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Cyclosporin A enhances colchicine-induced apoptosis in rat cerebellar granule neurons

[1]Anna Maria Canudas, [1]Elvira G Jordà, [1]Ester Verdaguer, [1]Andrés Jiménez, [2]Francesc Xavier Sureda, [1]Víctor Rimbau, [1]Antoni Camins & *,[1]Mercè Pallàs

[1]Unitat de Farmacologia i Farmacognòsia, Facultat de Farmàcia, Universitat de Barcelona, Nucli Universitari de Pedralbes, Barcelona E-08028, Spain and [2]Unitat de Farmacologia, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, C./St Llorenç 21 43201 Reus (Tarragona), Spain

1 Cyclosporin A (CsA, 1–50 μM), an immunosuppressive drug with known neurotoxic effects, did not decrease the viability of primary cultures of rat cerebellar granule neurons (CGN) or induce apoptotic features. However, CsA specifically enhanced the cytotoxicity and apoptosis induced by colchicine (1 μM).

2 Flavopiridol, an inhibitor of cyclin-dependent kinases (CDKs), prevented the neurotoxic effects of colchicine plus CsA. At 0.1–5 μM, it also showed antiapoptotic effects, as revealed by propidium iodide staining, flow cytometry and counting of cell nuclei.

3 Roscovitine (25–50 μM), a selective cdk1, 2 and 5 inhibitor, showed an antiapoptotic effect against colchicine- and colchicine plus CsA-induced apoptosis.

4 CsA increased the expression of cdk5 and cdk5/p25 mediated by colchicine, a CDK involved in neuronal apoptosis. After treatment of CGN with colchicine plus CsA, the changes in the p25/p35 ratio pointed to cdk5 activation.

5 Immunohistochemical results showed a nuclear localization of cdk5 after neurotoxic treatment, which was prevented by cdk inhibitors. Thus, we propose a new mechanism of modulation of CsA neurotoxicity mediated by cdk5.

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Keywords: Cyclosporin A; colchicine; neurotoxicity; flavopiridol; roscovitine; Cdk5

Abbreviations: AIF, apoptotic inducing factor; CDK, cyclin-dependent kinase; CGN, rat cerebellar granule cells; CsA, cyclosporin A; FSC, forward scatter; OD, optical density; PBS, phosphate-buffered solution; PI, propidium iodide; PTP, permeability transition pore; PVDF, polyvinylidene fluoride; SSC, side scatter

Introduction

Cyclosporin A (CsA) is an immunosuppresive drug that is widely used in organ transplantation and the treatment of autoimmune disorders. The immunosuppressive action of CsA results from binding to cyclophilin, an intracellular immunophilin. The cyclosporine/immunophilin complex inhibits the activity of the calcium-activated phosphatase calcineurin, thus resulting in the accumulation of phosphorylated calcineurin substrates in the cell, including the nuclear factor of activated T cells, which is active only in the nonphosphorylated state.

In addition to its therapeutic use as an immunosuppressive agent, CsA is considered a potent inhibitor of the mitochondrial permeability transition pore (PTP), a multiprotein complex involved in the apoptotic process (Massicot *et al*., 1997). Several proteins have been related to the mitochondrial PTP, namely the mitochondrial voltage-dependent anion channel, the adenine nucleotide translocator, the mitochondrial benzodiazepine receptor, members of the Bax/Bcl 2 families and cyclophilin D. In certain conditions, mitochondria releases through PTP, substrates such as cytochrome *C* and the apoptotic inducing factor (AIF), which modulate the apoptotic process (Leist & Jaattela, 2001; Chang *et al*., 2002).

Given its effects on mitochondrial PTP, CsA shows neuroprotective effects against neurotoxic agents such as glutamate (Brutovetsky & Dubinsky, 2000) and methylmercury (Limke & Atchison, 2002). However, the antiapoptotic effects of CsA are more pronounced in the presence of caspase inhibitors (Chang & Johnson, 2002). On the other hand, it has been reported that CsA enhances rather than inhibits MPP^+-induced apoptosis in SH-SY5Y neuroblastoma cells (Fall & Bennett, 1998) and the NMDA neurotoxic effect in cortical cultures (McDonald *et al*., 1997). CsA induces apoptosis in mixed neuron/glia (McDonald *et al*., 1996). This may account for the clinically relevant direct neurotoxic effects of CsA, and suggests that it could modulate the neurotoxicity of several agents.

Since the data on the effects of CsA in neuronal preparations are controversial, we examined the neurotoxic effects of CsA in cultures of rat cerebellar granule cells (CGN). The culture of CGN is a widely used model in neuropharmacology to study the pathways of drug-induced apoptosis. For instance, CGN have been used to assess the neurotoxic effects of glutamate (Slagsvold *et al*., 2000), kainic acid (Verdaguer *et al*., 2002a, b), MPP^+ and colchicine (Gorman *et al*., 1999).

Furthermore, as stated above, CsA enhances MPP^+-induced apoptosis in neuroblastoma cells. Previous studies

*Author for correspondence; E-mail: pallas@ub.edu

have shown that colchicine-induced apoptosis in CGN is mediated through cytochrome *C* release and caspase-3 activation (Gorman *et al*., 1999; Volbracht *et al*., 2001; Kim *et al*., 2002). Here we tested whether CsA also modulates colchicine-induced apoptosis.

Methods

Experiments were performed in accordance with the ethical code of the Spanish legislation on Animal Protection and the European Union Directive.

Materials

The following pharmacological agents were used: colchicine, CsA and propidium iodide (PI) from Sigma Chemical Co. (St Louis, MO, U.S.A.); benzyloxycarbonyl-valyl-alanyl-aspartyl fluoromethyl ketone (z-VAD.fmk) from Bachem AG (Bubendorf, Switzerland) and roscovitine from Calbiochem (Darmstadt, Germany). Cell culture media and foetal calf serum (FCS) were from GIBCO (Life Technologies, Paisley, U.K.); cell culture salts, enzymes, Mowiol® 4-88 and Triton X-100 were purchased from Sigma; other chemical reagents of analytical grade were from Panreac Química (Barcelona, Spain). Flavopiridol was a generous gift from Aventis Pharmaceuticals Inc. (Bridgewater, NJ, U.S.A.).

CGN cultures

Primary cultures of rat cerebellar granule neurons were prepared from 7-day-old Sprague–Dawley rat pups, as described elsewhere (Verdaguer *et al*., 2002b). Briefly, the cerebella were freed of meninges, minced, trypsinized and treated with DNAse. Cells were dissociated by repeated pipetting, and separated from nondissociated tissue by sedimentation. The cell density was adjusted to 8×10^5 cells ml^{-1} and cells were plated on poly-L-lysine-coated 96-well plates or glass coverslips in 24-well plates, at a density of 300,000 cells cm^{-2}. Cultures were grown in Eagle's basal medium containing 10% FCS, 2 mM L-glutamine, 0.1 mg ml^{-1} gentamicin and 25 mM of KCl. Cytosine arabinoside (10 μM) was added 16–18 h after plating, to inhibit the growth of non-neuronal cells. Cultures prepared by this method were enriched by more than 95% in granule neurons, assessed by GFAP immunocytochemistry.

Treatment of CGN and survival assay

After 7 days *in vitro* (DIV), CGN were incubated for 24 h in complete medium containing CsA (ranging from 1 to 50 μM). As reported by Bonfoco *et al*. (1995), colchicine induces apoptosis in CGN after 24 h of incubation. We tested the neurotoxic effects of colchicine (1 μM) and colchicine plus CsA (10 μM) after 24 h, in the presence or absence of z-VAD.fmk, flavopiridol and roscovitine.

To assess the loss of cell viability, we used the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) method (Hansen *et al*., 1989). MTT was added to the cells at a final concentration of 250 μM and incubated for 1 h to allow the reduction of MTT to produce a dark blue formazan product. Media were removed, and cells were dissolved in dimethylsulfoxide. The formation of formazan was measured by absorbance change at 595 nm, using a microplate reader (BioRad Laboratories, CA, U.S.A.). Viability results were expressed as percentages. The absorbance measured from nontreated cells was taken to be 100%.

Analysis of apoptosis rate by flow cytometry

Apoptosis was measured 24 h after initiating treatments. The culture medium was removed, cells were collected from culture plates by gentle pipetting and washed with phosphate-buffered solution (PBS). PI (10 μg ml^{-1}) and Triton X-100 (0.1%, v v^{-1}) were added 30 min before cytofluorometric analysis. Flow-cytometer experiments were carried out using an Epics XL flow cytometer (Beckman Coulter Inc., Fullerton, CA, U.S.A.). The instrument was set up with the standard configuration: excitation of the sample was performed using a 488 nm air-cooled argon-ion laser at 15 mW power. Forward scatter (FSC), side scatter (SSC) and red (620 nm) fluorescence for PI were acquired. Optical alignment was based on optimized signal from 10 nm fluorescent beads (Flow-check fluorospheres, Beckman Coulter Inc.). Red fluorescence was projected on a 1024 monoparametrical histogram. Aggregates were excluded by gating single cells by their area vs peak fluorescence signal. Apoptosis was evaluated using appropriate software (WinMDI).

Detection of apoptotic nuclei by PI staining

PI staining was used to obtain morphological evidence of apoptosis (Atabay *et al*., 1996). CGN were grown on poly-L-lysinized glass coverslips and, after treatment, fixed in 4% paraformaldehyde/PBS, pH 7.4 for 1 h at room temperature. After washing with PBS, they were incubated for 3 min with a solution of PI in PBS (10 μg ml^{-1}). Coverslips were mounted in Mowiol® 4–88. Stained cells were visualized under UV illumination at $\times 20$ magnification (Eclipse TE-200, Nikon Corp., Japan) and their images were captured using a digital camera.

The apoptotic cells showed shrunken, brightly fluorescent, apoptotic nuclei with higher fluorescence and condensed chromatin than nonapoptotic cells. Apoptotic nuclei were scored by counting at least 500 cells for each sample in triplicate.

Assay of caspase-3 enzymatic activity

We used the colorimetric substrate Ac-DEVD-*p*-nitroaniline (Oncogene Research Products, Boston, MA, U.S.A.) for the determination of caspase-3 activity using the following method: 24 h after treatment with drugs, CGN were collected in lysis buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 0.1 mM EDTA, pH 7.4). A measure of 0.5 μg μl^{-1} of protein was incubated with 200 μM Ac-DEVD-*p*-nitroaniline in assay buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, 0.1 mM EDTA, pH 7.4) in 96-well plates at 37°C for 24 h. Absorbance of the cleaved product was measured at 405 nm in a microplate reader (BioRad).

Western blot analysis

Aliquots of treated or control cells, containing 30 μg of protein per sample, were placed in sample buffer (0.5 M Tris-HCl pH 6.8, 10% glycerol, 2% (w v^{-1}) SDS, 5% (v v^{-1}) 2-β-mercaptoethanol, 0.05% bromophenol blue) and denatured by boiling at 95–100°C for 5 min. Samples were separated by electrophoresis on 10% acrylamide gels. Thereafter, proteins were transferred to polyvinylidene fluoride (PVDF) sheets (Immobilon™-P, Millipore Corp., Bedford, MA, U.S.A.) using a transblot apparatus (BioRad). Membranes were blocked overnight with 5% nonfat milk dissolved in TBS-T buffer (Tris 50 mM; NaCl 1.5%; Tween 20, 0.05%, pH 7.5). They were then incubated with a primary antibody against cdk5 and p25/35 (1 : 1000, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). After 90 min, blots were washed thoroughly in TBS-T buffer and incubated for 1 h with a peroxidase-conjugated IgG antibody (Amersham Corp., Arlington Heights, IL, U.S.A.). Immunoreactive protein was visualized using a chemiluminescence-based detection kit following the manufacturer protocol (ECL kit; Amersham Corp.). Routinely, protein load was monitored using phenol red staining of the blot membrane.

Cleavage of p35 to p25 by calpains increases cdk5 activity (Patrick *et al*., 1999; Kusakawa *et al*., 2000) and alters the intracellular localization of the cdk5/p25 protein complex. Changes in the optical density (OD) ratio between p25 and p35 bands was taken as an index of cdk5 activation The activity of cdk5 was partially predicted by the levels of p35 and p25 proteins.

Immunocytochemistry against CDK5

CGCs were grown on sterile glass slides. After stimuli, cells were washed twice in PBS and fixed in 4% paraformaldehyde/PBS, pH 7.4 for 1 h at room temperature. Cells were preincubated for 30 min with PBS containing 0.3% Triton X-100 and 30% normal horse serum at room temperature. After blocking, cells were incubated with an antibody against CDK5 (Santacruz, 1 : 100) overnight at 4°C. Cells were then washed extensively and incubated with secondary antibody, for 1 h at room temperature. Coverslips were thoroughly washed and mounted in Mowiol® 4-88 and immunosignal analysis was performed using fluorescence microscopy at $\times$100 magnification (Eclipse, Nikon).

Statistical analysis

Data are given as mean $\pm$ s.e.m. from at least three experiments on 3–6 independent cultures. Data were analyzed by ANOVA, followed by Tukey–Kramer multiple comparison test.

Results

Treatment of CGN with CsA is not neurotoxic

Exposure of CGN to CsA alone (1–50 μM) for 24 or 48 h did not induce neuronal cell death, since MTT values did not significantly differ from control (Figure 1). Treatment of CGN for 24 h with various concentrations of CsA (1–50 μM) did not increase the number of condensed nuclei or apoptotic cells, as measured by flow cytometry (Figures 1 and 6). No other signs of neurotoxicity were detected by microscopic observation of the treated cultures (Figure 7a).

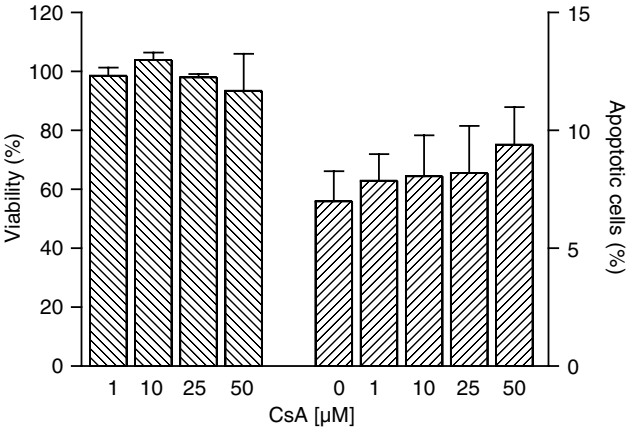

Figure 1 Lack of effect of CsA (1–50 μM, 24 h) on viability (left axis) or the fraction of apoptotic cells (percentage of hypodiploid cells measured by cytometric analysis; right axis). Each point is the mean $\pm$ s.e.m. of four wells from 3–5 cultures.

Effects of CsA on colchicine neurotoxicity

We tested the effects of CsA at concentrations up to 50 μM on 1 μM colchicine-induced neurotoxicity. Colchicine is a neurotoxin that causes cytoskeletal alterations through the inhibition of microtubule assembly. Treatment of CGN with 1 μM colchicine decreased MTT values by 35%. At 1–50 μM, CsA increased colchicine neurotoxicity and induced a maximal MTT decrease of 55%, $P<0.01$ (Figures 2a and 7a).

CsA enhances colchicine-induced apoptosis in CGN

CsA induces apoptosis in several neuronal preparations (Serkova *et al*., 2000). To test whether CsA (at concentrations ranging from 1 to 50 μM) has an apoptotic effect on CGN, we quantified the hypodiploid DNA content using flow cytometry. Apoptosis is associated with lower DNA fluorescence in flow-cytometric analysis, which is regarded as a useful feature for its quantitative detection. However, when incubated together with 1 μM colchicine, CsA significantly increased the colchicine-induced apoptosis in CGN (about 45% in colchicine-treated cells and 63% in colchicine plus CsA (10 μM)) (Figure 2b). Representative histograms from flow cytometry of several samples are shown in Figure 7b.

In another series of experiments, the nuclei of cultured CGN were visualized by means of PI staining and fluorescence microscopy. This is a reliable method for evaluating nuclear morphology in neuronal cells. Control cultures also showed a certain number of apoptotic cells, but normally not more than 7% (Figures 2b and 7c). In contrast, the number of 1 μM colchicine-treated cells showing condensation of nuclear chromatin was higher (about 48%), a morphological sign associated with apoptosis. In the presence of CsA, the number of apoptotic nuclei was significantly higher (Figures 2b and 7c).

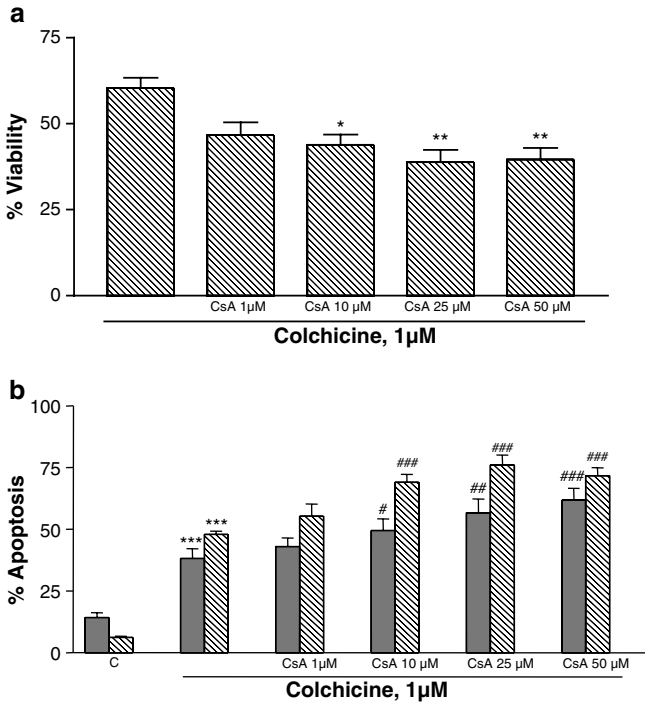

Figure 2 Effect of CsA (1–50 μM) on the neurotoxic effect of a 24 h treatment with colchicine (1 μM) on CGN. (a) Viability studies. Data are the mean$\pm$s.e.m. of the percentage change vs control cells, arbitrarily set at 100%. (b) Analysis of colchicine-induced apoptosis in the various conditions tested. Bar chart shows the percentage of cells rated as apoptotic, by flow-cytometric analysis (hypodiploid cells: black bars) or by morphologic analysis (slashed bars). Results are the mean$\pm$s.e.m. of four wells from 3–5 cultures. Cells were exposed to colchicine (1 μM) for 24 h, in the presence or absence of CsA. The statistical analysis was carried out with one-way ANOVA followed by Tukey's test, $^{***}P<0.001$ vs control values. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs colchicine values.

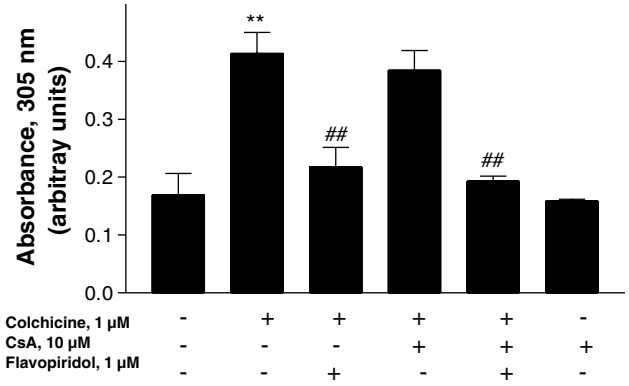

Figure 3 Caspase-3 activity in CGN exposed to 1 μM colchicine, colchicine plus CsA or CsA alone. Results are the mean$\pm$s.e.m. of three cultures. The statistical analysis was carried out with the one-way ANOVA, followed by Tukey's test. $^{**}P<0.01$ vs control values, $^{\#\#}P<0.01$ vs colchicine values.

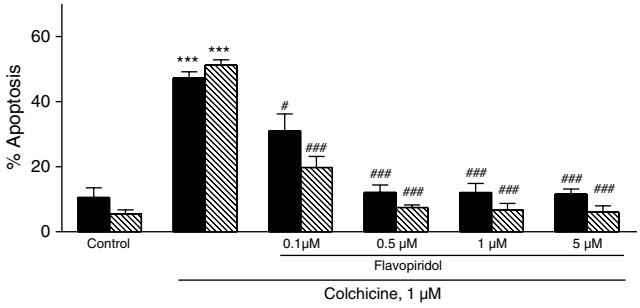

Figure 4 Antiapoptotic effect of flavopiridol (0, 1–5 μM) on 1 μM colchicine-induced changes in the percentage of cells rated as apoptotic, by means of flow-cytometric analysis (hypodiploid cells, black bars) or by morphologic analysis (slashed bars). Results are mean$\pm$s.e.m. of 4–6 independent cultures. The statistical analysis was carried out with the one-way ANOVA followed by Tukey's test. $^{***}P<0.001$ vs control values; $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ vs colchicine values.

Colchicine induces caspase-3 activation in CGN

It is well known that colchicine-induced apoptosis in CGN is prevented by caspase inhibitors (Gorman *et al*., 1999). The addition of z-VAD.fmk (100 μM) to the culture medium decreased neurotoxic effects and the percentage of the hypodiploid population significantly when compared to colchicine plus 10 and 25 μM CsA (Figure 1). Therefore, we measured caspase-3 activity in CGN treated with 1 μM colchicine. As expected, caspase-3 activity dramatically increased (180%) detected after 24 h incubation. When CsA 10 μM was co-incubated with colchicine, caspase-3 activity did not increase further (Figure 3).

Neuroprotective effects of cdk inhibitors against colchicine and colchicine plus CsA-induced neurotoxicity in CGN

The neuroprotective effects of flavopiridol were also evaluated. Flavopiridol (0.1–5 μM) reduced the neurotoxicity induced by colchicine in all the parameters measured: viability, apoptosis rate by flow cytometry and nuclear condensation (Figures 4 and 7). As stated above, CGN viability decreased to 60% after colchicine plus CsA treatment. Flavopiridol significantly protected cultures from cell death induced by colchicine plus CsA (Figure 5a) in a dose-dependent manner. Flavopiridol clearly protected against colchicine plus 10 μM CsA-induced apoptosis measured by flow cytometry, decreasing the hypodiploid peak to control values (Figure 5b). In addition, nuclear staining studies and morphological apoptotic features revealed that flavopiridol reduced colchicine plus CsA-induced neurotoxicity, as measured by PI positive nuclei (Figure 5b). Figure 7 shows representative flow-cytometric histograms and nuclear microphotographs of PI staining of CGN cultures in various experimental conditions.

Moreover, preincubation with roscovitine (25–50 μM) reduced the apoptotic action of colchicine- or colchicine plus CsA-induced apoptosis measured by flow cytometry or PI-positive nuclei quantification (Figure 6).

CsA enhances the expression of cdk5 induced by colchicine

We evaluated the effect of colchicine on the expression of cdk5, a cyclin kinase involved in cytoskeletal maintenance

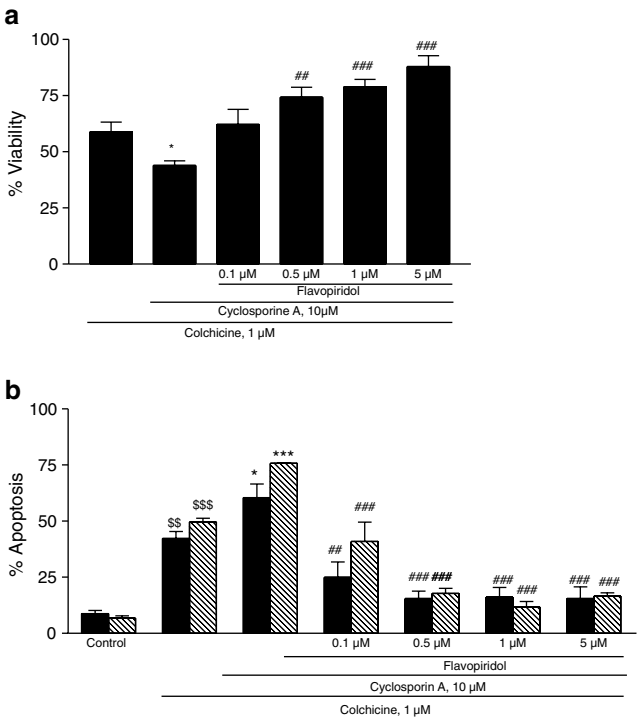

Figure 5 Neuroprotective effect of flavopiridol on 1 μM colchicine plus CsA (10 μM)-induced toxicity on CGN. (a) Viability studies. Data represent the mean $\pm$ s.e.m. of four independent experiments (cultures) performed in quadruplicate, and are expressed as the percentage change vs control cells, arbitrarily set at 100%. (b) Antiapoptotic effect of flavopiridol (1–5 μM) on 1 μM colchicine plus CsA-induced changes in the percentage of cells rated as apoptotic, by flow-cytometric analysis (hypodiploid cells, black bars) or by morphologic analysis (slashed bars). Results are mean $\pm$ s.e.m. of 4–6 independent cultures. The statistical analysis was carried out with the one-way ANOVA followed by Tukey's test. $^{\$\$}P<0.01$, $^{\$\$\$}P<0.001$ vs control values; $^{**}P<0.01$, $^{***}P<0.001$ vs colchicine values; $^{\#\#}P<0.01$,$^{\#\#\#}P<0.001$ vs colchicine plus CsA values.

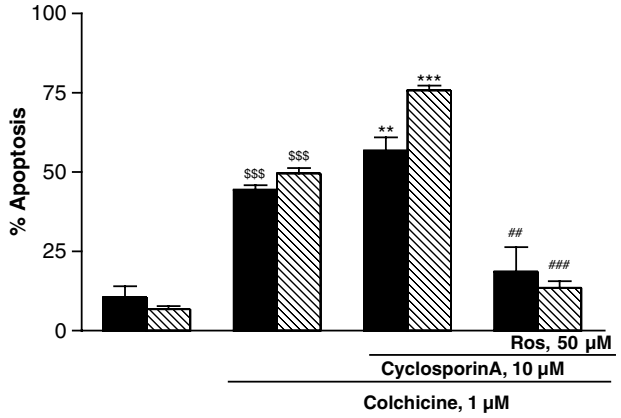

Figure 6 Bar chart showing the antiapoptotic effect of roscovitine (50 μM) on 1 μM colchicine plus CsA-induced changes in the percentage of cells rated as apoptotic, by flow-cytometric analysis (hypodiploid cells, black bars) or by morphologic analysis (slashed bars). Results are mean $\pm$ s.e.m. of 4–6 independent cultures. The statistical analysis was carried out with the one-way ANOVA followed by Tukey's test. $^{\$\$}P<0.01$, $^{\$\$\$}P<0.001$ vs control values; $^{*}P<0.05$, $^{***}P<0.01$ vs colchicine values; $^{\#\#}P<0.01$,$^{\#\#\#}P<0.001$ vs colchicine plus CsA values.

(Dhavan & Tsai, 2001), and the possible modification of its activity/expression after cotreatment with CsA. Cdk5 protein levels were assessed by Western blotting after 24 h incubation with 1 μM colchicine. Colchicine raised cdk5 levels, which were further increase by coincubation with CsA (10 μM) (Figures 7 and 8). On the other hand, CsA 10 μM alone slightly increased cdk5 expression. Pretreatment with 1 μM flavopiridol or 50 μM roscovitine decreased cdk5 expression both in colchicine-treated cells and in colchicine plus CsA-treated CGN. These findings suggest that an increase in cdk5 activity is involved in the enhancer action of CsA on colchicine-induced apoptosis in CGN, and this involvement was further examined. In this regard, cleavage of p35 to p25 was studied as a parameter of cdk activation. p25 levels increased, which was accompanied by a diminution in p35 levels in colchicine- and colchicine plus CsA-treated cells. The ratio p25/p35 returned to control value in the presence of 1 μM flavopiridol or 50 μM roscovitine (Figure 8). We further investigate the distribution of cdk5 by immunohistochemistry. Figure 8 showed that cdk5 was enriched in nuclear and perinuclear regions after colchicine and colchicine plus CsA treatment, indicating a possible nuclear apoptotic pathway implicated in the neurotoxic effect of colchicine and colchicine plus CsA. In presence of flavopiridol or roscovitine, the nuclear migration was prevented.

Discussion

Neurotoxicity is a serious central nervous system affectation that occurs in CsA-treated patients, with an incidence of 10–30% in organ-transplanted patients (Gijtenbeek *et al*., 1999; Bechstein & Tullius, 2000). Its symptoms are tremor, somnolence, visual disturbances, hallucinations and seizures (Gijtenbeek *et al*., 1999), and they have been related to the ability of CsA to induce apoptosis in several types of neurons. A number of hypotheses have been proposed to describe the possible pathways involved in CsA-induced apoptosis in neurons and in non-neuronal cells (McDonald *et al*., 1996; 1997). It has been described that CsA-induced apoptosis may be mediated by the p53 induction and activation of c-jun (Pyrzynska *et al*., 2002), the generation of nitric oxide (Ikesue *et al*., 2000) and intracellular calcium increase (Kaminska *et al*., 2001). Furthermore, in SH-SY5Y cells, CsA enhances MPP^+ toxicity, a neurotoxic compound that is used *in vitro* as a model of Parkinson's disease in animals (Fall & Bennett, 1998). In contrast, a study performed in striatal neurons demonstrated that, at low concentrations, CsA protects from the toxicity of 3-nitropropionic acid, which mimics Huntington's disease in animal models, although, at high concentrations, it does not prevent neuronal damage (Leventhal *et al*., 2000). Therefore, low concentrations of CsA may show transient but protective effects due to the blockade of the mitochondrial PTP, whereas, at higher concentrations, may cause neurotoxicity. The mechanisms of CsA-mediated toxicity in neuronal and glial cells should be understood to prevent neuronal CsA adverse events.

To our knowledge, our results demonstrate for the first time that, in CGN, CsA promotes the neuronal death induced by colchicine. However, CsA by itself, in our cell culture conditions, did not show any cytotoxic effect. One of the

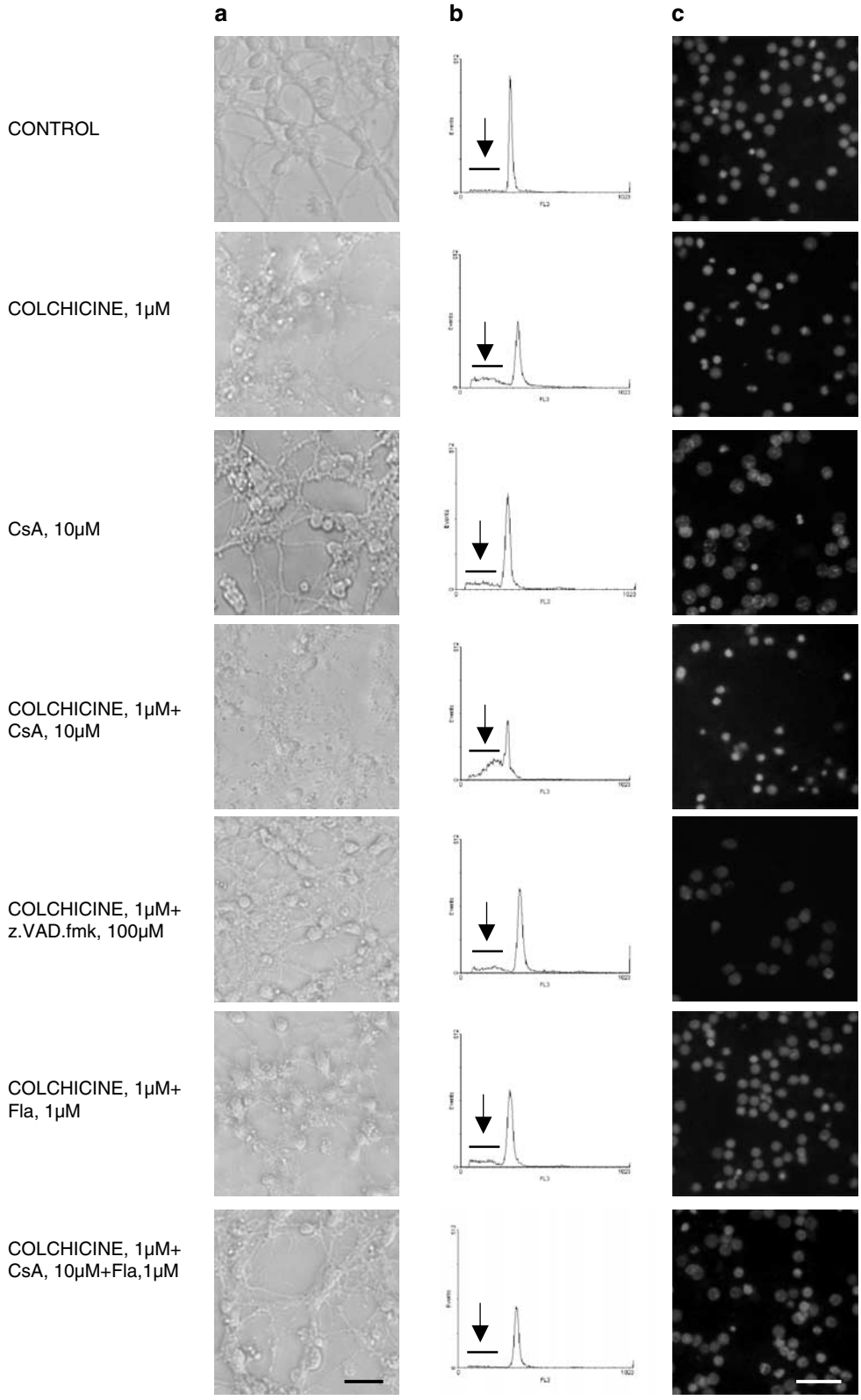

Figure 7 Effect of the various treatments assayed on CGN. (a) Representative phase-contrast images of CGN cultures in the presence of several drug treatments. (b) Flow-cytometric histograms of PI-stained cells after the same treatments. Arrow indicates the population considered as apoptotic. (c) Fluorescence photomicrographs showing chromatin condensation in permeabilized CGN. Calibration bar, 10 μM.

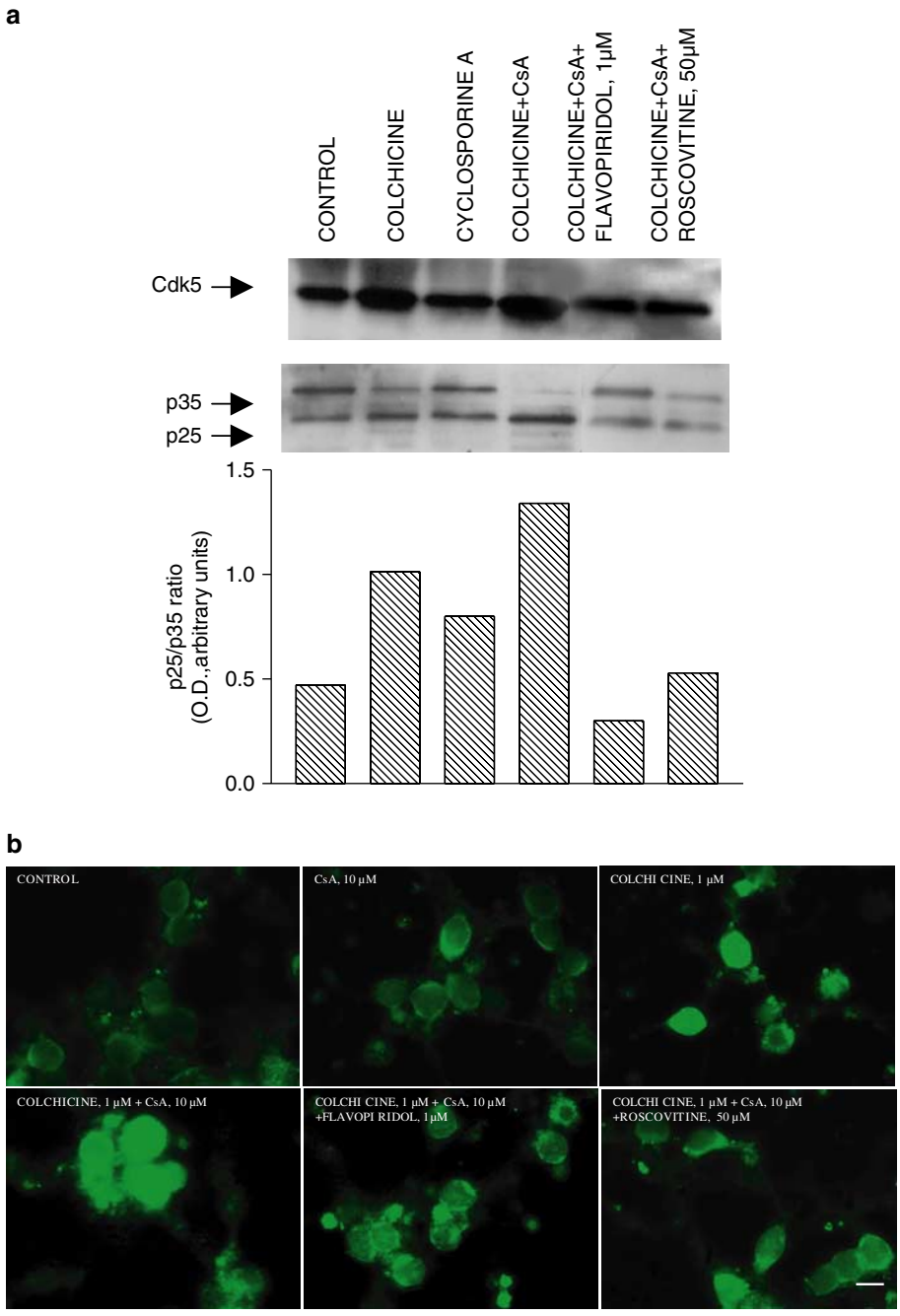

Figure 8 (a) Upper panel: Western blot of Cdk5. Evidence that Cdk5 is overexpressed in CGN after colchicine (1 μM), CsA (10 μM) and colchicine plus CsA treatment, and blocked in the presence of flavopiridol (1 μM) and roscovitine (50 μM). Lower panel: representative Western blot analysis of p35/p25 immunoreactivity in CGN in various experimental conditions. The bar chart shows semiquantitative p25/p35 ratio (OD, arbitrary units) in the same experimental conditions. (b) Immunostaining of CGN for cdk5 demonstrated an increase on nuclear labeling. The pre-incubation with flavopiridol or roscovitine reduced the nuclear localization of cdk5. Calibration bar, 100 μM. All experiments (Western blot and immunostaining) were carried out at least in duplicate on three different cultures.

possible mechanisms whereby CsA enhances colchicine-induced apoptosis may involve mitochondrial alteration (Serkova *et al*., 2000). Several studies support the hypothesis that alteration of ATP levels is responsible for kidney, liver and intestine toxicity caused by CsA (Uemoto *et al*., 1989; Ruiz-Cabello *et al*., 1994; Gabe *et al*., 1998).

Another possible mechanism involved in the enhancement by CsA of colchicine-induced apoptosis may be the increase of the intrinsic apoptotic pathway. In agreement with other studies, we show that colchicine-induced apoptosis results in part from the activation of the intrinsic pathway (Zamzani & Kroemer, 2001). In this intracellular pathway, mitochondria release proapoptotic signals (e.g., cytochrome *C*) and activate downstream effectors in neurons such as caspase-3 (Marks *et al*., 1998). Our results showed that colchicine increased caspase-3 activity, and that z.VAD.fmk abolished the neuro-

toxic effects of colchicine on CGN. However, although z.VAD.fmk protected CGN from colchicine plus CsA neurotoxicity, the presence of CsA did not further increase caspase-3 activity in colchicine-treated cultures, suggesting an alternative pathway involved in CsA plus colchicine-induced apoptosis in CGN. However, it should be noted that mitochondria are also involved in caspase-independent neuronal injury (Joza *et al*., 2002; Zhang *et al*., 2002; Zhu *et al*., 2003).

In fact, our results suggest an independent mitochondrial pathway that may participate in the enhancement by CsA of colchicine-induced apoptosis in CGN and that involves cdk5 activation. Cdk5 is an atypical cyclin-dependent kinase (CDK), widely distributed in the brain, but it is not involved in cell cycle regulation (Henchcliffe & Burke, 1997; Dhavan & Tsai, 2001; Leclerc *et al*., 2001; Knockaert *et al*., 2002). High expression of this protein may be a common feature of apoptosis in neuronal and non-neuronal cells (Smith & Tsai, 2001; Smith *et al*., 2001). Certain data suggest the participation of cdk5/p25 in neuronal apoptotic death in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (Takahashi *et al*., 2000; Alvarez *et al*., 2001; O'Hare *et al*., 2002; Lau *et al*., 2002). Our hypothesis is based on the fact that flavopiridol, a pan inhibitor of cdks, and roscovitine, a more selective cdk5 inhibitor (Meijer *et al*., 1997; 1999; Sedlacek, 2001, Zhai *et al*., 2002), reduces both the neurotoxic effect of colchicine- and colchicine plus CsA-treated cells, indicating that cdk5 activation is related to neuronal cell death triggered by colchicine or colchicine plus CsA, in agreement with Kerokoski *et al*. (2001; 2002), who showed that CsA slightly increased the levels of cdk5 expression and activity in hippocampal neurons, despite the low levels of p25.

Activation of cdk5 and cleavage of p35 to p25 are strongly correlated and the activity of cdk5 may be partially predicted by the levels of p35 and p25 proteins (Patrick *et al*., 1999; Kusakawa *et al*., 2000; Lee *et al*., 2000; Kerokoski *et al*., 2001). Cdk5 binds to p25 and this complex has a nuclear/perinuclear localization (Weishaupt *et al*., 2003). The misallocation of cdk5 due to the proteolysis of p35 may lead to the phosphorylation of several substrates that are involved in neuronal cell death. CsA alone, colchicine and both together altered the ratio p25/p35, slightly increasing the p25 fraction. Our data indicated an increase in the levels of p25 that caused prolonged activation and inappropriate localization of cdk5 can be observed by immunocytochemistry results, thus mediating the neurotoxic effect of colchicine and the enhancement of these effects by CsA (Weishaupt *et al*., 2003). There is a cdk5-dependent phosphorylation and inhibition of MEF2 (monocyte enhancer factor), a transcription factor that modulates survival/death functions in neurons. MEF2 expression is also inhibited by CsA (Mao & Wiedman, 1999). Thus, we suggest a nuclear pathway by which colchicine alone and colchicine plus CsA trough cdk5/p25 activation induce neuronal apoptosis, probably inhibiting neuronal survival factors.

In summary, CsA enhances the neurotoxicity of colchicine through an increase in the expression of cdk5/p25, which is essential for apoptosis (Patrick *et al*., 1999). Our data provide further evidence that CsA may enhance the proapoptotic effects of other drugs in neurons.

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