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# p73 participates in male germ cells apoptosis induced by etoposide

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**ABSTRACT:** Etoposide is a commonly used drug in testicular cancer chemotherapy. However, the molecular pathways that activate germ cell apoptosis in response to etoposide are poorly understood. The aim of this study was to evaluate the participation of p73, a member of the p53 family, in apoptosis induced by etoposide in male germ cells. First, we used GC2-spc cells—a male germ cell model—to evaluate apoptotic signaling after treatment of etoposide. We found an important increase in p73 protein levels, along with the c-Abl kinase, its physiological activator, in response to etoposide. This increase was accompanied by a decrease in cell viability and activation of caspase-3. Pifithrin (PFT) treatment prevented p73 increase and apoptosis induced by etoposide. Also, the *in vitro* knockdown of p73 or p53 by shRNA, significantly prevented the decrease in cell viability after etoposide treatment. In an *in vivo* model—21-day-old rat testes—we observed an up-regulation of the protein levels of p73 and phosphorylated p73—at c-Abl site Tyr99—in response to the etoposide injection. STI571 (a pharmacological inhibitor of c-Abl) or PFT co-injection prevented etoposide-induced up-regulation of phospho-p73 and pro-apoptotic TAp73 isoform levels. Moreover, caspases-3, -8, -9 activation and germ cell death induced by etoposide were significantly decreased by these drugs. These results support the notion that the c-Abl/p73 pathway is activated in germ cells after etoposide treatment, triggering apoptosis, possibly assisting p53.

**Key words:** Spermatogenesis / p73 / apoptosis / germ cells / etoposide

## Introduction

Testicular cancer is the most common type of cancer affecting men between 15- and 35-year-old (Bosl and Motzer, 1997). In 1975, the incidence in the USA was 3.5 cases per 100 000 (Flechon *et al.*, 2008), although in 2004, it had increased to 5.8 cases per 100 000. Nevertheless, even in advanced stages of tumor progression, most cases of testicular cancer are curable. Treatments include surgery, radiation and chemotherapy or usually a combination of these methods. The most commonly used drugs for testicular cancer are Vepesid or VP-16 (Etoposide), Platinol (cisplatin) and Blenoxane (bleomycin sulfate). Etoposide is a podophyllotoxin semi-derivative agent that is used in a variety of chemotherapeutic treatments, including therapy for pediatric tumors. Previous reports have proposed that etoposide's antimitotic activity is mediated by its interaction with topoisomerase II, an ATP-dependent nuclear enzyme that regulates DNA topology by transiently breaking and joining double-stranded DNA (Bromberg *et al.*, 2003). It has been shown that etoposide induces apoptosis, dependent or independent on p53, in several cultured cancer cell lines and it is widely used in different protocols for testicular cancer (Sturgeon *et al.*, 2008). Previous evidence has shown that spermatogonia and spermatocytes are the major cell types undergoing apoptosis induced by etoposide in both adult and pubertal rats (Sjoblom *et al.*, 1998; Stumpp *et al.*, 2004; Ortiz *et al.*, 2009). The first widely used chemotherapy was a combination of cisplatin, vinblasltin and bleomycin (PVB) (Einhorn and Donohue, 1977), but a later study by Lederman *et al.* showed the benefit of etoposide combined with cisplastin and bleomycin (BEP) with respect to treatment with PVB. This chemotherapy leads to a 95% regression in patients (Williams *et al.*, 1987) and furthermore, BEP therapy was shown to be less toxic than PVB (Bosl and Motzer, 1997). Nowadays, etoposide is the first line drug used against testicular cancer. However, the molecular mechanisms by which etoposide induces germ cell apoptosis remain unknown.

p73 is a transcriptional factor, member of the p53 family and shares aminoacidic and structural similarity with p53, especially within its DNA binding domain (Levrero *et al.*, 1999). In view of this, p73 can transactivate several target genes in common with p53, and induce apoptosis through Bax translocation and p53 up-regulated modulator

of apoptosis (PUMA) activation (Melino et al., 2004; Ramadan et al., 2005; Soond et al., 2007; Ortiz et al., 2009). Nevertheless, it has been shown that p73 can also activate other genes and trigger apoptosis independently of the status of p53 (De Laurenzi et al., 2000; Schmelz et al., 2005). p73 is expressed in several isoforms by alternative use of its promoter, generating TAp73 isoforms that contain the transactivation domain, or ΔNp73 isoforms, lacking the transactivation domain. It has been shown that although the TAp73 isoforms induce apoptosis, ΔNp73 isoforms counteract this activity and function as pro-survival proteins (Belloni et al., 2006; Ramadan et al., 2005). c-Abl tyrosine kinase phosphorylates p73, thereby activating it, in response to several genotoxic stimuli such as oxidative damage or DNA breaks (Agami et al., 1999; Gong et al., 1999).

We have previously shown that etoposide induces apoptosis in meiotic spermatocytes in pubertal rat testes, accompanied by an increase of p53 protein levels, along with caspase-3,-8 and -9 activation. In addition, p73 is also expressed in adult mammalian testes, where it appears to have a role in apoptosis caused by irradiation (Hamer et al., 2001), but its role in male germ apoptosis after genotoxic stimuli has never been addressed. In this context, pharmacological inhibition of c-Abl with the tyrosine kinase inhibitor STI571 (Gleevec, Imanitib) has been shown to be an excellent tool to study the role of p73 in vivo and in vitro conditions (Alvarez et al., 2008; Cancino et al., 2008). Thus, the goal of this study was to elucidate the role of p73 in apoptosis triggered by etoposide in rat germ cells, using an in vitro and in vivo model.

# Materials and Methods

## Cell culture and cell viability assays

GC2-spc cells were maintained in Dulbecco Modified Eagle Medium supplemented with 10% FBS plus 100 U/ml penicillin and 100 U/ml streptomycin in a humidified atmosphere containing 5% $CO_2$ at 37°C (Hofmann et al., 1994). To measure cell viability, GC2 cells were plated in 96-well plates at 10 000 cells/well and then assayed at different times. Cells were treated with 10–100 μM Etoposide, 100 μM Pifithrin (Sigma, St Louis, MO, USA) (PFT) or a combination of both drugs. After incubation at 37°C, MTS substrates (Cell TITER 96 Aqueous Assay, Promega, Madison, WI, USA) were added according to protocol, and absorbance at 490 nm was measured in an enzyme-linked immunosorbent assay (ELISA) reader (Boots CellTech Diagnostics Inc., Slough, UK).

## Animals

Twenty-one-day-old male Sprague–Dawley rats were acquired from the Animal Facility of our Faculty. The rats were housed under a 12L:12D cycle, with water and food provided ad libitum. The rats were killed by cervical dislocation. Investigations were conducted in accordance with the rules laid down by the Consortium for Developing a Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching and by our National Research Council. All animal protocols were endorsed by the Chilean National Fund of Science and Technology (FONDECYT).

## Intratesticular injections

Twenty-one-day-old male rats were anesthetized with xylazine:ketamine: (10 and 75 mg/kg) injected intracavitally into the abdomen. Both testes were exteriorized through a low midline incision and in each testis 10 μl of etoposide (Merck, Darmstadt, Germany), dissolved in PBS at a concentration of 100 μM, infused via a 30G needle, inserted through the tunica albuginia with the tip resting in the testicular interstitium. Following drug delivery, the testes were returned to the peritoneal cavity, and the incision was closed. Twenty-four hours later, both testes were removed (Ortiz et al., 2009). In each experiment one testicle was fixed for histology and the other used for protein extraction. As a control, PBS alone was injected into both testes. For c-Abl inhibition we used STI571 (Novartis Pharma, Basel, Switzerland) dissolved in NaCl 0.9% at a final concentration of 50 μg/ml. We also used PFT at a final concentration of 100 μM in PBS. Ten microliter of each drug was independently co-administered with 100 μM etoposide. After drug delivery, the testes were returned to the peritoneal cavity and the incision was closed. As a control, NaCl 0.9% or PBS alone was injected into the testes. Three different rats were used for all experiments and they were sacrificed 24 h after injection for the assessment of germ cell apoptosis.

## Histology and apoptosis determination

Testes were fixed in Bouin's solution and embedded in paraffin. Sections were counterstained with periodic acid-Schiff and hematoxylin for the assessment of germ cell apoptosis. We have previously showed that pycnotic germ cells express apoptotic markers such as active caspase-3 and stain positively for TUNEL (Moreno et al., 2006). The apoptotic index was calculated as the average number of apoptotic (pycnotic) germ cells per seminiferous tubule cross-section. Three testicular histological sections were taken per rat (three rats total) and a minimum of 100 randomly selected tubules were counted in each tissue section (a total of 900 tubules were recorded per treatment). The data represent the mean ± SE.

## Inmunofluorescence

GC-2 cells were plated on polylysine-coated coverslips at 30 000 cells/coverslip. After 1 day in DMEM medium, cells were treated with 100 μM Etoposide for 4 h. Cells were fixed in cold Methanol for 10 min at −20°C and washed three times 10 min in PBS. Three percent BSA in PBS for 1 h was used to block. Primary antibody against active caspase-3 (Cell Signaling, Danvers, MA, USA) was applied at a concentration of 2 μg/ml and incubated overnight at 4°C in a humidified chamber. Then, slides were washed three times for 10 min in PBS containing 0.1% Tween-20. Secondary antibody anti-rabbit conjugated with Alexa Fluor-594 (1/500) (Molecular Probes, OR, USA) was applied for 2 h at room temperature. Samples were counterstained with 10 μg/ml 4′,6-diamidino-2-phenylindole (DAPI, Sigma) and washed three times for 10 min in PBS containing 0.1% Tween-20. Light and fluorescence images were captured under an Olympus BX51 microscope and analyzed with Adobe® Photoshop 7.0 (Adobe System Incorporated, USA).

## Immunohistochemistry

p73 and c-Abl were localized in paraffin embedded cross-sections of rat testis fixed in 4% paraformaldehyde. The samples were treated with 3% $H_2O_2$ in PBS for 5 min and then blocked for 5 min with 4% BSA. Slides were washed twice for 5 min in a Tris–HCl buffer, pH 7.6 with 0.3 M NaCl and 0.1% Tween 20 and incubated overnight at 4°C in a humidified chamber with primary antibodies against p73 or c-Abl (2 μg/ml) in 4% BSA–PBS. Biotinylated secondary antibody, streptavidin–biotinylated–peroxidase complex, amplification reagent (biotinyl tyramide) and peroxidase-conjugated streptavidin were applied step-by-step for 15 min each. Afterwards, incubation slides were washed twice in a buffer for 3 min each. Finally, a substrate-chromogen solution consisting of concentrated Tris–HCl and 0.8% $H_2O_2$ (substrate) and 3,3-diaminobenzidine tetrahydrochloride solutions (chromogen) were applied for 5 min and washed in distilled water. Samples were observed under a phase contrast
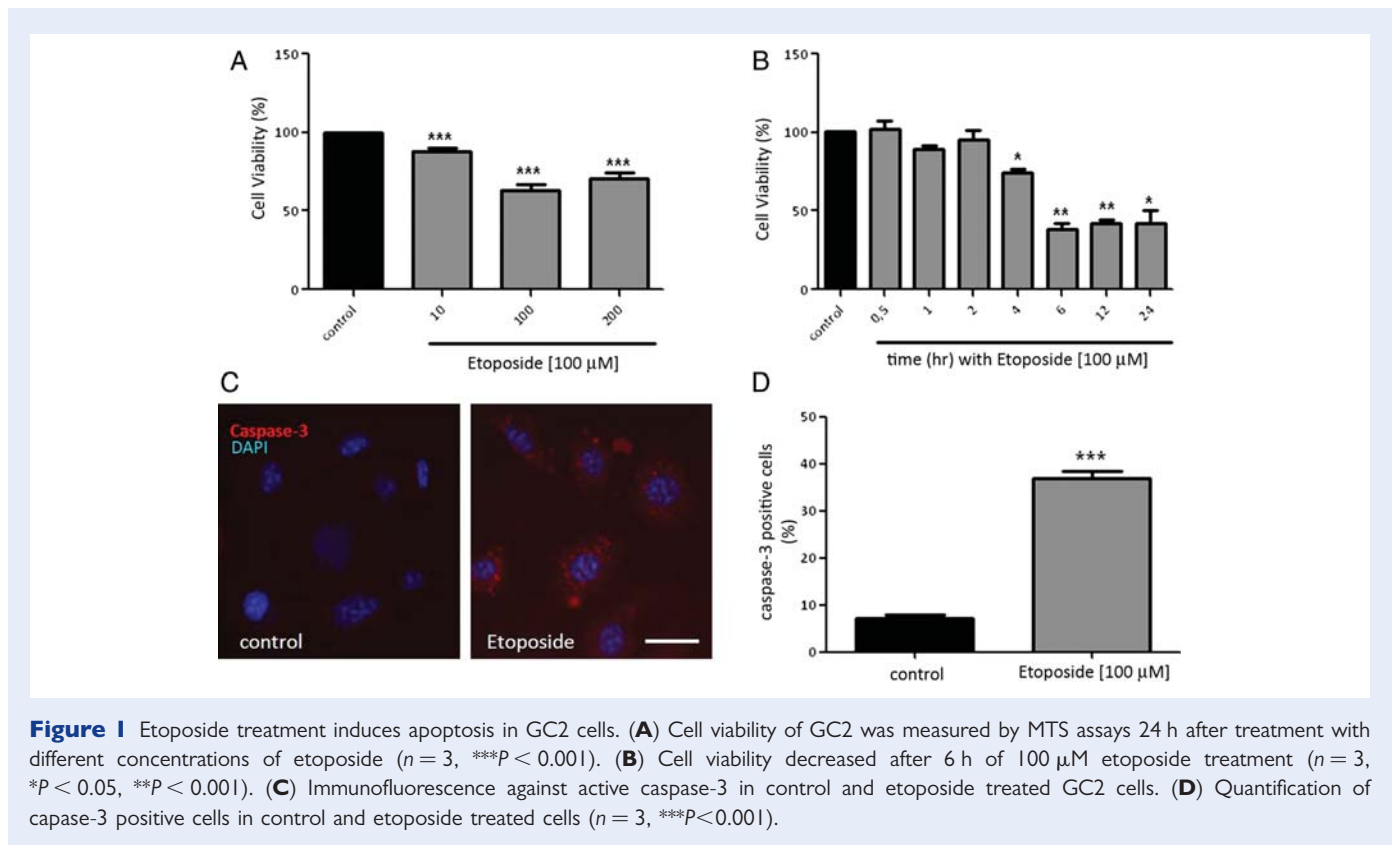
**Figure 1** Etoposide treatment induces apoptosis in GC2 cells. (**A**) Cell viability of GC2 was measured by MTS assays 24 h after treatment with different concentrations of etoposide ($n = 3$, ***$P < 0.001$). (**B**) Cell viability decreased after 6 h of 100 μM etoposide treatment ($n = 3$, *$P < 0.05$, **$P < 0.001$). (**C**) Immunofluorescence against active caspase-3 in control and etoposide treated GC2 cells. (**D**) Quantification of capase-3 positive cells in control and etoposide treated cells ($n = 3$, ***$P < 0.001$).

microscope (Optiphot-2, Nikon, Japan) and photographed with a digital camera (CoolPix 4500, Nikon, Japan).

## Protein extraction and western blot assay

Seminiferous tubules were separated by continuous pipetting in 1.5 ml KHB (Krebs−Henseleit buffer plus 1% BSA) medium [2 g/l D-Glucose, 0.141 g/l Magnesium Sulfate (Anhydrous), 0.16 g/l NaH$_2$PO$_4$, 0.35 g/l KCl and 6.9 g/l NaCl] with 15 μl of a collagenase solution (0.5 mg/ml) added. Tubuli were decanted whereas maintaining Leydig and blood cells suspended in the medium, which was consequently discarded. Protein extraction was performed by homogenization of the isolated seminiferous tubuli obtained from 21-day-old rat testis or the GC2 cells harvested in a buffer containing 1 M NaCl, 1 mM EDTA, 10 mg/ml PMSF, 1% Triton X-100, 20 mM Tris−HCl pH 7.4 and centrifuged for 10 min at 1000 g. The samples were run on a 10% polyacrylamide gel (SDS−PAGE) under reducing and denaturing conditions, and transferred to a PDVF membrane at 400 mA for 2 h. PDVF membranes were blocked with 5% (w/v) non-fat milk, 0.1% Tween in PBS, pH 7.4 and then incubated overnight at 4°C with the following antibodies: rabbit polyclonal anti p73 (2 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti p73 phosphorylated at Tyr99 (dilution 1/1000; Cell Signaling, Danvers, MA, USA), rabbit polyclonal anti c-Abl (2 μg/ml, Santa Cruz Biotechnology) or anti-β-actin (1 μg/ml; Sigma), as a loading control. Membranes were then incubated with a secondary antibody conjugated with horseradish peroxidase (KPL, Gaithersburg, MD, USA) diluted 1:3000 in blocking solution for 1 h at room temperature, and complexes were detected by ECL (Pierce Biotechnology, Rockford, IL, USA). Bands obtained were analyzed measuring the pixels with Adobe® Photoshop 7.0 (Adobe System Incorporated, USA) and normalized to β-actin. Each experiment was carried out in triplicate and performed at least three times.

## Caspase activity measurements

Caspase activity assays were performed as previously described (Codelia *et al.*, 2008). Briefly, seminiferous tubules were separated by continuous pipetting in 1.5 ml KHB (Krebs−Henseleit buffer plus 1% BSA) medium with 15 μl of a collagenase solution (0.5 mg/ml) added. Tubuli were decanted while maintaining Leydig and blood cells suspended in the medium, which was consequently discarded. Isolated seminiferous tubules were homogenized in a buffer containing 1 M NaCl, 1 mM EDTA, 10 mg/ml PMSF, 1% Triton X-100, 20 mM Tris−HCl pH 7.4. The activity of each caspase was determined by using colorimetric substrates labeled with chromophore p-nitroaniline (pNA) for caspase-3 (Ac-DEVD-pNA), caspase-8 (AC-IETD-pNA) and caspase-9 (AC-LEHD-pNA), Calbiochem (Darmstadt, Germany). pNA is released upon caspase cleavage and produces a yellow color that was measured at 405 nm. Absorbance proportionally increases depending on the amount of caspase activity present in the sample. The amount of product generated was calculated by extrapolation of a standard curve of free pNA. One international unit (IU) was defined as the amount of caspase hydrolyzing 1 μmol of pNA/min at 25°C. Results of specific activity are expressed in units of enzyme per milligram of tissue (U/mg protein). The results are presented as the mean of three different rats. Protein concentration was measured by using the BCA method (Pierce, Rockord, IL, USA).

## shRNAs and transfections

The shRNA-p53, shRNA-p73 and non-silencing control short hairpin mRNAs (shRNAs) vector were a kind gift from Dr Thorsten Stiewe (Philipps-University Marburg, Marburg, Germany). The respective target sequences were as follows: shRNA-p53 GTCTGTTATGTGCACGTAC, shRNA-p73 GGCAGAGTGTGGTTGTGCC and TTCTCCGAACGTG TCACGT for control shRNAs. Transient transfections of GC-2 were performed with LipofectAMINE 2000 reagent according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA).
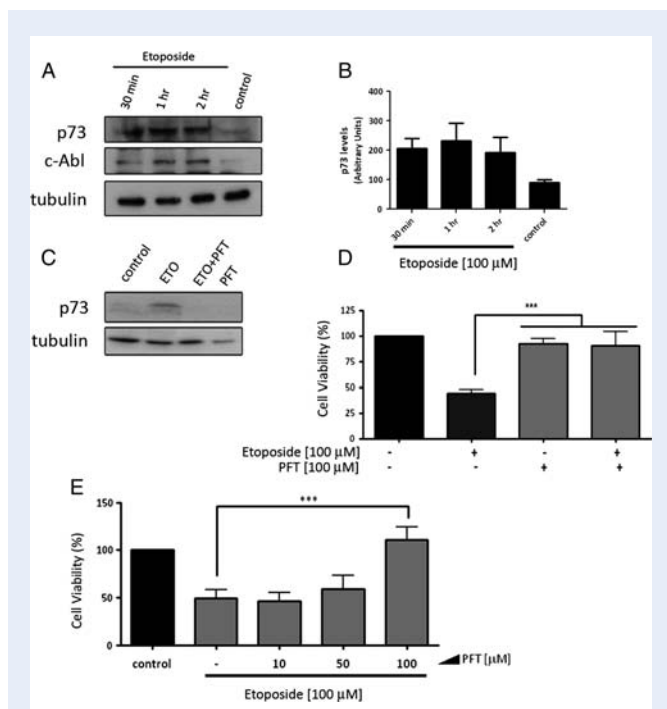
**Figure 2** Etoposide treatment up-regulates p73 levels in GC2 cells. (**A**) Levels of p73 in GC2 cells were evaluated at different times of incubation with 100 μM etoposide. (**B**) Protein quantification shows a clear increase in p73 protein levels after etoposide treatment. (**C**) The increase of p73 protein levels caused by etoposide was significantly prevented by 100 μM PFT co-treatment. (**D**) Cell viability decreases after etoposide treatment, which was prevented by PFT, correlating with the p73 levels observed in (C) ($n = 3$, ***$P < 0.001$). (**E**) Cell viability in the presence of 100 μM etoposide alone or in the presence of increasing concentrations of PFT. All experiments were performed in triplicate (mean ± SE).

## Statistical analysis

For mean comparisons, we used analysis of variance (ANOVA). When the ANOVA test showed statistical differences, the Tukey *post hoc* test was used to discriminate between groups. Statistical significance was considered as $P < 0.05$ (Sokal, 1995). Statistical analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Results are given as mean ± Standard Error (SE).

# Results

## Etoposide induces apoptosis and c-Abl/p73 activation in GC2 cells

To evaluate the effects of etoposide on the c-Abl/p73 signaling, we used—as an *in vitro* cell line model of germ cells— the GC-2 cell line. GC-2 cells are a good *in vitro* model of spermatocytes since they show meiosis markers at 37°C (Hofmann *et al.*, 1994). First, we evaluated if etoposide-induced apoptosis in GC-2 cells. As expected, etoposide caused a significant decrease in cell viability in a concentration-dependent manner after 6 h of incubation with the drug, as evaluated by the MTS assay (Fig. 1A and B, *$P < 0.05$). This drop in cell survival
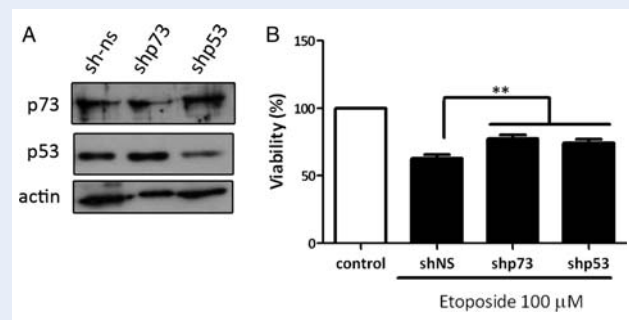


**Figure 3** Decrease of p73 and p53 protein levels prevent etoposide-induced apoptosis in GC-2 cells. GC-2 cells were transfected with short hairpin RNA against p73 (shRNA-p73) or p53 (shRNA-p53). (**A**) Transfection with shp53 decreases protein levels of p53, whereas only a modest effect was observed with shp73 on p73 protein levels. Nonsense short hairpin (sh-ns) did not affect protein levels of p73 or p53. β-actin was used as a loading control. (**B**) Viability of GC-2 cells, transfected with shp53 or shp73, is significantly higher than those transfected with sh-ns in the presence of etoposide ($n = 3$, **$P < 0.01$). Bars show the mean ± SE.

was accompanied by an increase in caspase-3 activation (Fig. 1C and D), suggesting GC2 cells died by apoptosis.

Then, we analyzed if there was a concomitant increase in p73 protein levels after etoposide treatment. Results showed that 100 μM etoposide treatment up-regulated p73 protein levels significantly after 30 min of incubation (Fig. 2A and B). Levels of the tyrosine kinase c-Abl, the physiological activator of p73, were also increased after etoposide treatment (Fig. 2A), suggesting the activation of the c-Abl/p73 module in response to etoposide. Next, we evaluated if the pharmacological inhibition of p73 could prevent etoposide-induced apoptosis in GC-2 cells. PFT is a classical pharmacological p53 inhibitor (Murphy *et al.*, 2004), however, a recent report shows that PFT also causes a decrease in p73 levels (Davidson *et al.*, 2008). We observed that PFT administered together with etoposide prevented up-regulation of p73 levels, as well as the decrease in cell viability induced by etoposide after six hours of treatment (Fig. 2C, D and E, ***$P < 0.001$). PTF alone did not alter the levels of p73. In order to specifically reduce the levels of p73 or p53, we used shRNA. Results showed that even though shp73 treatment resulted in a modest reduction of protein levels (Fig. 3A), the cell viability of transfected cells treated with etoposide was significantly higher than those transfected with a nonsense shRNA (Fig. 3B, **$P < 0.01$). shp53 treatment clearly reduced p53 protein levels, which was in accordance with an increase in cell viability after etoposide treatment, as compared with those cells transfected with a nonsense shRNA (Fig. 3A and B, **$P < 0.01$). Thus, our results indicate that both p73 and p53 participate in etoposide-induced apoptosis in GC-2 cells.

## Etoposide induces c-Abl/p73 in pubertal rat testis

In order to evaluate the participation of p73 in apoptosis induced by etoposide *in vivo*, we performed intratesticular injections of etoposide in 21-day-old rats. At this age, physiological apoptosis occurs at a low
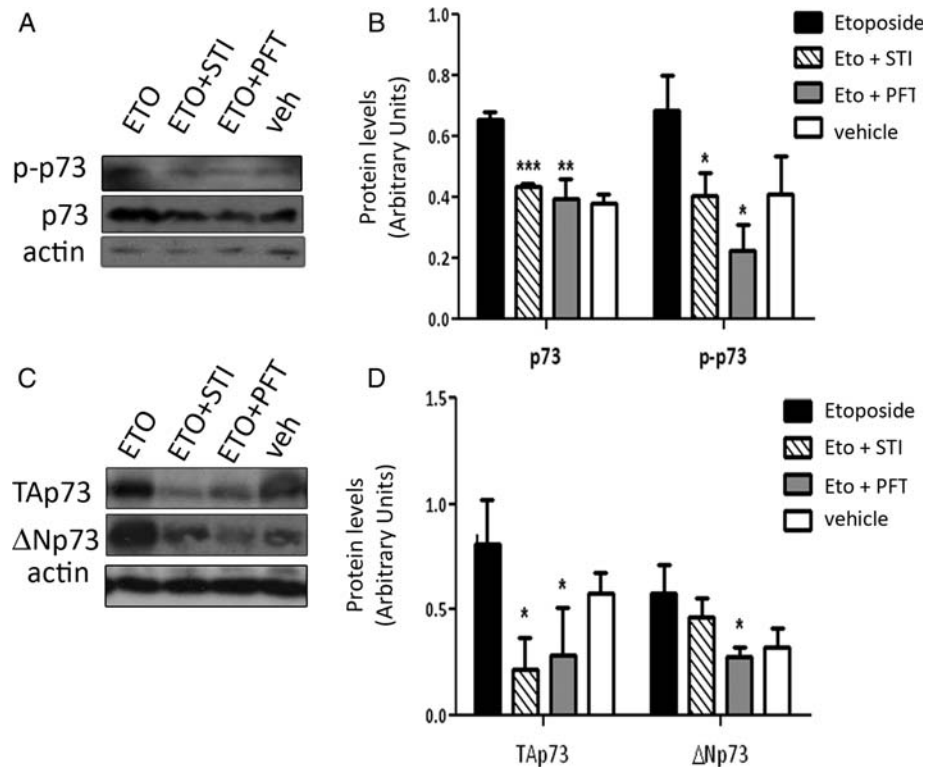
**Figure 4** Tyrosine kinase inhibitor, STI571 and classical p53 inhibitor, PFT prevent etoposide-induced p73 activation in rat testes. (**A**) Rat testes treated with etoposide showed an increase in Tyr99 phosphorylated p73 (p-p73) and total p73, in comparison with those treated with vehicle. This effect was prevented when etoposide was injected along with both STI571 and PFT. (**B**) Quantification of p-p73 and total p73 western blots. β-actin was used as a loading control ($n = 3$, $*P < 0.05$, $***P < 0.001$). (**C** and **D**) Rat testes treated with etoposide showed an increase in both TAp73 and ∆Np73 protein levels. This effect was prevented mainly in Tap73 when etoposide was injected along with both STI571 or PFT, and DNp73 with PFT ($n = 3$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). Bars show the mean ± SE.

level, allowing for easy detection of any changes of these levels (Moreno et al., 2006; Lizama et al., 2009). Our previous studies showed that a single intratesticular injection of 10 μl of a solution containing 200 μM etoposide into 21-day-old rats, induced a significant increase in germ cell apoptosis 24 h after treatment (Ortiz et al., 2009), associated with an increase in p53 protein levels. Here, we observed that 100 μM etoposide significantly increased levels of total p73 as well as p73 phosphorylated at Tyr99—a c-Abl phosphorylation site—supporting the hypothesis of c-Abl/p73 signaling activation by drug treatment (Fig. 4A and B, $*P < 0.05$). In addition, STI571 prevented the increase of the phosphorylated form of p73, as well as the TAp73 and ∆Np73 isoforms induced after etoposide treatment. About 100 μM PFT prevented up-regulation of total p73 and phosphorylated p73 (Fig. 4A and B). Even more, PFT was also able to prevent the increase of TAp73 and the ∆Np73 isoform after etoposide treatment (Fig. 4 C and D,). In order to define which cell type expresses p73 and c-Abl in 21-day-old rat testis, we performed immunohistochemistry. Results showed that p73 had a ubiquitous distribution, both cytoplasmic and nuclear, in vehicle-treated testes of 21-day-old rats (Fig. 5B). The immunoreaction against p73 increased after etoposide treatment in all cell types, including apoptotic (pycnotic) cells (Fig. 5A, arrow). On the contrary, c-Abl showed a cytoplasmic localization in both germ and Sertoli cells

(Fig. 5C). After etoposide treatment, the c-Abl immunoreaction significantly increased in the cytoplasm of most testicular cells, but mainly in late pachytene spermatocytes (Fig. 5C, arrowhead) Controls without the primary antibody did not show any reaction (Fig. 5D, insert).

Finally, we evaluated the effects of STI and PFT treatment in etoposide-induced apoptosis in germ cells. As we have shown previously (Ortiz et al., 2009), etoposide induces a significant increase in the number of apoptotic germ cells 24 h after treatment, along with an increase in caspase-3, -8 and -9 activity, as compared with rats treated with vehicle only (Fig. 6A–C, $*P < 0.05$). The increase in caspase activity 24 h after etoposide treatment was prevented by co-injection with 50 μg/ml STI571 or 100 μM PFT (Fig. 6C). In addition, the presence of STI571 or PFT prevented the increase of germ cell apoptosis (apoptotic index) induced by etoposide treatment (Fig. 6A and B). STI or PFT alone did not show any effect on germ cell apoptosis (Fig. 6B). Thus, up-regulation of p73 is associated with apoptosis induction in male germ cells.

These results show the activation of p73 after etoposide treatment in vivo, and more specifically the activation of its pro-apoptotic isoform TAp73, which can be modulated using the c-Abl inhibitor STI571 and PFT. This supports the participation of p73 in the apoptosis caused by etoposide in male germ cells.
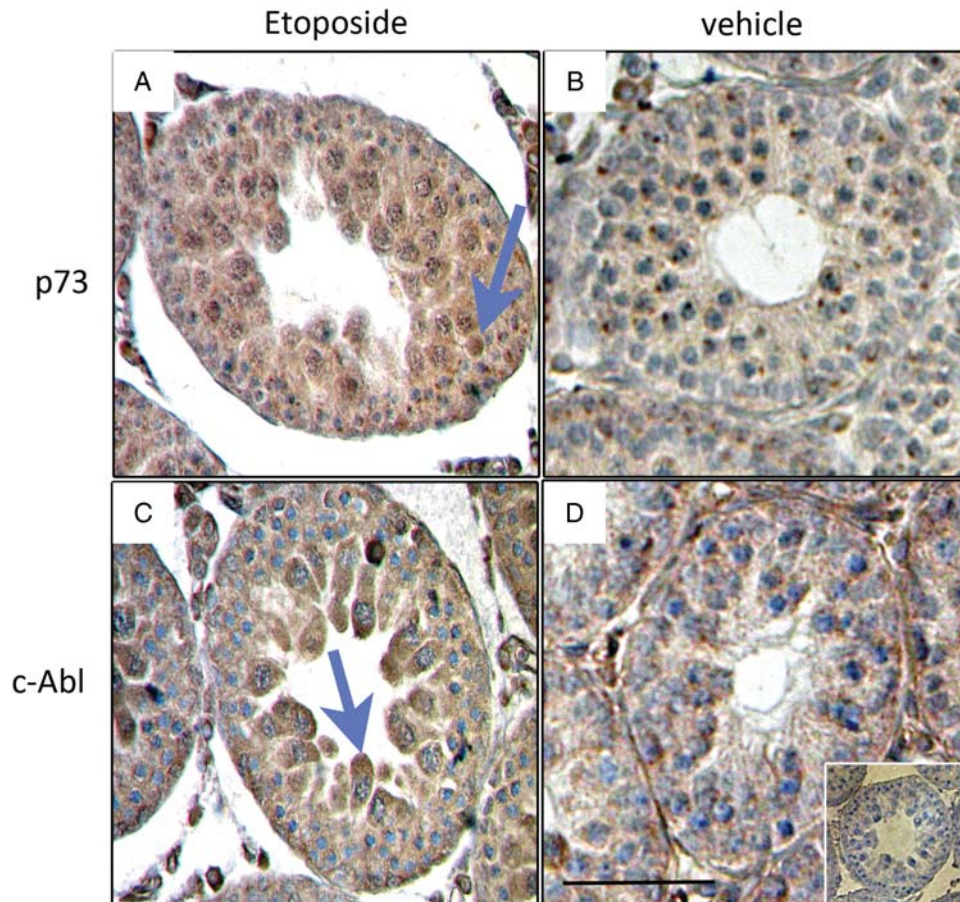
**Figure 5** Localization of p73 in vehicle and etoposide treated 21-old-day rat testes. (**A**) p73 shows a strong immunoreaction after etoposide treatment in all cell types, including apoptotic (pycnotic) cell (arrows). (**B**) p73 has a ubiquitous distribution, both cytoplasmic and nuclear, in vehicle-treated 21-day-old rat testes. (**C**) c-Abl shows a cytoplasmic localization in both germ and Sertoli cells. The immunoreaction is stronger in the cytoplasm of late pachytene spermatocytes. (**D**) c-Abl distribution in vehicle-treated rat testes. Control without the primary antibody did not show any reaction (insert). Bar 100 μm.

# Discussion

Etoposide (a pharmacological inhibitor of toposisomerase II), along with cisplatin and bleomycin, are the most common drugs utilized in testicular cancer chemotherapy (Flechon *et al.*, 2008), but their effects on germ cell death are poorly understood. The data shown in the present work suggest that c-Abl and p73 play a role in etoposide-induced germ cell apoptosis, which can be counteracted by using STI571 (Imanitib, Gleevec) or PFT.

Recently, we showed that etoposide induces an increase in caspase activity, particularly caspase-9, in pubertal rat testis (Ortiz *et al.*, 2009). In addition, spermatocyte apoptosis was accompanied by an increase in BAX and PUMA mRNA levels, along with the increase in p53 levels. In this work, we have extended our results and shown that p73, a member of the p53 family, could assist p53 in germ cell demise after etoposide treatment. It has been shown that p73 is involved in both developmental and apoptotic processes, and that it can act both dependently and independently of p53 (Melino *et al.*, 2003; Billon *et al.*, 2004; Ramadan *et al.*, 2005). Hamer *et al.* (2001) showed the participation of the c-Abl/p73 pathway in

radiation-induced apoptosis in germ cells, but no studies have been carried out for etoposide or other anti-cancer drugs in the testis. In the present work, we show that both *in vivo* and *in vitro*, etoposide induces an increase at the protein levels of p73 and c-Abl, suggesting a role for the signaling module in both systems. *In vitro*, by using shRNA, we showed that p73 and p53 are required for apoptosis induced by etoposide in GC-2 cells. Even though the reduction in protein levels of p73 was not as drastic as it was for p53, cell viability was significantly higher than cells transfected with the nonsense shRNA after etoposide treatment. Interestingly, viability in cells transfected with shp73 and shp53 was similar, despite the fact that the reduction of p53 protein was more dramatic than that observed for p73. The fact that we did not obtain viability levels similar to controls, may be due to inefficient transfection of these cells, meaning the amount of transfected cells is low in relation with the cells affected by etoposide, which results in a dilution of the cell survival effect.

STI571 specifically inhibits the c-Abl tyrosine kinase and Bcr-Abl tyrosine kinase, a constitutively expressed, abnormal tyrosine kinase resulting from the Philadelphia chromosome (Ph+), an abnormality associated with chronic myeloid leukemia (Druker, 2001). However,
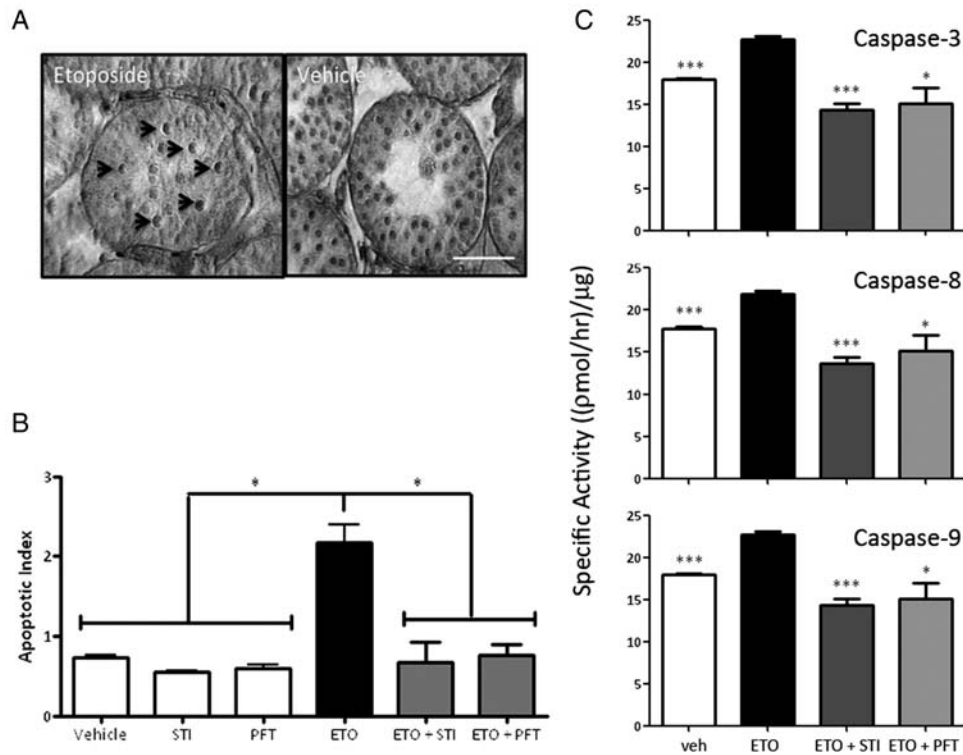
**Figure 6** Etoposide-induced germ cell apoptosis is prevented by tyrosine kinase inhibitor, STI571 and classical p53 inhibitor, PFT. (**A**) Photomicrography showing pycnotic (apoptotic) germ cell (arrowheads) in etoposide treated rat testis. Bar 100 μm. (**B**) Etoposide significantly increased the number of apoptotic cells, which was prevented in the presence of 50 μM STI571 or 100 μM PFT ($n = 3$, *$P < 0.05$). (**C**) Caspase-3, -8 and -9 activity significantly increased in 21-days-old rat testes after 100 μM etoposide treatment. Caspase activation was prevented when 50 μM STI571 or 100 μM PFT was added along with etoposide (*$P < 0.5$, ***$P < 0.001$, $n = 3$). All experiments were performed in triplicate. Bars show the mean ± SE.

the concentrations required for inhibition of the Bcr-Abl tyrosine kinase also inhibit the c-kit, platelet derived growth factor (PDGF) and ABL-related gene (ARG) receptors (Buchdunger *et al.*, 2000). Previous works have shown that STI571 delayed the formation of the germ-line stem cell pool, reduced proliferation of type A spermatogonia and induced germ cell apoptosis when administered to 5–6-day-old male rats, suggesting that STI571 inhibits pro-survival signals (probably PDGF or c-kit) in germ cells (Nurmio *et al.*, 2007, 2008). However, in the present work, we showed that STI571 has a pro-survival activity, probably by the inhibition of the apoptotic effect of c-Abl/p73. A reasonable explanation for this apparent contradiction is that STI71 has a differential effect, depending on the developmental age and cell type. It is possible that meiotic spermatocytes are not fully dependent on PDGF signaling in order to survive, as are mitotically dividing spermatogonia. In this context, it is feasible to hypothesize that whereas STI571 induces apoptosis in spermatogonia, it protects spermatocytes from apoptosis. Our *in vivo* results clearly show that STI571 protects germ cells from apoptosis induced by etoposide by preventing c-Abl/p73 pathway. Even more, we also show here that etoposide induces activation of c-Abl/p73 in GC-2 cells. However, STI571 itself induces an increase in apoptosis (data not shown), suggesting that this compound inhibits tyrosine kinase-dependent survival signals (probably PDGF or c-kit) in this cell line, similar to those observed in young rats (Nurmio *et al.*, 2007, 2008).

Therefore, further studies are necessary in order to study this dual effect of STI571 on different cell types in pubertal and adult testes.

We show that p73 is involved in germ cell apoptosis, probably assisting p53 in response to DNA damage. Interestingly, we found that PFT, a classical p53 inhibitor, was capable of preventing the increase of p73 levels caused by etoposide. The molecular mechanism of action of PFT is not very clear, but it has been reported that PFT inhibits the action of p53 by blocking its nuclear transport (Komarova *et al.*, 2003; Murphy *et al.*, 2004). Other studies show that it can also inhibit p73 activity (Davidson *et al.*, 2008) and apoptosis independently of p53 (Sohn *et al.*, 2009). Further studies are needed to understand how PFT can apparently modulate p53 and p73 activities and apoptosis in germ cells. In spite of this, we were able to show a direct correlation between the drop in p73 levels, the decrease in caspase activation and the inhibition of germ cell apoptosis both *in vivo* and *in vitro*, supporting the participation of p73 in this process.

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## Authors' roles

V.A.C.: data collection and analysis; M.C.: data collection; A.R.A.: data analysis, financial support, writing and final approval of manuscript; R.D.M.: experimental design, data analysis, financial support, writing and final approval of manuscript.

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