Differential regulation of p73 variants in response to cisplatin treatment in SH-SY5Y neuroblastoma cells

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Abstract. The present study aims to investigate the role of p73 in response to cisplatin treatment in p53 wild-type neuroblastoma SH-SY5Y cells. Results showed that cisplatin induced a dose-dependent up-regulation of p53, p73, and a number of p53-responsive genes. Interestingly, endogenous Δexon2p73-expression was down-regulated by cisplatin treatment. Neither p21 nor GADD45 induction was observed in p53-deficient Lan-1 cells, although endogenous TAp73 expression was markedly induced. In the presence of cisplatin, exogenous TAp73 overexpression in SH-SY5Y cells induced p21 up-regulation without altering the apoptotic sub-G1 cell population. Moreover, siRNA-mediated suppression of TAp73 expression did not alter the sub-G1 population. Collectively, our results suggest that wt-p53 SH-SY5Y cells respond to cisplatin by inducing p73 isoform regulation and sustaining p53-dependent apoptosis that is independent of TAp73α.

Introduction

Neuroblastoma (NB) is a malignant solid tumor of early childhood which is derived from neural crest precursor cells. Of the antitumor agents used in NB induction chemotherapy, cisplatin is known to exert cytotoxic effects as a result of DNA lesions involving the formation of intra-strand adducts. The resulting inhibition of DNA synthesis is associated with transcription arrest and subsequent cell-cycle inhibitory effects and apoptosis. Unlike most human tumors, NB cells do not exhibit mutated p53 at diagnosis. NB tumors from heavily treated patients can acquire high-level drug resistance during cytotoxic therapy. The subsequent loss of p53 function (1,2) is thought to be possibly due to a series of mutations (3). The p73 protein, which is a structural and functional homologue of the p53 protein, is capable of activating specific target genes and inducing cell-cycle arrest and apoptosis (4,5). Moreover, *TPp73* encodes several different isoforms generated by the use of a second promoter or alternative 3'-end splicing (6). The N-terminus of the protein exists in various shorter isoforms that lack a transactivation (TA) domain. The dominant negative inhibitor, ΔNp73α, and the full-length TAp73α are the most studied isoforms to date. Despite the fact that it is located on chromosome 1p36.3, a chromosome which undergoes frequent loss of heterozygosity in some cancers such as NB, the *TPp73* gene is rarely mutated in human tumors (7). Recently, it was reported that the TAp73 protein can induce apoptosis through different mechanisms. For that matter, TAp73 in Saos-2 osteosarcoma cells lacking p53 elicits endoplasmic reticulum stress due to the overexpression of scotin, an apoptosis mediator (8). Besides, p73 could activate the death receptors through PUMA transactivation and via Bax mitochondrial translocation (9). Other investigators found that, in the U373MG astroma cell line lacking endogenous p53 ($p53^-/p73^+$), TAp73 can sentisize cells to apoptosis through Fas signaling pathway and showed that this apoptosis depends on caspase activation but is not due to variations in death-inducing signaling complex components (10). Importantly, ΔNp73 is known to play a role in various human tumors (11) whereas TAp73 may be involved in development and differentiation processes in normal cells, particularly in neurons (12). In previous studies, we reported that ΔNp73 is the only accumulated isoform in undifferentiated NB (13), and that TAp73α cooperates with wt-p53 to efficiently induce apoptosis (14).

This study focuses on the role of p73 isoforms in response to cisplatin induced-cytotoxicity in malignant human neuroblasts. In this respect, it has already been shown that TAp73 inhibition increases chemoresistance in tumor cells (15). In the specific context of undifferentiated NB tumors, known to acquire chemoresistance during treatment and to overexpress the ΔNp73 isoform, one plausible hypothesis is that TAp73 and ΔNp73 might induce chemosensitivity and chemoresistance respectively, regardless of p53 status. To validate this hypothesis, we investigated the impact of p73α overexpression following the transient transfection of ΔNp73α and TAp73α

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in both the wild-type p53-expressing human NB cell line, SH-SY5Y, and the p53-deficient cell line, Lan-1.

Materials and methods

Neuroblastoma cells, culture, cisplatin treatment and transfection. The parental neuroblastoma SH-SY5Y cell line was purchased from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). Cells were grown under standard conditions in Dubelcco's modified Eagle's medium (DMEM) (Gibco) supplemented with 2 mM L-glutamine, 10% fetal calf serum, and gentamicin (Panpharma) 10 μg/ml. The Lan-1 cell line (kind gift of Dr Nicole Gross, Pediatric Oncology Research, Lausanne, Switzerland) was grown in RPMI medium supplemented with 2 mM L-glutamine, 0.1 M HEPES, 10% fetal calf serum, and gentamicin 10 μg/ml. Cell cultures were maintained at 37˚C in a humidified 5% CO_2 atmosphere.

For transfection experiments, cells were seeded onto 6-well plates. At a density of approximately 60% confluence, cells were transfected with 4 μg of recombinant vector pcDNA3 expressing either full-length p73α (TAp73α), N-terminally truncated p73 (ΔNp73α), full-length TAp73ß (TAp73ß), or the dominant negative form of p53, p53DD, using Lipofectamine 2000 reagent according to the supplier's instructions (Invitrogen). To ascertain the transfection efficiency, cells were transfected in parallel experiments with pEGFP-C1 vector (Clontech) and 30 h post-transfection, cisplatin was added to cell culture media and cells were further grown for 24 h before being collected for FACS, Western blot and RT-PCR analyses. For kinetic experiments, cells were collected at the time indicated in Fig. 2C and the induction of target genes was monitored by RT-PCR.

Cell viability assay. SH-SY5Y cells were treated for 24 h with different cisplatin concentrations. Drug-induced toxicity was assessed by the colorimetric MTS assay carried out in 96-well plates containing 100 μl of culture medium. At the time-point of treatment, 20 μl of CellTiter 96® Aqueous One solution cell proliferation assay reagent (Promega) was added to each well and incubated at 37˚C for 2 h. The absorbances were determined at 490 nm with a 96-well plate reader (MRX, Dynatech Laboratories). All samples were tested and measured in triplicate using untreated cells as a 100% survival control. Mean values and standard deviations were determined. Background absorbance was determined with a blank solution containing 100 μl of DMEM and 20 μl of CellTiter solution, and this value was substracted from all experimental values obtained.

siRNA transfections. Double-stranded TAp73-siRNA oligonucleotides (for sequence, see ref. 15) and p53-siRNA were purchased from Qiagen (SI02655170 for p53-siRNA). SH-SY5Y cells (500 000 cells into 6-well plates) at 40-50% confluency were treated or not with cisplatin 10 μM and then transfected for 48 h with siRNA-luciferase or siRNA-TAp73 (final concentration of 200 and 500 nM) using Oligofectamin (Invitrogen). Then cells were collected for cell-cycle analysis by FACS and for immunodetection by Western blotting.

Flow cytometry. Cell-cycle distributions were measured by fluorescence-activated cell sorting (FACS) analysis. Cells were collected and fixed in 70% ethanol at -20˚C for at least 30 min. Cells were washed twice with PBS and incubated for 30 min at room temperature in PBS containing 100 μg RNase A and 10 μg propidium iodide. DNA content and cell-cycle analysis were assessed using FACScalibur.

Western blot analysis. For total protein extracts, cells were harvested and lysed in 1X Laemmli buffer supplemented with protease inhibitor cocktail (Roche). For cell fractionation, cells were harvested in A buffer (HEPES 10 mM pH 7.8, KCl 10 mM, $MgCl_2$ 2 mM, EDTA 0.1 mM, protease inhibitor cocktail). After a 10-min incubation time at 4˚C, NP40 was added (0.6%) and cells were centrifuged (2800 g for 5 min). The supernatant corresponds to the cytoplasmic extract. Pellet was suspended in B buffer (HEPES 50 mM pH 7.8, KCl 50 mM, NaCl 300 mM, EDTA 0.1 mM, protease inhibitor cocktail), and incubated for 30 min at 4˚C. After centrifugation, supernatant corresponds to the nuclear extract. Protein lysates were submitted to 7.5 or 10% SDS-PAGE, and then transferred onto nitrocellulose filters. After saturation, the membranes were incubated with primary antibody diluted in PBS 0.1% Tween-20, 3% skim-milk. The primary antibodies used were anti-p73 polyclonal antibody (1/1000, gift from Sanofi, Labèges), anti-p53 monoclonal antibody (DO-7, 1/1000, Dako), anti-$p21^{WAF1}$ monoclonal antibody (Ab-1, 1/200, Oncogene), anti-GADD45 polyclonal antibody (H-165, 1/200, Tebu-bio), anti-PUMA polyclonal antibody (1/1000, Usbiological), anti-MDM2 monoclonal antibody (Ab-1, 1/1000, Oncogene), and anti-ß-actin monoclonal antibody (C4, 1/1000, Chemicon).

Semi-quantitative RT-PCR analysis. Total RNA was isolated using RNAble reagent (Eurobio). Reverse transcription was performed from 1 μg total RNA using Superscript II RNase H-reverse transcriptase (Gibco BRL). PCR reactions were performed on 1 ng of cDNA using Platinum Taq polymerase (Invitrogen) in a PTC100 thermocycler (MJ Research). The primer sequences were as follows: $p21^{WAF1}$ forward primer, 5'-GCGACTGTGATGCGCTAATG-3', and reverse primer, 5'-AGAAGATCAGCCGGCGTTTG 3'; GADD45 forward primer, 5'-GAAGACCGAAAGGATGG-3', and reverse primer, 5'-GGGAGATTAATCACTGG-3'; p73 (exon 1-3) forward primer, 5'-CGGGACGGACGCCGATG-3', and reverse primer, 5'-GGTGGAAGACGTCCATGCTGGAA-3'; p73 (exon 3bis-5) forward primer, 5'-ACAAACGGCCCGCATGT TC-3', and reverse primer, 5'-CTTGGCGATCTGGCAGTA G-3'; p53 forward primer, 5'-CCCCTCCTCAGCATCTTAT CC-3', and reverse primer, 5'-CACCTCAAACGTGTTCCGT CC-3'; GAPDH forward primer, 5'-CTGCACCACCAACT GCTTAG-3', and reverse primer, 5'-AGGTCCACCACTG ACACGTT-3'. PCR products (1/10) were loaded onto 6% polyacrylamide gel electrophoresis and relative expression was analyzed using GAPDH as internal control.

Results

Cisplatin induces p53/TAp73 target gene expression in SH-SY5Y ($p53^+$, $p73^+$) cells but not in Lan-1 ($p53^-$, $p73^+$) cells. We first analyzed the expression of p53, p73, and p53/p73

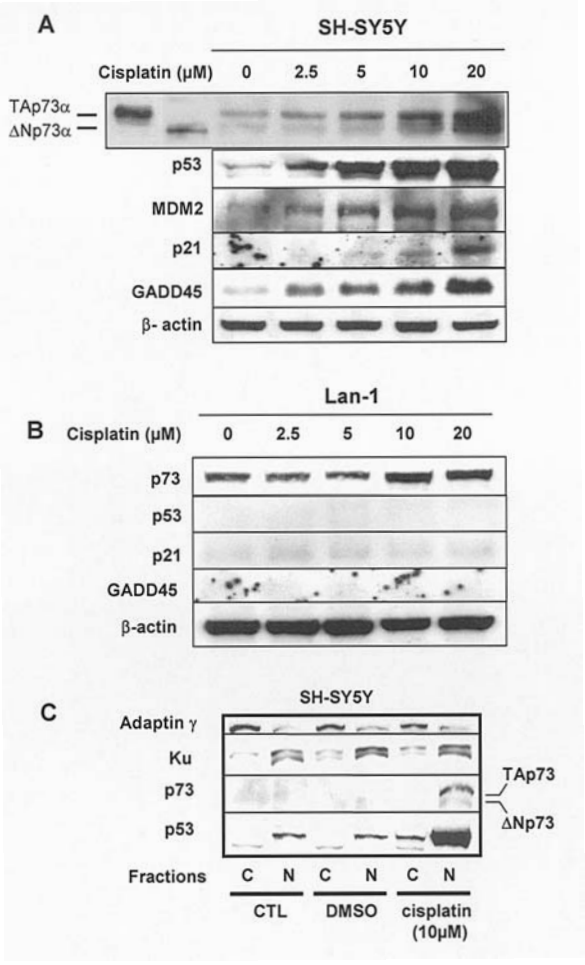

Figure 1. p53/p73 target genes are induced by cisplatin in p53-proficient cells but not in p53-deficient cells. Cells were treated with increasing doses of cisplatin for 24 h, and protein extracts were submitted to Western blotting and p53/p73 target genes were analyzed in p53 wt SH-SY5Y cells (A) and in p53-deficient Lan-1 cells (B). (C) Cell fractionation experiments were performed as described in Materials and methods with (from left to right) parental cells (CTL), cells treated with Dimethyl sulfoxyde (DMSO) and treated with cisplatin 10 μM; C, cytoplasmic fraction; N, nuclear fraction.

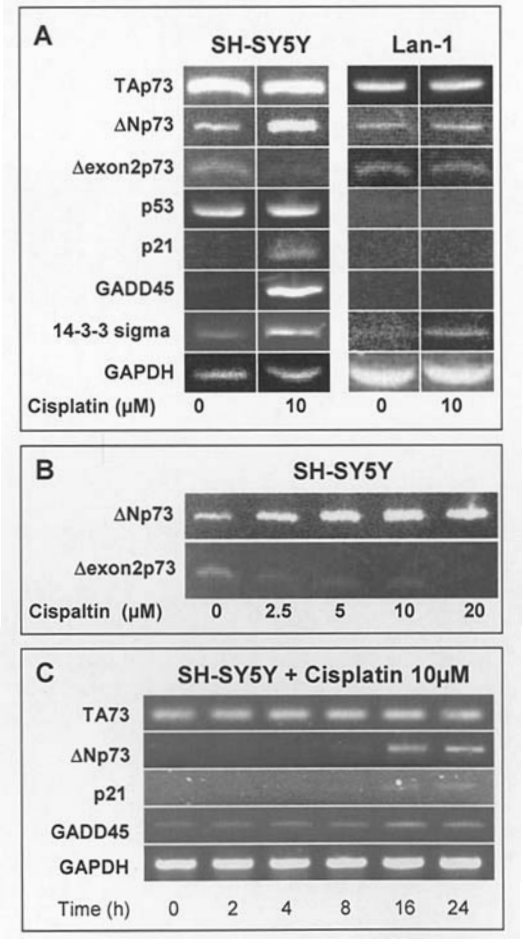

Figure 2. Differential regulation of p73 isoforms in SH-SY5Y cells in response to cisplatin. (A) SH-SY5Y and Lan-1 cells were treated with different doses of cisplatin for 24 h. RNA was extracted and submitted to semi-quantitative RT-PCR experiments. (B) The amounts of ΔNp73 and Δexon2p73 transcripts induced by cisplatin. (C) Kinetic measure by RT-PCR of target gene expression in cells treated with cisplatin 10 μM.

target genes in response to 24-h treatment with cisplatin at various concentrations. In SH-SY5Y cells ($p73^+$; $p53^+$), cisplatin induced p53 and p73 accumulation in association with a dose-dependent increase in the target genes involved in cell-cycle arrest (p21), DNA repair (GADD45) and the regulation of p53/p73 activity (MDM2) (Fig. 1A). Conversely, none of these p53-target genes was expressed in the p53-deficient Lan-1 cells, despite the presence of endogenous p73 (Fig. 1B).

Given that p53 and p73 exert their transcriptional activity in the nucleus, subcellular distribution in SH-SY5Y cells was used as a marker for these activities. Cell fractionation was analyzed by marking cytoplasmic and nuclear fractions with gamma-adaptin and ku, respectively. Following cisplatin treatment, p53 and p73 were located mainly in the nuclear fraction (Fig. 1C), suggesting that these stabilized proteins might act as efficient transcription factors.

We then wondered whether induction might occur at transcriptional level. Semi-quantitative RT-PCR showed that, in response to cisplatin, p53 and TAp73α expression in SH-SY5Y cells was stable at the mRNA level, strongly suggesting that protein accumulation is a result of protein stabilization rather than the degree of gene transcription (Fig. 2A). In contrast, p21, GADD45, and 14-3-3σ were significantly induced. Transcription of the N-terminal truncated isoform of p73 (ΔNp73) was strongly up-regulated in a dose-dependent manner but, strikingly, the level of Δexon2p73 transcript had decreased (Fig. 2A and B), showing thus a complex regulation among various p73 N-truncated transcripts in response to cisplatin genotoxicity. To test whether or not the endogenous ΔNp73 is induced earlier than 24 h of treatment, transcript levels were measured by RT-PCR at several time-points in SH-SY5Y cells treated with 10 μM of cisplatin. As shown in Fig. 2C, the level of ΔNp73 expression was appreciably induced after 16 h of treatment and appeared to be the same at 24 h.

Cisplatin induces apoptosis in SH-SY5Y cells but not in Lan-1 cells. Cisplatin toxicity was studied in the two cell lines using an MTS cytotoxicity assay. Cisplatin induced dose-dependent cytotoxicity in SH-SY5Y cells (Fig. 3A) but not in Lan-1 cells (data not shown). Analysis of flow cytometry showed that cisplatin treatment induced a significant increase in the sub-G1 fraction, which is indicative of cell apoptosis (Fig. 3B and C). No such increase was observed in Lan-1

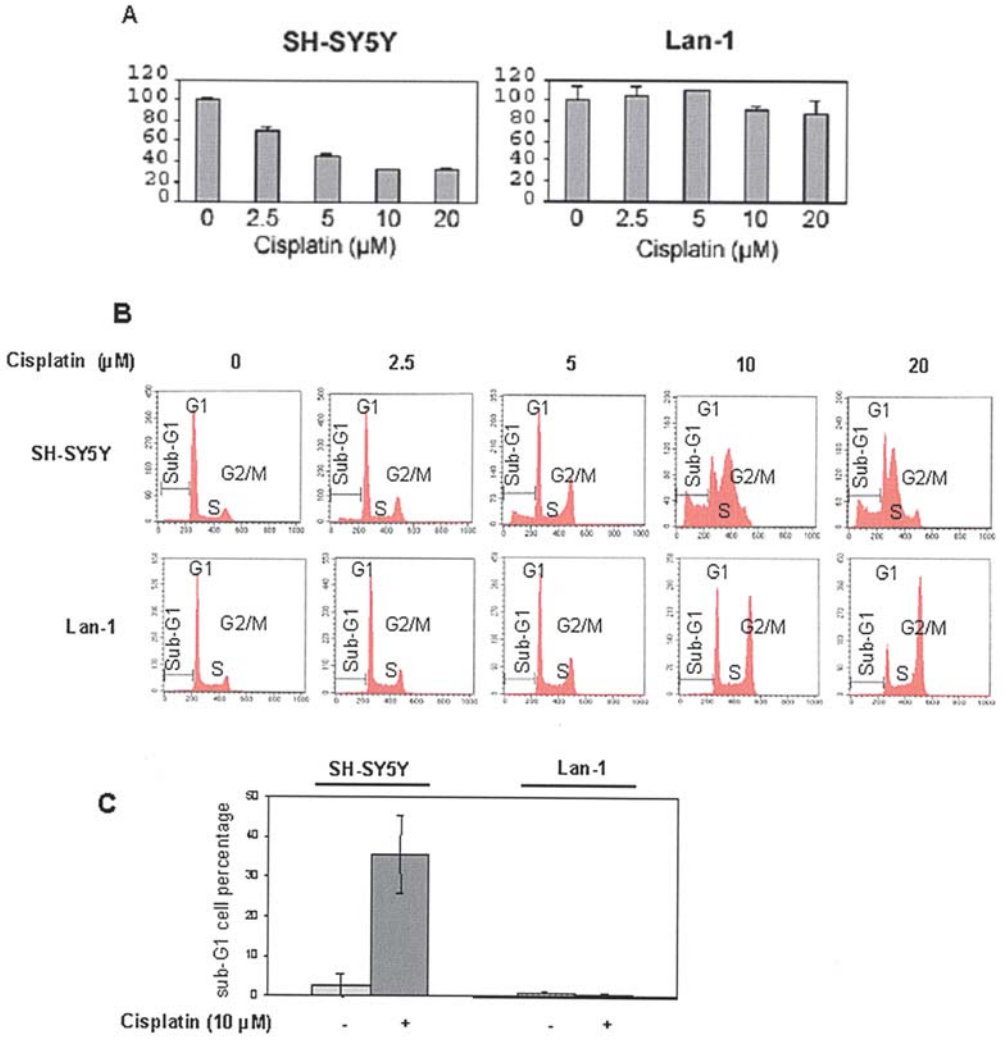

Figure 3. Cytotoxicity and apoptosis induced by cisplatin in SH-SY5Y cells. (A) SH-SY5Y and Lan-1 cells (left and right panel respectively) were treated with different doses of cisplatin for 24 h and submitted to cytotoxicity MTS test. The data are expressed as mean from triplicate assays. (B) Cell cycle profile analysis. SH-SY5Y and Lan-1 cells were treated with increasing doses of cisplatin for 48 h and submitted to flow cytometry analyses. (C) Histogram reporting the mean value of the percentage of sub-G1 cell population obtained in three or two independent experiments for SH-SY5Y and Lan-1 cells respectively.

cells (Fig. 3B and C). Moreover, the proportion of S-phase cells increased gradually in line with increased concentrations of cisplatin in SH-SY5Y cells while, conversely, Lan-1 cells were enriched in the G2/M phase (Fig. 3B).

TAp73α and ΔNp73α differentially regulate p53/p73 target genes in SH-SY5Y cells but are not involved in cisplatin-induced apoptosis. We then examined the effects of TAp73 and ΔNp73 overexpression on p53 and p53/p73 target gene expression using transient transfection with recombinant plasmids.

First the transactivation efficiency of TAp73 or ΔNp73 was measured and expressed as percentage of GFP cells transfected with pEGFP-C1 vector. The transfection efficiency was found to be nearly 100% in SH-SY5Y cells and 70% in Lan-1 cells (data not shown). Moreover, semi-quantitative RT-PCR was performed to examine endogenous ΔNp73 expression following exogenous TAp73 overexpression. Indeed, ΔNp73, a dominant negative regulator containing TAp73-responsive elements in its promoter, is known to be induced by TAp73 and p53 (16,17). As expected in the absence of cisplatin, endogenous ΔNp73 mRNA expression was induced upon TAp73 expression vector when compared to transcript levels in empty-vector or ΔNp73-transfected cells (Fig. 4A, upper panel, left side); this ΔNp73 transcript level was found to be increased in cisplatin-treated cells transfected with TAp73-expression vector (Fig. 4A, upper panel, right side).

In the absence of cisplatin, p53 protein accumulation was induced by TAp73α as well as by ΔNp73α overexpression, as has been previously reported using the adenoviral system (14). This accumulation in untreated SH-SY5Y cells might be the result of stabilization induced by competition for MDM2 as p73 is known to form a stable complex with MDM2 (18,19). p53 accumulation was strongly enhanced in response to treatment with cisplatin. As expected, in the absence of cisplatin, p21 up-regulation was totally inhibited by ΔNp73α the dominant negative inhibitor of p53/TAp73. Instead, in

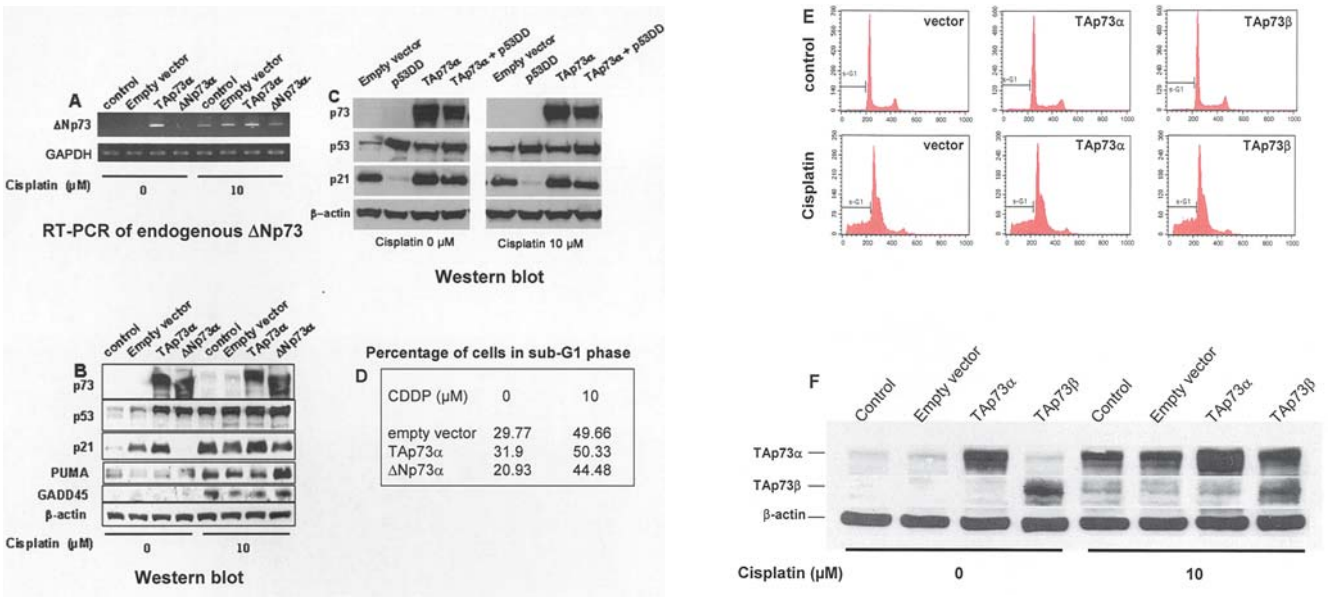

Figure 4. Effect of an ectopic expression of either TA or ΔNp73α in SH-SY5Y cells in response to cisplatin. Effect of TA- and ΔNp73 in SH-SY5Y cells treated with cisplatin on p53 target genes. (A) Induction of endogenous ΔNp73 in cisplatin-treated cells. SH-SY5Y cells were transiently transfected with empty vector or vector expressing either TAp73α or ΔNp73α, and were treated or not with 10 μM cisplatin for 24 h and analyzed by semi-quantitative RT-PCR. (B) Protein samples were analyzed by Western blotting for the expression of p73, p53 and target genes. (C) SH-SY5Y cells were transiently transfected with TAp73α expressing vector, p53DD expressing vector, or both. (D) Measurement of the cell percentage in sub-G1 phase by FACS. (E) Cell cycle profile of cells transfected with either TAp73α or TAp73ß expressing vectors. (F) Western blotting showing the transfection control in SH-SY5Y cells with TAp73α and TAp73ß.

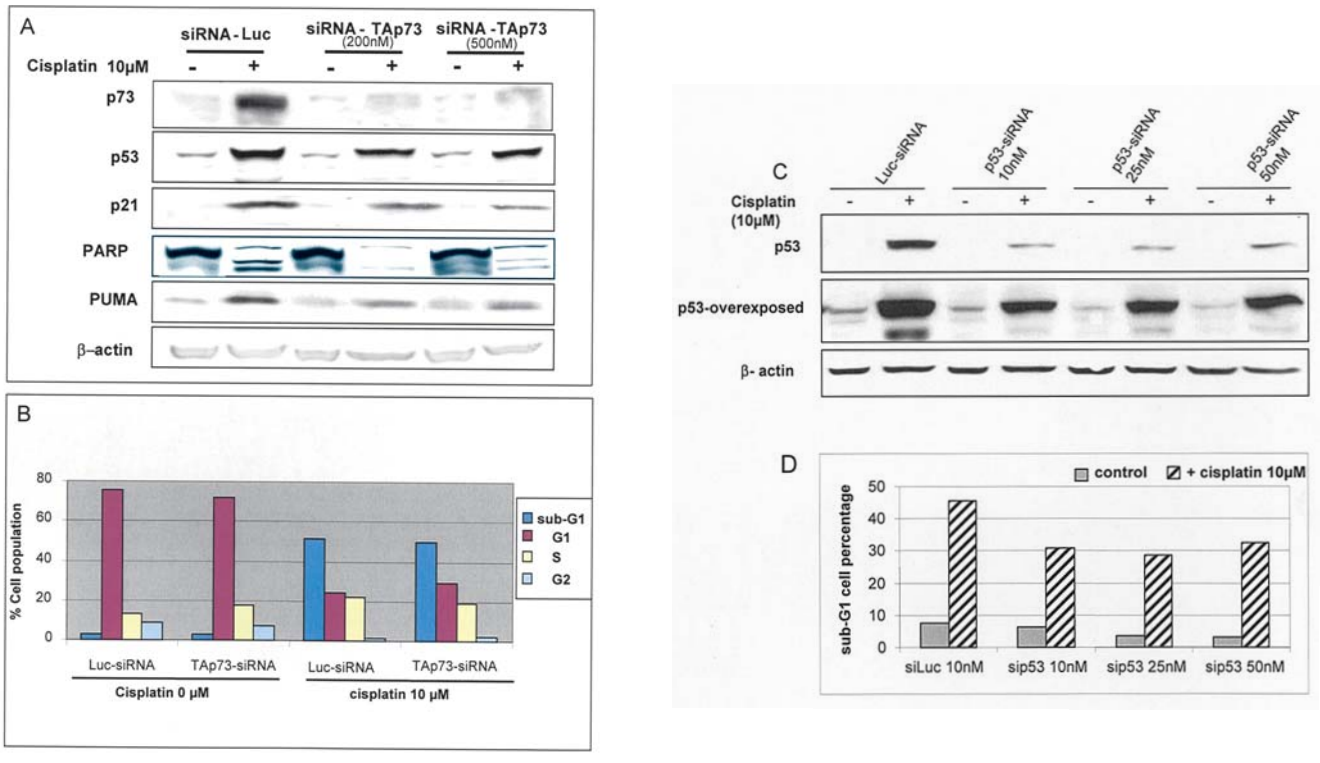

Figure 5. Blocking TAp73 expression with siRNA does not change sub-G1 phase. (A) SH-SY5Y untreated cells or treated with cisplatin 10 μM were transfected with non-target Luc-siRNA and TAp73-siRNA for a final concentration as indicated. At 48 h post-transfection, cells were lysed by Laemmli buffer and the total extracted protein was submitted to Western blotting for p73, p53, p21, PARP and PUMA expression analyses. (B) TAp73-independent apoptosis shown as histogram obtained by cytofluorometry using FACS analysis from cisplatin-treated SH-SY5Y cells. At 48 h post-transfection, nuclear DNA content was analyzed by FACS. The percentage of cells in each phase, was presented (sub-G1, G1, S and G2 as gray, white, striped and black box respectively). (C and D) SH-SY5Y cells were treated with p53-siRNA in the absence or presence of cisplatin 10 μM compared to control Luc-siRNA and analyzed by Western blotting (upper panel) or FACS (bottom panel). Two exposition times are shown: 3 min (upper) or 1 h (middle); the amount of p53-siRNA is indicated and ß-actin was used as loading control. (D) Percentage of sub-G1 fraction in SH-SY5Y treated (striped black box) with p53-siRNA (sip53) or with luciferase-siRNA (siLuc) or untreated cells (grey box).

the presence of cisplatin, the strong degree of p21 induction observed with TAp73α was only partially inhibited by ΔNp73 (Fig. 4B). Moreover, overexpressed ΔNp73α rather than down-regulating PUMA expression, seemed to enhance it. In cisplatin-treated TAp73α overexpressing cells, however, PUMA and GADD45 expression levels were similar to those in the control or empty vectors, suggesting that, even in the presence of genotoxic stress, TAp73 is not involved in the induction of the expression of these proteins (Fig. 4B).

In order to inhibit the DNA-binding ability of p53, cisplatin-treated cells were cotransfected with TAp73α and p53DD, the dominant negative form of p53. When p53DD alone was overexpressed, it inhibited the basal level of p21 expression, showing that transfection-induced p21 expression is dependent on p53 transcriptional activity (Fig. 4C). In TAp73/p53DD cotransfected cells, however, p53DD only partially inhibited p21 expression, suggesting that the remaining p21 expression might be the result of TAp73 transcriptional activity (Fig. 4C).

The effect of TA and ΔNp73α on cell-cycle distribution was further analyzed by flow cytometry. p73 overexpression did not alter the sub-G1 fraction in TAp73α-SH-SY5Y-transfected cells when compared to cells transfected with an empty vector (32% versus 30% without cisplatin and 50% versus 49% with cisplatin, respectively, See table in Fig. 4D), once again supporting the hypothesis that TAp73α is not involved in cisplatin-induced apoptosis. In contrast, ΔNp73 overexpression slightly protected cells from apoptosis in the absence of cisplatin (21% versus 30% of cells in the sub-G1 phase). This effect was less marked following treatment with cisplatin (sub-G1 fraction of 44% in ΔNp73α transfected cells compared to 49% in empty vector cells).

Finally, as the TAp73ß isoform has been shown to be more transcriptionally active than TAp73α, we analyzed the effect of its overexpression on apoptosis induction. Like TAp73α, TAp73ß did not increase the sub-G1 cell population either with or without cisplatin (Fig. 4E).

Silencing TAp73α expression with siRNA did not interfere with the sub-G1 fraction. To further rule out any involvement of TAp73 in the cell-death pathway, we wondered whether silencing endogenous TAp73 expression in wild-type p53 SH-SY5Y cells ($p53^+/p73^+$) would result in the inhibition of apoptosis. We thus used Western blot analysis to test the efficiency of TAp73-siRNA in SH-SY5Y cells treated with 10 μM cisplatin compared to that in untreated cells. The upper panel of Fig. 5A shows that, as expected, strong cisplatin-induced TAp73 expression in cells transfected with the non-target control, Luc-siRNA, decreased dramatically in intensity in cells transfected with TAp73-siRNA (both with 200 and 500 nM). This inhibition was specific to p73 as p53-protein expression continued to be induced by cisplatin (Fig. 5A, second panel). It is interesting to note that cisplatin stimulated TAp73 and p53-protein expression (Fig. 1A). p53-protein expression was also stimulated by TAp73 over-expression (Fig. 4B). It can be concluded, therefore, that the p53 expression observed in Luc-siRNA following cisplatin treatment was the result of both direct drug induction and TAp73 activation. In TAp73-siRNA transfected cells, the TAp73-mediated p53 induction was inhibited by siRNA. Consequently, the expression profile of PUMA matched that of p53 in these cells, indicating that PUMA regulation is TAp73-independent (Fig. 5A, lane TAp73-siRNA compared to control Luc-siRNA). Flow cytometry revealed that the sub-G1 fraction in cells treated with TAp73-siRNA remained unchanged when compared to that of the Luc-siRNA control, (Fig. 5B); once again, this finding indicates that apoptosis in cisplatin-treated SH-SY5Y cells is independent of p73. Then, to check the p53 involvement in cisplatin-triggered apoptosis, double-stranded p53-siRNA at different doses was transfected using HiPerfect transfection agent into SH-SY5Y cells. In the absence of cisplatin, p53 inhibition was efficient from 10 nM (Fig. 5C, upper panel). To better estimate the decrease of p53 protein expression, the overexposed Western blotting revealed by chemiluminescence showed that the inhibition was similar whether cells were treated with p53-siRNA at 10, 25 or 50 nM (Fig. 5C, middle panel). Notably, this inhibition was ineffective when cells were treated with cisplatin (Fig. 5C, lane cisplatin-treated versus untreated cells). Consequently, the amount of sub-G1 fraction measured by FACS in cisplatin-treated cells decreased by approximately 1/3 only as compared with control luciferase-siRNA (Fig. 5D).

Discussion

p53-p73 interactivity has been shown to promote drug-induced apoptosis in other malignant cells (20,21). The findings of the present study show that cisplatin induced differential regulation of p73 variants. TAp73α and ΔNp73α were both up-regulated while p73Δexon2 was down-regulated. The down-regulation of *p73Δexon2* transcript that is controlled by the same promoter as for TAp73 isoform is, to some extent, to be expected given its anti-apoptotic role in agreement with a previous study (22).

The fact that Δexon2p73 was down-regulated by cisplatin in a dose-dependent manner and that ΔNp73, in contrast, was up-regulated, highlights the complex regulation of p73-isoform expression. Our evidence suggests that the Δexon2p73/ΔNp73 ratio might be a possible cisplatin-sensitivity marker.

As far as ΔNp73α is concerned, we would expect this isoform to increase chemoresistance. Our findings show that overexpressed ΔNp73α inhibited the expression of certain p53/p73 target genes such as p21, and moderately reduced the sub-G1 fraction in untreated SH-SY5Y cells. In cisplatin-treated cells, however, this antagonistic effect decreased, suggesting that this anti-apoptotic function is exerted under slight but not high stress conditions that force cells to die (cisplatin, herein). In addition, the fact that PUMA was not down-regulated by overexpressed ΔNp73α, suggests that the 'dominant negative' role of ΔNp73α is dependent on the target promoter. In this regard, Vossio *et al* (23), in a series of p53/ΔNp73 cotransfection experiments, showed that ΔNp73α overexpression decreased p53-induced activation of p21 but had no effect on p53-induced activation of other apoptotic genes such as p53AIP. Moreover, the expression of various genes, such as *Pig3* and *cyclin G,* can be increased by ΔNp73 in a p53-independent fashion (24), thus suggesting that PUMA regulation is independent of p53 in neuroblastoma cells.

We recently found that ΔNp73α is capable of transactivating the neurogene *BTG2* promoter specifically in wild-type p53

neuroblastoma cells, but not in mutated p53 cells, and that this transactivation is specific to neuroblastoma as it is not observed in MCF-7, a breast cancer cell line (25).

Given the dominant negative role of ΔNp73 on p53 downstream genes, the inhibition of p21 expression following transient ΔNp73 overexpression occurred as expected. However, following treatment with cisplatin, p21 was still significantly expressed despite p53 inhibition by p53DD, suggesting that, in this case, TAp73 may be involved in the regulatory process. p21 protein expression, however, was not observed in p53-deficient Lan-1 cells despite endogenous p73 expression. A recent report demonstrated that the p73 protein may be a direct target for CDK complexes (26). The p21 protein, although accumulated, may be inactive due to its interaction with Akt (27).

As far as the involvement of TAp73 in apoptosis is concerned, our previous study demonstrated that an overexpression of adenoviral-TAp73 induced a considerable increase in the sub-G1 population (14,28). However, in this particular study, FACS analysis showed that ectopic TAp73α expression in SH-SY5Y cells did not interfere with apoptosis either with or without cisplatin. This discrepancy might be due to the fact that transfection experiments induce lower TAp73α protein levels than adenoviral infection. Nonetheless, even in Lan-1 cells ($p73^+$, $p53^-$), neuroblasts did not undergo apoptosis but rather accumulated in the G2 phase, suggesting once again that TAp73 is not involved in cisplatin-mediated apoptosis.

This particular finding argues against an expected role of TAp73 in the response to cisplatin-induced DNA damage, at least in cells expressing wt-p53. Using the RNA interference approach, Irwin *et al* (15), showed that the inhibition of TAp73 expression did not significantly restore viability in cisplatin-treated wt-p53 U2OS cells. In contrast, the same authors also showed that TAp73 disruption enhanced chemoresistance in mutated p53 SW480 cells. Collectively, the findings of these studies, combined with our findings, suggest that TAp73 is not involved in the cisplatin-response in cells lacking functional p53 (Lan-1 cells herein, for example) because of the need for p53/p73 cooperation or in cells expressing functional wild-type p53 (SH-SY5Y cells herein, U2OS cells in Irwin's study). However, Gong *et al* (29) reported that, in HCT116-3(6) cells with a functional *MLH1* gene but not in HCT116 lacking the *MLH1* gene, c-Abl and p73 is implicated in a mismatch-repair-dependent apoptosis pathway which contributes to cisplatin-induced cytotoxicity.

Other evidence has been documented in a recent study indicating that p73 as well as p63 was not required for the p53-dependent (or p53-independent) apoptosis of T cells (30). In this particular study, the transient overexpression of a mutated transcriptionally inactive form of TAp73α (p73α R293H, donated by Dr Caput) had the same cellular effect as overexpressed wt-TAp73α (data not shown), arguing in favor of the lack of TAp73α involvement in cisplatin response. However, it has been reported that, in other cancer cell lines such as breast carcinoma cells that lack drug-induced p53 activation (e.g. MCF7/ADR^{IGR} and MDA-MB157), TAp73 can assume the function of p53 in apoptosis (31). Therefore, the role of TAp73 in apoptosis induction in p53-deficient cancer cells might be considered as tissue-specific.

The lack of TAp73α involvement prompted us to test the potential role of TAp73ß, an alternative C-terminus splice, in chemosensitivity. In contrast to Stiewe *et al* (32), who demonstrated that TAp73ß was more efficient at inducing apoptosis than TAp73α, we observed no difference between the effects of the two overexpressed isoforms, suggesting that the C-terminus domain of TAp73 is not a determining factor in cisplatin-induced p73 activity.

Considering that overexpressed MycN in Lan-1 cells might interfere with apoptosis signaling pathways, this particular cellular model might not be a good control model for p53-negative neuroblastoma cells. Moreover, there might be a link between TAp73 and MycN as the human *p73* promoter contains two MycN sites located between positions -119 and -2714 (33). It has been reported that MycN modulates the expression of p73 transcripts in SK-N-AS and SH-EP1 neuroblastoma cell lines, which have been transiently transfected with the pCMV*myc* expression plasmid (34). Besides, as an apoptosis signaling pathway involves both p53 and MycN (35), it is logical to speculate a possible link between TAp73 and MycN (36). To rule out any misinterpretations that could arise from this possible association, we used TAp73-siRNA transient transfection to abrogate TAp73 expression in isogenic SH-SY5Y cells ($p53^+/p73^+$) treated with cisplatin. Our results show there was no change in apoptosis in comparison to cells transfected with a Luc-siRNA non-target control, further supporting the idea that p53-induced apoptosis in p53 wild-type neuroblastoma cells does not need p73. The fact that, cisplatin may form with DNA huge adducts, p53-siRNA cannot access the target mRNA sequence, so that p53 expression is still enhanced in cisplatin-treated cells. Recent findings have shown that cisplatin neurocytotoxicity is the result of different apoptosis pathways that involve specific molecules in a complex network (37). However, the diverse role of p73 isoforms in the physiological condition of neuronal cells remains to be elucidated.

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