

# **Caspase 8/10 are not mediating apoptosis in neuroblastoma cells treated with CDK inhibitory drugs**

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## **ABSTRACT**

Olomoucine and Roscovitine are pharmacological inhibitors of cyclin-dependent kinases (CDK) displaying a promising profile as anticancer agents. Both compounds are effective inducers of apoptosis in a human neuroblastoma cell line, SH-SY5Y. The characterization of this process had suggested the involvement of an extrinsic pathway (Ribas and Boix, 2004), which depends on either Caspase 8 or Caspase 10 activation. However, neither Caspase 8 nor Caspase 10 is expressed in SH-SY5Y cells because of gene silencing. Upon Olomoucine or Roscovitine treatment, no re-expression of Caspase 8 or Caspase 10 was found. Therefore, in SH-SY5Y cells, this type of drugs is not triggering a canonical, Caspase 8/10-mediated, extrinsic apoptotic pathway.

*Key words:* Olomoucine, Roscovitine, Caspase, SH-SY5Y, Jurkat

## 1. Introduction

Olomoucine and Roscovitine are drugs with an inhibitory effect on cyclin-dependent kinases (CDK). They are characterized by inhibiting CDK1, CDK2 and CDK5, but not CDK4 and CDK6. As a consequence, both compounds stop cell cycle and cell proliferation. In addition, apoptosis is also induced in many cell types. These cellular effects oppose neoplastic development and, as a consequence, neoplasia becomes an obvious target for these drugs (Knockaert et al., 2002).

Little is known about the cellular events connecting drug-induced CDK dysfunction and apoptosis. In order to investigate these events, we have characterized the apoptotic process triggered by Olomoucine (200 $\mu$ M) and Roscovitine (50 $\mu$ M) in SH-SY5Y cells (Ribas and Boix, 2004). Our results have suggested the possibility of apoptosis being initiated at a membrane death receptor (Fas-like) and being transmitted by Caspase 8/10. However, Caspase8 and Caspase10 proteins are not expressed in SH-SY5Y cells because of gene silencing due to DNA methylation (Eggert et al., 2001). Therefore, we hypothesized Olomoucine and Roscovitine could be inducing the re-expression of Caspase 8, Caspase 10 or both, thus triggering apoptosis from an undetermined death receptor. By means of Western blot studies and, particularly, by a kinetic analysis of Caspase 8/10 activation, this hypothesis has been ruled out.

## 2. Materials and Methods

Roscovitine and Iso-Olomoucine were purchased from Calbiochem (San Diego, CA, USA). Olomoucine was acquired from Tocris (Bristol, UK). zVADfmk (Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; Catalog # N-1510) was supplied by Bachem (Bubendorf, Switzerland). CellTiter 96® kit, containing the MTS reagent (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxy methoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt), was provided by Promega (Madison, WI, USA). Cell viability (%) was measured by the capability of cells in culture to reduce MTS. Cell death (%) = 100 - cell viability (%).

SH-SY5Y cells were grown in DMEM medium supplemented with 2 mM L-Glutamine. Jurkat cells were grown in RPMI medium. Final media contained 10% volume of fetal calf serum. The liquid media and the fetal calf serum were supplied by Invitrogen (Barcelona, Spain). Cell cultures were maintained in a 95% air, 5% CO<sub>2</sub>, water saturated atmosphere at 37°C. Drugs were dissolved in DMSO (Dimethyl Sulfoxide) to provide the stock solutions of Olomoucine (50 mM), Iso-Olomoucine (50 mM), Roscovitine (20 mM) and zVADfmk (20 mM). From the stock solutions, drugs were added to the cultures at the final concentrations reported in the text and figures. Anti-Fas (human, activating), clone CH11, monoclonal antibody was supplied by Upstate biotechnology (Charlottesville, VA, USA) and used at the final concentrations further reported.

Cells extracts for Western blot analysis were performed as follows. Cells were washed with PBS (Phosphate Buffered Saline) at 4°C and lysed with a lysis-buffer containing 1% Nonidet P-40 in 50 mM Tris/HCl (pH 7.2), 5 mM EDTA, 1 mM PMSF (Phenylmethanesulfonyl Fluoride), 2 µg/ml Aprotinin, 1 µg/ml Leupeptin and 1 µg/ml Pepstatin. Pellets were gently disrupted by pipetting and extraction let to proceed on ice for 5 min. Following a centrifugation at 0 °C and 12000 g for 15 min, a clear supernatant devoid of nuclei was obtained. Protein concentration was determined by means of the DC Protein Assay (BioRad, Hercules, CA, USA) and equal protein loads were subjected to electrophoresis in 12% polyacrylamide gels. Then, proteins were transferred to PVDF (Polyvinylidene difluoride) membranes (Amersham, Buckinghamshire, UK) by means of a MINI® trans-blot module from BioRad. Membranes were reacted with the following antibodies: Anti-Caspase 8 (1:250 dilution; Catalog # AM46T) from Oncogene Research Products (San Diego, CA, USA); Anti-Caspase 10 (1:1000 dilution; Catalog # M059-3) from MBL (Woburn, MA, USA); Anti-αTubulin (1: 2000 dilution; Catalog # T5168) from Sigma (St. Louis, MO, USA). Secondary peroxidase conjugated antibodies were also from Sigma. Immunoblots were finally developed by means of the SuperSignal® West Dura reagent from Pierce (Rockford, IL, USA).

The kinetics and extent of effector Caspase activation (Caspase 3 and Caspase 7) was determined by the proteolysis of AcDEVDafc (Acetyl-Asp-Glu-Val-Asp-7-amino-4-(trifluoromethyl) coumarin), a fluorogenic substrate measuring DEVDase activity. Assays on cultured cells in 96 multiwell plates were performed as we have reported before (Yuste et al., 2001; Ribas and Boix, 2004). The same procedure was applied to quantify the proteolysis of AcIETDafc (Acetyl-Ile-Glu-Thr-Asp-7-amino-4-(trifluoromethyl) coumarin), a fluorogenic substrate for either Caspase 8 or Caspase 10 (IETDase activity). AcDEVDafc and AcIETDafc were obtained from Enzyme Systems Products (Livermore, CA, USA).

### 3. Results

In order to test if Olomoucine (200  $\mu$ M) or Roscovitine (50  $\mu$ M) were inducing apoptosis through the re-expression of Caspase 8 or Caspase 10 in SH-SY5Y cells, our first approach was to characterize the content of Caspase 8 and Caspase 10 zymogens in these cells facing these drugs. At 9 h of treatment most cells are either apoptotic or committed to apoptosis, this fact defined the time course shown in Fig. 1. Even when films were overexposed, no band compatible with Caspase 8 zymogen was detected. Concerning Caspase 10 zymogen detection, the lack of specificity of some commercial antibodies had been reported (Kischkel et al., 2001). We tested some of those reported as specific and, indeed, they usually were in Jurkat extracts but not in SH-SY5Y ones (Fig. 1). However, despite of the background, no band with the mobility expected for Caspase 10 zymogen was found. We wondered if the activation by proteolysis of the zymogens could mask the re-expression of Caspase 8/10. To control this event, we repeated the time course analysis in the presence of 100  $\mu$ M zVADfmk, a general inhibitor of caspases. As seen in Fig. 1, the presence of zVADfmk made no difference. In conclusion, neither Caspase 8 nor Caspase 10 seemed to be re-expressed upon Olomoucine or Roscovitine treatments.

To better support this result, we decided to perform an alternative approach, the kinetic analysis of caspase activation by these drugs (Fig. 2). Caspase 8 and Caspase 10 share very similar affinities towards the aminoacids they target. The motif IETD is cleaved by both enzymes. As a consequence, the

cleavage and afc (7-amino-4-(trifluoromethyl) coumarin) fluorochrom release from the synthetic peptide Ac-IETD-afc, provides a method to detect and quantify Caspase 8/10 activation (IETDase activity). Analogously, Caspase 3 and Caspase 7 show a similar affinity for the motif DEVD. Both are known to be effector caspases placed downstream of the apoptotic cascade of caspase activation. As a consequence, the fluorescence released by the cleavage of the synthetic peptide AcDEVDafc measures the activation of these effector capases (DEVDase activity). In addition, it indicates that apoptosis is taking place in the cell culture.

We proceeded to determine IETDase and DEVDase activities in SH-SY5Y cell cultures treated with either Olomoucine (200  $\mu$ M) or Roscovitine (50  $\mu$ M). As shown in Fig. 2, apoptosis and DEVDase activity increased in a time-dependent manner while IETDase activity was absent. As a negative control, we assayed Iso-Olomoucine (200  $\mu$ M), an inactive isomer of Olomoucine, and as expected IETDase and DEVDase activities remained at basal levels (not shown). SH-SY5Y cells were also treated with a Fas activating antibody (CH11 clone). Different antibody concentrations were tested up to 250 ng/ml and neither IETDase nor DEVDase activities were seen (Fig. 2).

In order to control the specificity of our approach and the activity of the CH11 antibody, Jurkat cells were studied. Jurkat cells are known to posses a functional Fas system that induces apoptosis upon stimulation by this antibody. As seen in Fig. 2, Caspase 8/10 dependent IETDase activity was already elevated at 6 h and decayed at 24 h, a time characterized by prominent cell destruction in the Jurkat culture. This result was consistent with the DEVDase activity profile from effector caspases, placed downstream of Caspase 8/10. In these cells, we thought of exploring the effects of Olomoucine (200  $\mu$ M) and Roscovitine (50  $\mu$ M). We found the profile of IETDase activity paralleled that of DEVDase one. Both activities increased in a time-dependent manner, thus emphasizing the near to zero value of the IETDase activity displayed by SH-SY5Y cells under the same treatment. In conclusion, Olomoucine and Roscovitine induce apoptosis in SH-SY5Y without the involvement of Caspase 8/10.

In each experiment, the extent of cell death induced by a 24 h treatment was controlled by means of the MTS reduction assay. The ratios of cell death obtained in SH-SY5Y were:  $58.8 \pm 8.8\%$  for Olomoucine,  $46.7 \pm 8.4\%$  for Roscovitine and  $-5.5 \pm 1.5\%$  for CH11 antibody. In Jurkat cells, the ratios were:  $59.6 \pm 19.7\%$ ,  $61.3 \pm 9\%$  and  $70.5 \pm 4.5\%$  respectively. The values are the mean  $\pm$  S.E.M. from the experiments comprised in Fig. 2 and were consistent with those expected (Ribas and Boix, 2004).

#### 4. Discussion

In this report, we have used two independent methods to demonstrate the null implication of Caspase 8 and Caspase 10 in the apoptotic pathways triggered by Olomoucine and Roscovitine in SH-SY5Y cells. In humans, Caspase 8 and Caspase 10 are constitutive elements of the apoptotic pathways initiated at cell membranes by death receptors, the so called extrinsic pathways (Ashkenazi and Dixit, 1998). Therefore, the activation of a canonical, Caspase 8/10-mediated, extrinsic pathway is not the mode of action of two distinct CDK inhibitory drugs in this paradigmatic neuroblastoma cell line.

The procedure to determine caspase activation has been devised to be applied to multiwell plates (Yuste et al., 2001). As a consequence, pharmacological approaches envisaging multiple simultaneous determinations are easily performed. However, the specificity of fluorogenic peptide substrates has been questioned. To be conclusive, we believe a negative control like Iso-Olomoucine for Olomoucine is required. To assess specificity, we suggest the use of a positive control of the caspase activation subjected to study. Jurkat cells and CH11 antibody have provided the positive control of Caspase 8/10 activation in our experiments. Moreover, Jurkat cells have also generated new information. First, Olomoucine and Roscovitine are effectively inducing apoptosis in this lymphoid cell line. Second, these drugs are activating Caspase 8/10 and, therefore, their involvement cannot be ruled out in these cells. However, Caspase 8 has been reported to be activated downstream of Caspase 3 (Slee, et al., 1999). Therefore, Caspase 8/10 could merely be amplifying and not triggering the caspase cascade in Jurkat cells. In conclusion, Caspase 8/10 implication in apoptosis triggered by Olomoucine and Roscovitine in

Jurkat cells can be a matter of discussion and future research however, as emphasized before, it can be definitively discarded in SH-SY5Y cells.

## **Acknowledgements**

This work has been funded by "Instituto de Salud Carlos III" (PI041488, 2005-2007), Spain. X.G.-A. thanks University of Lleida (Programa de Grups Emergents, 2002) for financial support. J.R. is presently a predoctoral fellow from "Fundació Dr. Pifarré" (Hospital Univ. Arnau de Vilanova, Lleida).

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Fig. 1. Olomoucine and Roscovitine do not trigger Caspase 8/10 zymogen expression in SH-SY5Y cells.

A) SH-SY5Y cells were treated with 200  $\mu$ M Olomoucine for the times stated in the figure. Then, proteins were extracted and analyzed by Western Blot with primary antibodies against Caspase 8, Caspase 10 and  $\alpha$ Tubulin (in order to control the amount of protein loaded per lane). B) SH-SY5Y cells were also treated with 50  $\mu$ M Roscovitine and processed as described before. Note that some cells were simultaneously treated with zVADfmk (100  $\mu$ M) in order to prevent zymogens to undergo autoproteolysis or proteolysis mediated by other caspases. Extracts from Jurkat cells were used as positive controls.

Fig. 2. Olomoucine and Roscovitine do not trigger Caspase 8/10 proteolytic activity in SH-SY5Y cells.

Fluorogenic substrates of Caspase 8/10 (AcIETDafc, white bars) and effector Caspase 3/7 (AcDEVDafc, hatched bars) were used to determine the activation kinetics of these enzymes in SH-SY5Y and Jurkat cells treated with CH11 antibody (250 ng/ml), Olomoucine (200  $\mu$ M) and Roscovitine (50  $\mu$ M). Jurkat cells treated with the CH11 antibody were used as a positive control of Caspase 8/10 activation. The x axis is the time (h) elapsed after the treatment. The y axis is proteolytic activity measured in arbitrary fluorescent units. The bar value is the mean  $\pm$  S.E.M. of two independent experiments with four independent determinations per experiment.

Fig. 1.

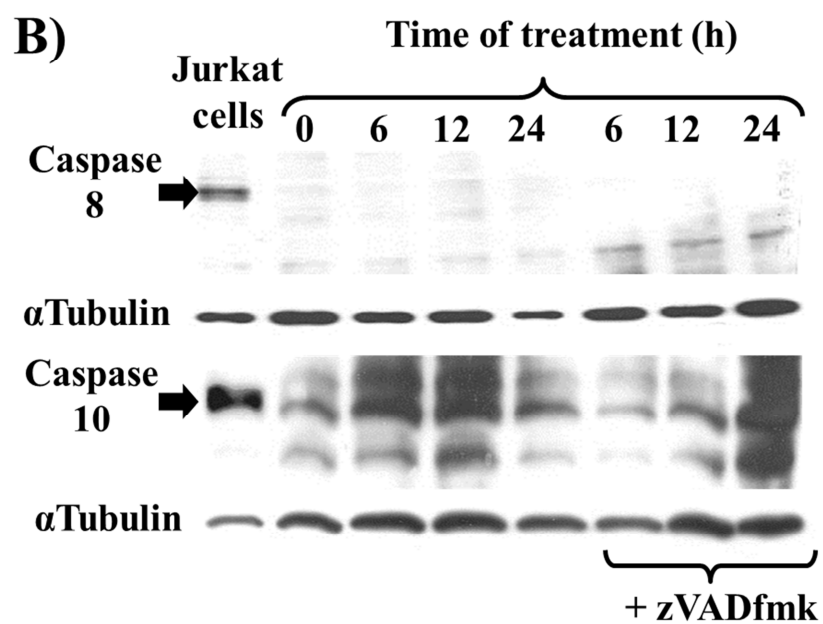
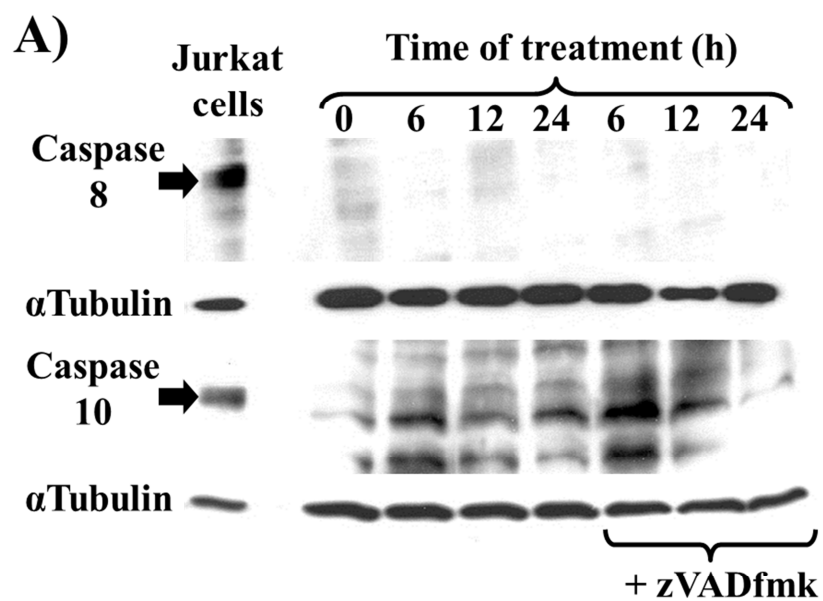


Fig. 2.

