (Fig. 1B). In conclusion, cell death, caspase activation, and cytochrome *c* release were completely eluding the control of Bcl-XL. Because an extrinsic pathway had been discarded (11), the results seemed to indicate a Bcl-2- and Bcl-XL-insensitive intrinsic pathway as the mode of apoptosis induction by roscovitine.

Before characterizing this issue further, we questioned the increased content of Bcl-2 and Bcl-XL in the transfected SH-SY5Y populations treated with roscovitine. Roscovitine is an inhibitor of CDK7 and CDK9 and, as a consequence, of transcription initiation and elongation (8). As we have reported previously, the basal expression of Bcl-XL protein is very low in SH-SY5Y cells. The elevated Bcl-XL content of

the transfected population did not decrease after treatment with roscovitine (50 $\mu\text{mol/L}$) for 48 h (Fig. 1C). Similar results were obtained in the Bcl-2-overexpressing SH-SY5Y cells (data not shown). Differing from their Mcl-1 relative, Bcl-2 and Bcl-XL proteins are characterized by a reduced turnover. For instance, after treatment with cycloheximide for 48 h on Bcl-2-transfected N18 neuroblastoma cells, there was no change in Bcl-2 protein levels (20).

Next, we investigated the possibility of CDK inhibitory drugs acting directly on mitochondria causing a membrane dysfunction, mitochondrial permeability transition, and swelling. Mitochondria from rat hepatocytes were isolated, incubated with these drugs, and checked for swelling as reported previously (16). Olomoucine and roscovitine were

used at apoptosis-inducing concentrations and 75 $\mu\text{mol/L}$ CaCl_2 as a positive control (Fig. 2). Neither olomoucine nor roscovitine disturbed mitochondrial permeability.

Searching for evidence of an intrinsic pathway in SH-SY5Y cells treated with olomoucine or roscovitine, we tested if Bax protein was translocating from the cytoplasm to mitochondria. This issue was approached by transiently transfecting SH-SY5Y cells with a GFP-Bax construct and visualizing the fluorescence pattern after a specific drug treatment (18). On translocation, the fluorescence changed from a diffuse, cytosolic pattern (Fig. 3A) to a spotted, mitochondrial pattern (Fig. 3B and C). This was quantified and found that olomoucine and roscovitine were significantly inducing Bax translocation (Fig. 3D). In conclusion, CDK inhibitory drugs were activating an intrinsic or mitochondrial pathway in SH-SY5Y cells.

Bax and Bak Proteins Mediate Apoptosis Triggered by CDK Inhibitory Drugs

To obtain a definitive proof of the involvement of the mitochondrial mechanisms of apoptosis in the mode of action of CDK inhibitory drugs, we tested the effects of these drugs on MEFs deficient in Bax, Bak, double deficient in both proteins, and their wild-type control (12, 13). Olomoucine and roscovitine caused cell death in a concentration-dependent manner on wild-type MEFs (Fig. 4A). Based on these results, olomoucine (400 $\mu\text{mol/L}$) and roscovitine (100 $\mu\text{mol/L}$) were chosen for the subsequent cell death studies in MEFs. Interestingly, no cell

death was observed in the double $\text{bax}^{-/-}\text{bak}^{-/-}$ MEFs (Fig. 4A). Next, we compared the four types of MEFs treated with olomoucine and roscovitine (Fig. 4B). The single deficiency of either Bax or Bak protein conferred resistance to both drugs. As shown, the single deficiency of Bak afforded a slightly greater protection than that of Bax. In summary, both Bax and Bak proteins are involved and required for olomoucine and roscovitine to cause cell death.

Although the mechanism of cell death was expected to be apoptotic, this has not been addressed in the experiments reported. Therefore, we analyzed cell morphology following treatment with these drugs for 48 h using fluorescence microscopy after double staining with bisbenzimidazole and propidium iodide (Fig. 5). As shown, CDK inhibitory drugs yielded typical apoptotic images in wild-type but not in $\text{bax}^{-/-}\text{bak}^{-/-}$ MEFs. In addition to morphology, we also checked a biochemical variable of apoptosis, precisely the activation of effector caspases (Fig. 6). The four types of MEFs were compared following a treatment of 24 h with olomoucine, roscovitine, and staurosporine. No caspase activation was found in $\text{bax}^{-/-}\text{bak}^{-/-}$ MEFs treated with any of the drugs. On the other hand, the single Bak deficiency inhibited effector caspase activation to a greater extent than Bax deficiency. This was coherent with the previous cell death data. In conclusion, an impaired intrinsic or mitochondrial pathway of apoptosis (double $\text{bax}^{-/-}\text{bak}^{-/-}$ deficiency) results in complete resistance of cells to apoptosis triggered by two CDK inhibitory drugs, olomoucine and roscovitine.

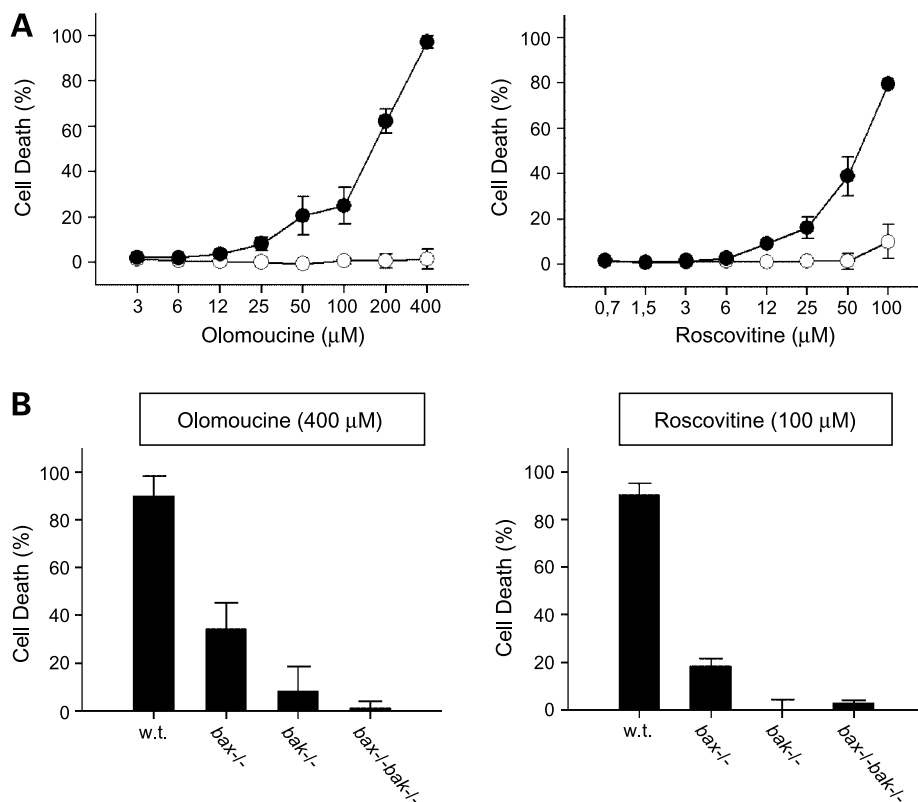


Figure 4. Cell death triggered by CDK inhibitory drugs is prevented by the double deficiency in Bax and Bak proteins. **A**, concentration dependency of olomoucine- and roscovitine-induced lethality was determined at 48 h treatment in wild-type and double $\text{bax}^{-/-}\text{bak}^{-/-}$ MEFs. **B**, wild-type, $\text{bax}^{-/-}$, $\text{bak}^{-/-}$, and $\text{bax}^{-/-}\text{bak}^{-/-}$ MEFs were treated for 48 h. Cell death was determined by the LDH release procedure. Mean \pm SE of at least two independent experiments. Each LDH determination was done in triplicate.

Discussion

As we have just shown, the deficiency of both Bax and Bak proteins totally protects from apoptosis triggered by CDK inhibitory drugs. This protection indicates that these pharmacologic agents are engaging an intrinsic or mitochondrial pathway to cause apoptosis in MEFs. Moreover, in SH-SY5Y cells, we have also provided evidence of Bax protein involvement and therefore an intrinsic pathway in response to these drugs.

As far as we know, this is the first report testing the effects of olomoucine and roscovitine on *bax*^{-/-}, *bak*^{-/-}, and *bax*^{-/-}*bak*^{-/-} MEFs. In one report, these MEFs were treated with ABT-737 plus roscovitine in the context of characterizing a synergism between both compounds (21). ABT-737 is a very promising compound that mimics the BH3 domain of proapoptotic Bad protein. Interestingly, double *bax*^{-/-}*bak*^{-/-} MEFs were completely resistant to the ABT-737 plus roscovitine treatment, similar to what we have found for olomoucine or roscovitine in a single-drug treatment. In that report, *bax*^{-/-} MEFs surpassed *bak*^{-/-} MEFs in apoptosis resistance. In contrast, we have found increased protection for *bak*^{-/-} MEFs facing either olomoucine or roscovitine alone. We believe this reflects a

difference in the mechanism to engage the intrinsic pathway. In this report, we have used lethal concentrations of roscovitine and olomoucine. In the ABT-737 report, roscovitine was used at 12 $\mu\text{mol/L}$ and combined with ABT-737. If used alone, roscovitine is sublethal at 24 to 48 h of treatment within a range of concentrations 10 to 15 $\mu\text{mol/L}$ in all cell lines we have tested thus far. This suggests that the results in the ABT-737 plus roscovitine report are probably informing more about the effects of ABT-737 than roscovitine. For instance, in neuroblastoma cell lines, we have also described a synergism between *R*-roscovitine (10 $\mu\text{mol/L}$) and Nutlin-3, a p53 activator compound (14). Remarkably, our Bcl-XL-overexpressing SH-SY5Y cells were protected from Nutlin-3 as well as the combined treatment of Nutlin-3 and roscovitine, thus indicating the predominance of the Nutlin-3 mode of apoptosis induction in the synergism.

Currently, most reports addressing the mode of apoptosis induction by roscovitine and related compounds seem to converge on the prominent role of transcription inhibition and Mcl-1 down-regulation (4–7). Mcl-1 is an antiapoptotic member of the Bcl-2 family; its down-regulation causes apoptosis. This supports our conclusion that an intrinsic pathway is underlying apoptosis by CDK

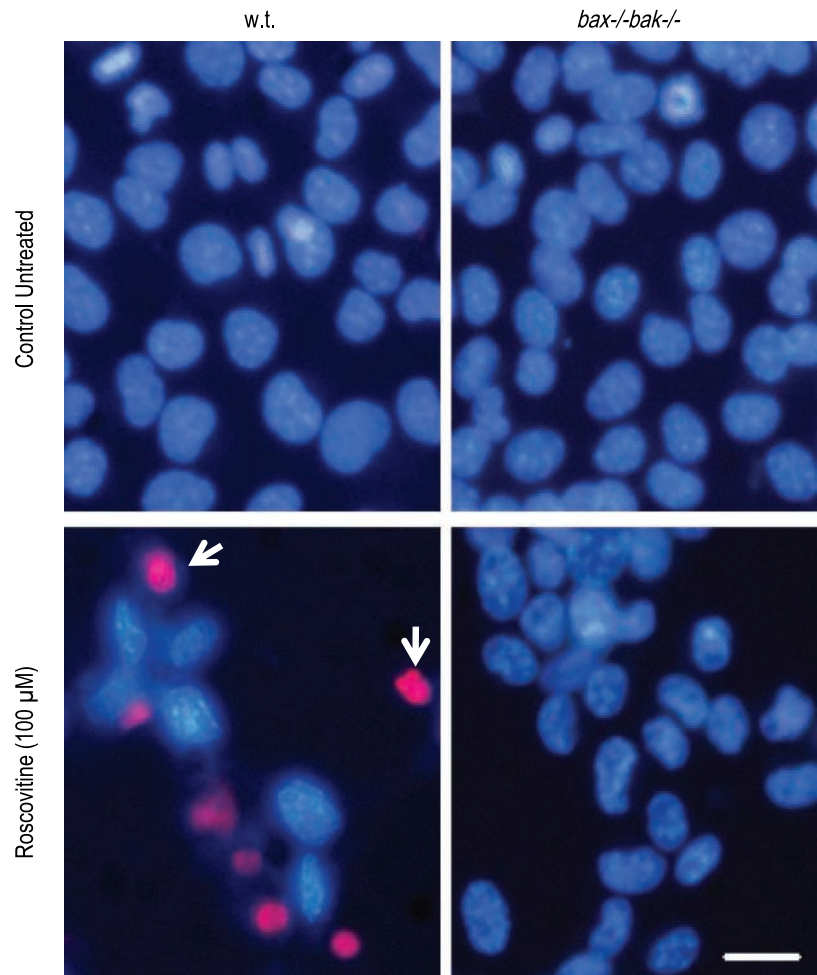


Figure 5. Apoptosis triggered by CDK inhibitory drugs is blocked by the double deficiency of Bax and Bak proteins. Double *bax*^{-/-}*bak*^{-/-} and wild-type MEFs were treated for 48 h. Fluorescence microscopy was done following a double staining with bisbenzamide and propidium iodide. Dead cells with typical apoptotic nuclei are observed (arrows). Bar, 20 μm .

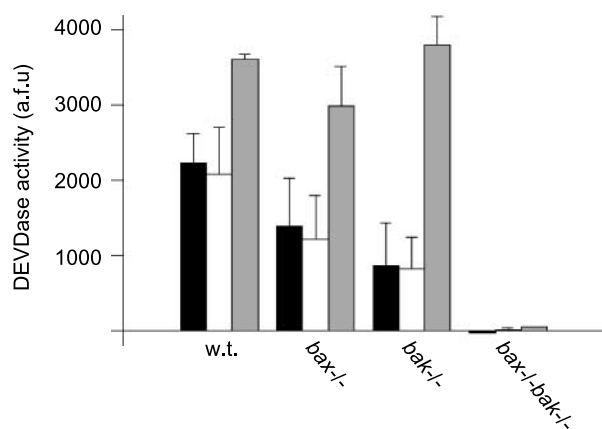


Figure 6. Activation of effector caspases by CDK inhibitory drugs is inhibited in Bax- and Bak-deficient MEFs. As stated, wild-type, *bax*^{-/-}, *bak*^{-/-}, and *bax*^{-/-}*bak*^{-/-} MEFs were treated for 24 h with 400 μmol/L olomoucine (black columns), 100 μmol/L roscovitine (white columns), and 1 μmol/L staurosporine (gray bars). The activity of effector caspases (DEVDase activity) was then measured in a.f.u. Mean ± SE of two independent experiments, each one containing six independent replicates.

inhibitory drugs. However, Mcl-1 overexpression seemed only to delay but not to inhibit the apoptotic process in Ramos cells challenged with *R*-roscovitine (22). Therefore, many questions remain concerning the apoptotic mechanisms at the mitochondria surface activated by CDK inhibitory drugs. For instance, in Fig. 1B, why is Bcl-XL unable to counteract not only apoptosis but also the pace of cytochrome *c* release in the cell population? What is the reason for Bcl-2, null (10, 22–24), and Mcl-1, scarce (22), protective effect on CDK inhibitory drug-induced apoptosis? In this report, we have discarded as a possible explanation both the reduction of Bcl-XL content due to transcriptional inhibition and the direct effect of these drugs on mitochondrial permeability transition.

In conclusion, we have no doubt that CDK inhibitory drugs are activating an intrinsic pathway to cause apoptosis. However, the null or reduced effect of antiapoptotic Bcl-2, Bcl-XL, and Mcl-1 proteins remains to be satisfactorily explained. We are convinced that the results we have just reported will contribute to circumscribe the future research on the mode of apoptosis induction by CDK inhibitory drugs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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