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Expression of *p53*-family members and associated target molecules in breast cancer cell lines in response to vincristine treatment

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Abstract

As the antimitotic agent vincristine (VCR) has been reported to induce a weak p53 response in some studies, we hypothesised that *p73* and *p63*, the recently described *p53* homologues, may replace *p53* in triggering apoptosis or cell cycle arrest effectors in VCR-treated cell lines. To address this issue, we measured *p53*, *p73* and *p63* mRNA and protein levels in two VCR-treated breast cancer cell lines, one *p53*-proficient (MCF7) and the other *p53*-deficient (MDA-MB157). We found an increase of *p53* mRNA and protein levels in VCR-treated MCF7 cells, while, as expected, no p53 protein was detected in VCR-treated MDA-MB157 cells. Surprisingly, the *p73* mRNA and protein expression levels decreased in both cell lines during VCR treatment, whereas p63 protein levels remained unchanged. In both cell lines, up-regulations of the canonical *p53*-target genes, such as *p21* and *GADD45*, were consistently observed. We conclude that, in response to VCR treatment: (1) *p53* is markedly induced in MCF7 cells, with the same extent than after DNA damaging drugs treatments; and (2) *p63* is not involved, while *p73* expression is down-regulated regardless of the *p53* status of the cell lines. Our results therefore suggest the involvement of a fourth member of the *p53* gene family, or the use of another pathway able to trigger canonical *p53*-target genes in response to VCR in *p53*-deficient cells.

Keywords: *p53*; *p63*; *p73*; Vincristine

1. Introduction

Breast cancers, the most frequent tumors in women, are usually treated by surgery and chemotherapy. In response to cellular stress caused by a number of anticancer agents, p53 protein accumulates and transactivates target genes such as *p21*, *14-3-3σ*, *GADD45*, and *bax*, known to be involved in cell cycle arrest, DNA repair, or apoptosis [1–4]. Although the *p53* pathway is instrumental for the response of breast cancer cell lines, such as MCF7, to intercalating and DNA synthesis inhibitors (adriamycin and cisplatin), it has been reported that this pathway is not always used in response to VCR treatment that has been shown to induce *p53*-target genes with weak p53 protein induction [5–9]. Moreover, in investigating the need for wild-type *p53* in the response of mouse embryonic fibroblasts to paclitaxel (trade mark Taxol)—an antineoplastic agent that stabilises cellular microtubules—Lanni *et al.* [10] pointed out that the drugs efficiency *in vivo* may be due not only to its microtubule-stabilising activity, but also to its ability to activate local release of an apoptosis-inducing cytokine such as TNFα, in a *p53*-independent manner. This evidence therefore suggest the involvement of *p53*-independent pathways in the response of tumours cells to some chemotherapeutic agents.

Two *p53* homologue genes, called *p73* and *p63*$^{p40/p51}$, have recently been discovered [11–14]. The *p73* gene is located at locus 1p36.33, and the *p63* gene is located at locus 3q27–28. The regions of partial homology between *p73*, *p63* and *p53* correspond to *p53* transactivation, DNA binding, and oligomerisation domains. Unlike *p53*, both *p73* and *p63* give rise to multiple isoforms due to alternative splicing and transcription from two different promoters. Some of these isoforms which lack the transactivation domain, the ΔN isoforms, exert a dominant negative action on the full-length isoforms (TA isoforms) [15].

When over-expressed in cultured cells, *p73* and *p63* have been demonstrated to be able to transactivate *p53* target genes (such as *p21*, *bax*, *MDM2*, *14-3-3σ*, *GADD45*) and

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induce apoptosis [13,14,16–22]. In response to some DNA damaging agents (actinomycin D, UV, doxorubicin, cisplatin, taxol, and mitomycin C), a lack of *p73* induction was initially observed in cancer cell lines [11,23,24], then, more recently, and in contrast, an up-regulation of p73 protein expression and/or its activity was demonstrated in response to taxol, cisplatin and radiotherapy [25–28]. Concerning *p63*, Katoh *et al*. [29] showed that TAp63γ protein accumulates and induces erythroid differentiation in response to DNA-damaging treatment triggered by UV radiation or actinomycin D, while a down-regulation of ΔNp63 in response to UV-B is required for epidermal apoptosis [30].

This study was conducted to assess the involvement of each gene of the *p53* family in VCR-treated breast cancer cells. We found that treated *p53*-proficient MCF7 cells followed the *p53* pathway, while down-regulation of *p73* expression was observed in both treated *p53*-proficient MCF7 cells and *p53*-deficient MDA-MB157 cells. No induction of p63 protein was found in either of the two cell lines. Data obtained in *p53*-deficient cells after VCR treatment therefore suggest that the *p73* induction is not required to induce *p53*-target genes.

2. Materials and methods

2.1. Cell culture conditions

MCF7 and MDA-MB157 cell lines were cultured as monolayers at 37° with 5% CO_2, and maintained by regular passage in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, 10 mM HEPES, 5 μg/mL of fungizone, 12.5 μg/mL of vancomycin, and 10 μg/mL of gentamicin.

Twenty-four hours after plating, cells were treated for 24 hr by vincristine, VCR, (Oncovin® 1 mg/mL, Pierre Fabre), adriamycin, ADR, (Adriblastine®, Pharmacia), and cisplatin, CDDP, (Cisplatine, Qualimed), before harvesting and counting by the trypan blue exclusion method. Upon high VCR doses, dead cells appeared as cells floating in the medium. A viability test was carried out on cells obtained following trypsinisation of cell monolayers from VCR-treated and control cells using a trypan blue exclusion test. Most of VCR-treated anchored cells, after trypsinisation were seen as cells excluding trypan blue (thus living cells). Percentage viability was deduced from cell counts.

2.2. DNA and RNA preparations

The cell monolayers fixed on the bottom of the flasks were trypsinised, the cells were counted and then lysed using sarkosyl-guanidinium-isothiocyanate (GTC). DNA and total RNAs were prepared using the GTC–CsCL gradient method. DNA was collected from the supernatant, dialysed and treated with proteinase K. After deproteinisation by phenol–$CHCl_3$, DNA was precipitated by ethanol and conserved in T.E. buffer (10 mM Tris–HCl pH 7.5; 1 mM EDTA). RNA was spun down at the bottom of the centrifuge tube, washed with 70% ethanol and precipitated with absolute ethanol. Following incubation with DNase RNase-free (GibcoBRL), it was purified by RNAzol® (Bioprobe).

2.3. p53 Gene sequencing

The *p53* gene was sequenced as previously described [31].

2.4. Semiquantitative determination of p53, p73, p21, GADD45 and 14-3-3σ transcript levels

cDNA was obtained by reverse transcription (RT) of RNA (2 μg), as previously described [32].

p53 cDNA was amplified using primers p1 and p2 located on the gene coding region (exons 6–8), *p73* using p3 and p4 (exons 6–8), *p73α* and *p73β* using p5 and p6 (exons 12–14), *ΔNp73* using p7 and p8, *TA + p73* using p9 and p10, *p21* using p11 and p12, *GADD45* using p13 and p14, and *14-3-3σ* using p15 and p16 (Table 1). In order to semiquantitatively determine the transcript levels of these genes, preliminary one-cycle stepwise amplifications were performed, and amplification kinetic curves were plotted, showing that a plateau phase was reached after a different number of cycles for each gene. The transcript level was therefore detected at a number of cycles preceding the plateau phase (Table 1).

cDNA (1 μL) was amplified in a 20 μL reaction mixture containing 100 ng of each primer in 10 mM Tris–HCl pH 9.0; 50 mM KCl; 0.1% TritonX-100; 1.75 mM $MgCl_2$; 200 μM dNTP; 1 μCi α^{33}P dATP and 1 U Taq DNA polymerase (Taq Gold Cetus Perkin-Elmer). The reaction mixtures were heated at 94° for 15 min, and amplifications were carried out in sequential cycles of 94° for 30 s, 59° for 1 min

Table 1
Description of primer pairs used in the RT-PCR method

Gene	Primers	Cycles
p53	p1: 5′ CCC CTC CTC AGC ATC TTA TCC 3′	25
	p2: 5′ CAC CTC AAA GCT GTT CCG TCC 3′	
p73	p3: 5′ TGG ATG ACC CTG TCA CCG GC 3′	32
	p4: 5′ TGC TCC CGG TAG TGG TCC TCA 3′	
	p5: 5′ CCG ACC CCA GCC TCG TCA 3′	32
	p6: 5′ CTG AGC CGC CGA TGG AGA T 3′	
	p7: 5′ CGG GAC GGA CGC CGA TG 3′	32
	p8: 5′ GGT GGA AGA CGT CCA TGC TGG 3′	
	p9: 5′ ACA AAC GGC CCG CAT GTT 3′	32
	p10: 5′ GAA CCG CTA GAC CGT CAT 3′	
p21	p11: 5′ GCG ACT GTG ATG GCG TAA TG 3′	22
	p12: 5′ AGA AGA TCA GCC GGC GTT TG 3′	
GADD45	p13: 5′ GAA GAC CGA AAG GAT GG 3′	28
	p14: 5′ GGG AGA TTA ATC ACT GG 3′	
14-3-3σ	p15: 5′ AAG GGC TCC GTG GAG AGG G 3′	25
	p16: 5′ AGA GGG GAA CTT TAT TGA GAG G 3′	
GAPDH	g1: 5′ CTG CAC CAC CAA CTG CTT AG 3′	22
	g2: 5′ AGG TCC ACC ACT GAC ACG TT 3′	

(*p53*), 65° for 1 min (*p73*), 60° for 1 min (*p21*), 55° for 1 min (*GADD45*), or 58° for 1 min (*14-3-3σ*), 72° for 2 min 30 s, and 72° for 7 min after the last cycle of amplification.

GAPDH gene was used as an internal control for amplification reactions. A multiplex amplification was difficult to perform due to the high levels of *GAPDH* transcripts and low transcript levels for some genes such as *p73*, *GADD45*, or *p53*. Therefore, for each sample, 2 μL of the transcript amplification product for the gene analysed and 2 μL of amplified *GAPDH* cDNA were mixed and electrophoresed on polyacrylamide gels [33]. Densitometric quantification was performed using the Joyce–Loebl chromoscan.

2.5. Immunoblotting

One million cells harvested after trypsinisation and mostly excluding trypan blue were lysed in 50 μL of Laemmli buffer (Biorad), and heated to 100° for 5 min. Lysates (10 μL) were electrophoresed on sodium dodecyl sulfate (SDS)–polyacrylamide gel, and then transferred to nitrocellulose membranes (Amersham) in a semidry transfer cell (Biorad). The filters were incubated with: 1 μg/mL of mouse anti-p53 (DO7, Dako), 2 μg/mL of mouse anti-p21 (Ab1, Oncogene Research), rabbit anti-p73α diluted to 1/1000 (generous gift of Caput and Sanofi Labège), 2 μg/mL of mouse anti-p63 (4A4, Santa Cruz), 1 μg/mL of rabbit anti-GADD45 (H165, Santa Cruz), and 2 μg/mL of goat anti-14-3-3σ (C18, Santa Cruz) antibodies. Proteins were visualised with an enhanced chemiluminescence detection system (Amersham). Membranes were then washed in 0.5% TBS-Tween, and incubated with mouse anti-actin antibody (C4, Chemicon) diluted to 1/5000 in order to quantify and normalise the results.

3. Results

3.1. Genomic p53 status in MCF7 and MDA-MB157 breast cancer cell lines

Our study was conducted in two cell lines, described as *p53*-proficient for MCF7, and *p53*-deficient for MDA-MB157 [34,35]. Sequencing performed on genomic DNA of the two cell lines resulted in a wild *p53* genotype for MCF7, as previously shown, and a mutated *p53* genotype for MDA-MB157 (deletion encompassing nucleotides 12184–12210 in exon 4). This deletion leads to disruption of p53 protein translation and results in the absence of p53 protein in MDA-MB157 cells (Fig. 1), as previously described [34–36]. To our knowledge, this is the first time that the *p53* status of the MDA-MB157 cell line has been clearly defined.

3.2. Over-expression of p53-target genes

Genotoxic lesions could lead to transcriptional activation of genes such as *p21*, *GADD45* and *14-3-3σ*, known to be canonical target genes of the *p53* gene. This activation results in cell cycle arrest followed by damaged DNA repair and/or apoptosis [1–3,37]. To test whether or not *p53*-target genes are induced in response to VCR, we treated MCF7 and MDA-MB157 cell lines with VCR concentrations yielding

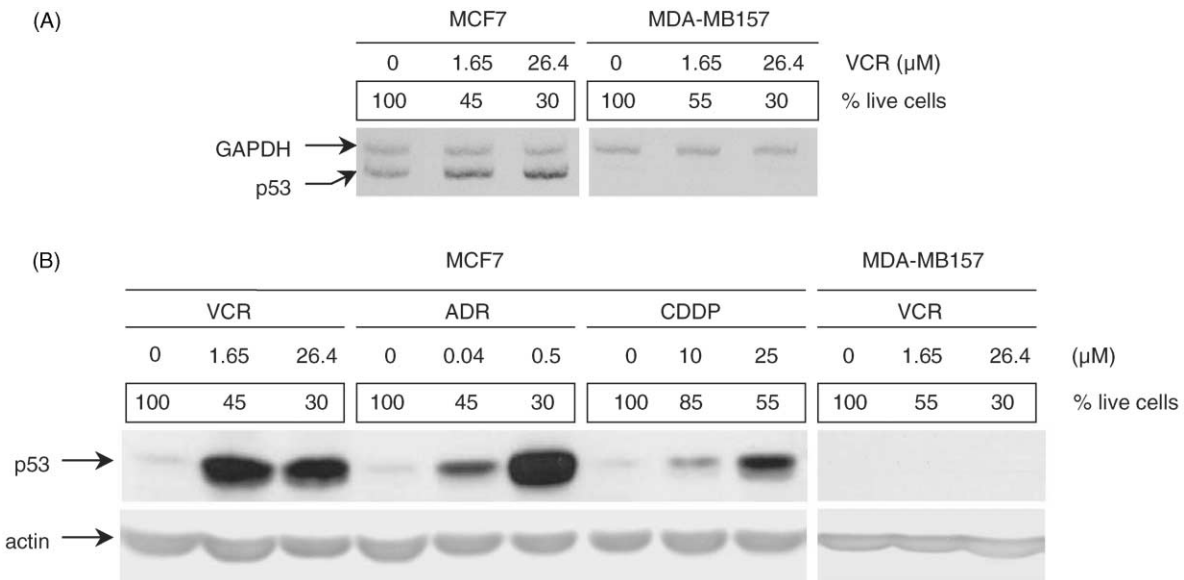

Fig. 1. p53 mRNA and protein expressions in MCF7 and MDA-MB157 cells treated with VCR, ADR or CDDP. (A) Semiquantitative RT-PCR analysis of p53 transcript level. *GAPDH* gene expression was used as internal control. A slight increase (2-fold) in *p53* gene transcript level was observed in MCF7 cells in response to VCR treatment, while no *p53* gene expression was detected in MDA-MB157 cells after 25 cycles of amplification. (B) Analysis of p53 protein expression by Western Blotting, using a monoclonal antibody (clone DO7, Dako). p53 Protein was accumulated in VCR-, ADR-, and CDDP-treated MCF7 cells, whereas no p53 protein was detected in the *p53*-deficient cell line MDA-MB157, even after prolonged exposure of the blot to film. The blot was then rehybridised with anti-actin monoclonal antibody (clone C4, Chemicon) as loading control.

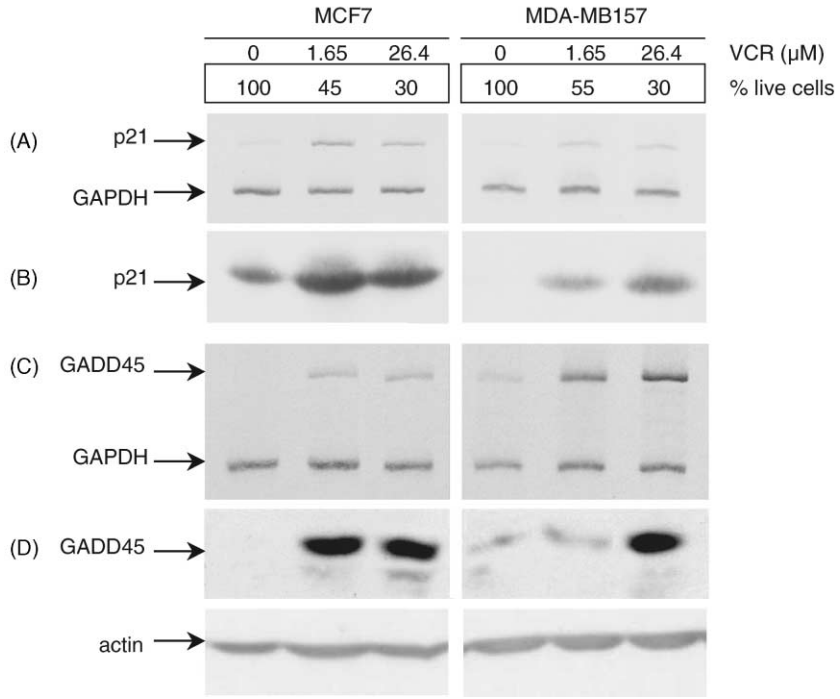

Fig. 2. *p21* (A and B) and *GADD45* (C and D) mRNA and corresponding protein expressions in VCR-treated MCF7 and MDA-MB157 cells. Semiquantitative RT-PCR analysis of gene transcripts using GAPDH gene expression as control (A and C). Protein expression was detected using a monoclonal antibody (clone Ab-1, Oncogene Research) for p21 (B), and a polyclonal antibody (H165, Santa Cruz) for GADD45 (D). (A) *p21* gene transcript level was increased in VCR-treated MCF7 and MDA-MB157 cells. (B) p21 protein level was strongly up-regulated in MCF7 and MDA-MB157 cells, in response to VCR treatment. (C) *GADD45* transcript level was increased in VCR-treated MCF7 and MDA-MB157 cells. (D) Immunoblotting revealed up-regulation of GADD45 protein in VCR-treated MCF7 and MDA-MB157 cells. The blot was rehybridised with anti-actin monoclonal antibody (clone C4, Chemicon) used as loading control for p21 and GADD45.

similar toxicities in the two cell lines, and measured the mRNA and protein expressions of these genes in both cell lines. mRNA level was assessed by a semiquantitative RT-PCR method using *GAPDH* gene expression as internal control; protein expression was analysed by Western blotting.

Analysis of *p21* following VCR treatment, showed up-regulation of *p21* mRNA levels, which was intense (10-fold) in MCF7 cells, and weak (3-fold) in MDA-MB157 cells (Fig. 2A, Table 2). Protein analysis revealed p21 accumulation in both cell lines (Fig. 2B, Table 2).

GADD45 gene expression analysis also revealed up-regulation of transcript and protein levels (Fig. 2C and D, Table 2) in MCF7 and MDA-MB157 cells, following antimitotic treatment.

14-3-3σ gene expression analysis in untreated cells showed detectable transcript levels in MCF7 cells, and barely detectable levels in MDA-MB157 cells. After VCR treatment, transcript levels were not significantly increased in MCF7 cells, in contrast with MDA-MB157 cells, although this increase was only detected after a large number of PCR cycles (Fig. 3A, Table 2). The 14-3-3σ protein level was significantly increased in VCR-treated MCF7 cells, but remained undetectable in MDA-MB157 cells (Fig. 3B, Table 2).

3.3. Up-regulation of p53 in VCR-treated MCF7 cells

We determined whether up-regulation of *p53*-responsive genes could be linked to activation of *p53*. Our results

Table 2
p53 Family members and target genes expressions in breast cancer cell lines treated with vincristine

	p53		p73				p63 Protein	p21		GADD45		14-3-3σ	
	RNA	Protein	p73α		p73β			RNA	Protein	RNA	Protein	RNA	Protein
			RNA	Protein	RNA	Protein							
MCF7	↗	↗↗	↘	↘	↘	n.d.	=	↗	↗↗	↗	↗↗	=	↗
MDA-MB157	0	0	↘	↘	↘	n.d.	=	↗	↗↗	↗↗	↗↗	↗	0

Transcript levels were assessed by semiquantitative RT-PCR. PCR fragments were separated on polyacrylamide gel and quantitated using a Joyce Loebl Chromoscan 3 and normalised to GAPDH. Proteins levels were measured by Western blotting. 0, no detectable expression; =, no change in expression; ↗, moderately increased expression; ↗↗, markedly increased expression; ↘, slightly decreased expression; n.d., not determined.

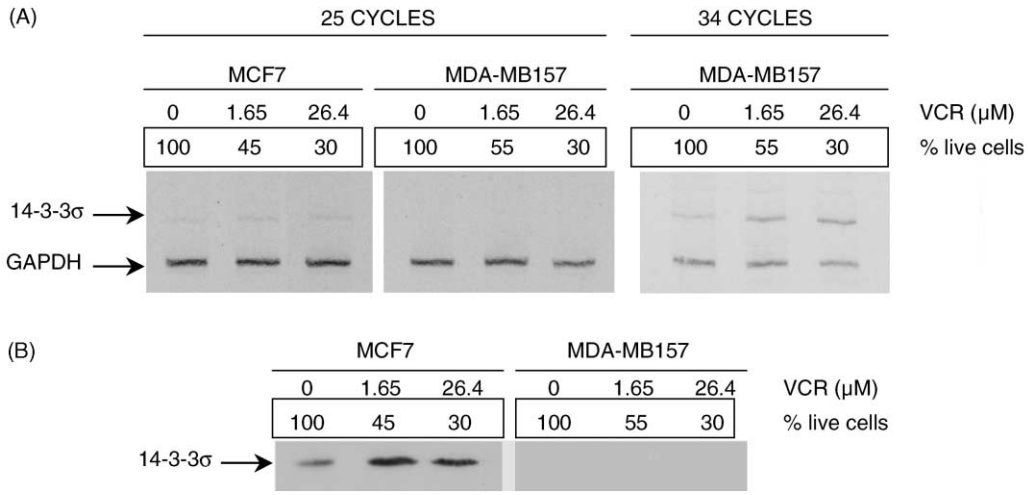

Fig. 3. *14-3-3σ* mRNA and protein expressions in VCR-treated MCF7 and MDA-MB157 cells. (A) Semiquantitative RT-PCR analysis of *14-3-3σ* mRNA expression at 25 and 34 cycles of amplification. *GAPDH* gene expression was used as internal control. *14-3-3σ* transcript level was not modified in VCR-treated MCF7 cells, while *14-3-3σ* mRNA expression was undetectable in MDA-MB157 cells after 25 cycles of amplification. *14-3-3σ* transcript was detected in MDA-MB157 cells after 34 cycles of PCR, and its level was increased (3-fold) after VCR treatment. (B) Analysis of 14-3-3σ protein expression by Western Blotting, using a polyclonal antibody (C-18, Santa Cruz). Immunoblotting revealed a slight increase of 14-3-3σ protein in VCR-treated MCF7 cells, whereas no 14-3-3σ expression was detected in MDA-MB157 cells, probably due to a low constitutive level in this cell line.

showed a weak induction (2-fold) of *p53* mRNA in VCR-treated MCF7 cells, while no mRNA expression could be detected in VCR-treated MDA-MB157 cells (Fig. 1A, Table 2). Following VCR treatment, p53 protein expression analysis revealed p53 protein accumulation in MCF7 cells with the same extent than that found in MCF7 treated by DNA damaging agents (ADR and CDDP). No p53 protein was detected in MDA-MB157 cells, consistent with mRNA expression data (Fig. 1B, Table 2). Therefore, our data clearly show that VCR can induce marked p53 protein upregulation in *p53*-proficient cells. However, there is a very marked discrepancy between the modest up-regulation of *p53* mRNA expression (2-fold) and the huge induction of p53 protein (more than 100-fold), indicating stabilisation of p53 protein in VCR-treated MCF7 cells.

3.4. In response to VCR treatment, p73 expression is down-regulated in MCF7 and MDA-MB157 cells, but p63α expression is unchanged

When ectopically over-expressed *via* gene transfection in cultured cells, *p73* and *p63* were demonstrated to transactivate *p53*-target genes. To investigate whether treatment with VCR could influence *p73* expression, leading, in turn, to up-regulation of *p21*, *GADD45* and *14-3-3σ* genes, we assessed *p73* transcript levels in VCR-treated MCF7 and MDA-MB157 cells by semiquantitative RT-PCR. To detect the overall *p73* transcript levels, assays were performed with primers encompassing the DNA binding domain (exons 6–8), a domain which is common to all isoforms, including *p73α* and *p73β*, the isoforms described to be most intensely expressed in various models [38,39]. We used primers located in the 3′ end of the *p73* gene (exons 12–14) to determine the transcript level of each *p73α* or *p73β* isoform.

Surprisingly, the overall *p73* transcript level was found to be decreased by as much as 3-fold in VCR-treated MCF7 and MDA-MB157 cells (Fig. 4A, Table 2). A decrease of the same order of magnitude was also observed for *p73α* and *p73β* isoforms in both cell lines, thereby excluding alteration of C-terminal splicing as a cause for the decreased overall transcript level (Fig. 4B, Table 2). Immunoblotting was performed using a polyclonal antibody recognising the carboxy terminal domain of the p73α isoform, and samples of SK-N-AS transfected with full-length p73α or ΔNp73α as positive controls to discriminate between the two isoforms. Immunoblotting revealed down-regulation of the p73α protein level in MCF7 and MDA-MB157 cells in response to VCR treatment (Fig. 5A, Table 2), consistent with the decreased transcript levels (Figs. 4 and 6). The decreased *p73* expression indicates that induction of *p73* is not required for the cellular response to VCR in *p53*-proficient and *p53*-deficient cells. To investigate the possibility of down-regulation of *p73* mRNA species lacking the transactivation domain (ΔN), known to exert, after translation, a dominant negative activity on the full-length isoforms (TA isoforms), we determined *ΔNp73* mRNA and protein levels. *ΔNp73* isoforms were undetectable before and after VCR treatment in both cell lines (Figs. 5 and 6), excluding this latter hypothesis.

Of note, VCR-treated MCF7 and MDA-MB157 cell lines rapidly became rounded (data not shown). These morphological changes lead to disorganisation of the cellular monolayer and occur in parallel with down-regulation of full-length *p73* mRNA and protein.

p63, the third member of the *p53*-family, was tested as a putative candidate which could be activated during VCR

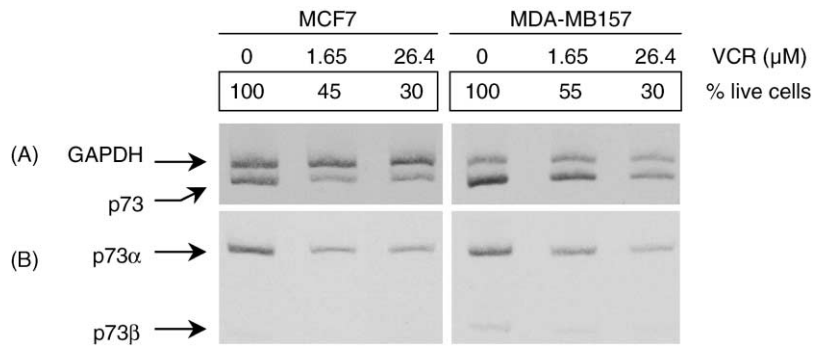

Fig. 4. *p73* mRNA expression in VCR-treated MCF7 and MDA-MB157 cells. (A) Semiquantitative RT-PCR analysis of *p73* gene transcript levels using primers located in DNA binding domain, common to all p73 transcript isoforms (exons 6 to 8). *GAPDH* gene expression was used as internal control. Overall *p73* transcript level was decreased in MCF7 and MDA-MB157 cells in response to VCR treatment. (B) Semiquantitative RT-PCR analysis of *p73α* and *p73β* transcripts using primers located in exons 12 and 14. After VCR treatment, *p73α* and *p73β* expression decreased in MCF7 and MDA-MB157 cells.

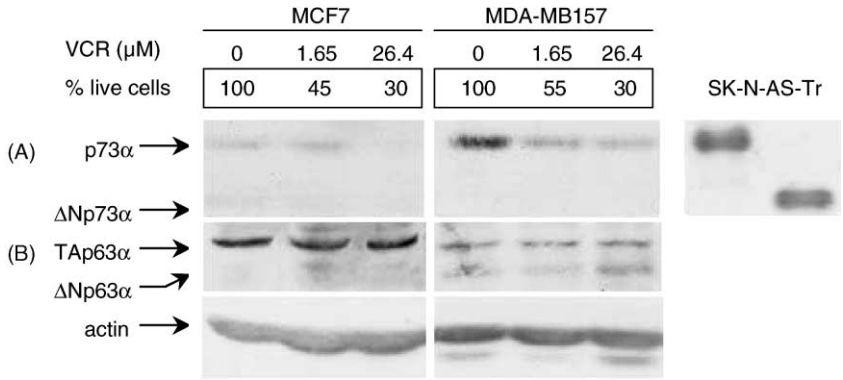

Fig. 5. p73 and p63 protein expressions in VCR-treated MCF7 and MDA-MB157 cells. (A) Analysis of p73α proteins expression by Western Blotting, using a polyclonal antibody (Sanofi, Labège). p73α Protein level decreased in VCR-treated MCF7 and MDA-MB157 cells. SK-N-AS cells transfected (SK-N-AS-Tr) with p73α or ΔNp73α were used as positive controls to discriminate between the two isoforms. (B) Analysis of p63α protein expression by Western Blotting, using a monoclonal antibody (clone 4A4, Santa Cruz). No variation was detected for TAp63α and ΔNp63α in VCR-treated MCF7 and MDA-MB157 cells. The blot was rehybridised with anti-actin monoclonal antibody (clone C4, Chemicon) used as loading control.

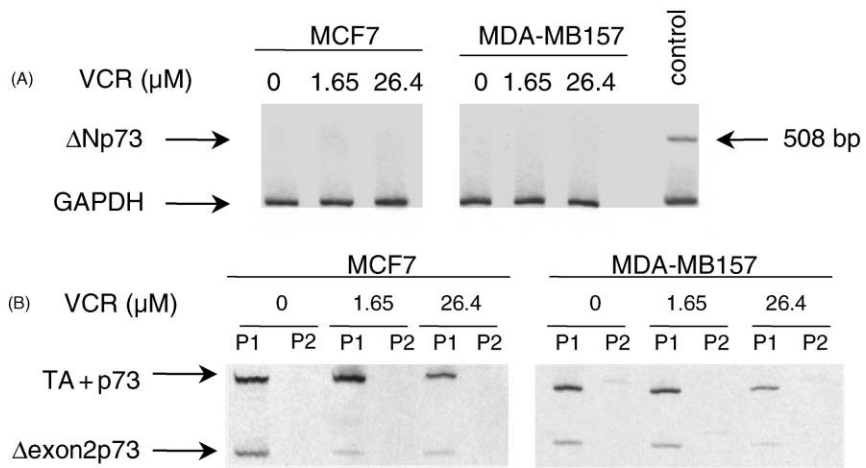

Fig. 6. Analysis of *p73* transcripts from different promoters in VCR-treated MCF7 and MDA-MB 157 cells. (A) *ΔNp*73 (TA-p73) mRNA expression in VCR-treated MCF7 and MDA-MB157 cells. A multiplex amplification was performed in the aim to detect *ΔNp73* and *GAPDH* transcripts levels. For *ΔNp73*, primers were located in exon 3 bis (exon specific for truncated form *ΔNp73*) and in exon 5. *GAPDH* gene expression was used as internal control. No *ΔNp73* mRNA was expressed in MCF7 and MDA-MB157 cells. A fragment of 508 bp corresponding to *ΔNp73* (*TA-p73*) mRNA could be detected in the positive control. (B) A multiplex amplification was performed in the aim to detect p73 isoforms transcribed from P1 and P2 promoters in VCR-treated MCF7 and MDA-MB157 cells. *p73* Isoform from P1 represents the *TA + p73* isoform, while *p73* isoform from P2 is a new *p73* isoform (D. Caput, personal communication). Two fragments were detected for P1 analysis in both cell lines: one of 232 bp corresponding to *TA + p73* isoform, and the second of 135 bp corresponding to the *p73* isoform lacking exon 2 (termed *Δexon2p73*). Note that, according to the results in Fig. 4, the level of *TA + p73*, which is the most abundant *p73* transcript, decreased in both cell lines treated with VCR. Fragments corresponding to *p73* transcription from P2 promoter were expressed at a very low level, and could not be detected on this figure.

treatment. By using a p63 monoclonal antibody (clone 4A4) for Western blotting, we found that both full-length TAp63α and ΔNp63α protein levels were not modified during VCR treatment, in either MCF7 or MDA-MB157 cells (Fig. 5B, Table 2).

Given the various data obtained, it is striking to observe an inverse correlation between *p53* and *p73* levels in *p53*-proficient and *p53*-deficient cell lines, as high p73α protein levels were detected in MDA-MB157 cells. This could suggest a cross-talk between *p73* and *p53*. However, these data need to be confirmed in a greater number of cell lines.

4. Discussion

The *p53* pathway may be used by cells submitted to cellular stress, and is proposed to be instrumental for cell cycle arrest in G1/S and G2/M phases, DNA repair or apoptosis. However, some anticancer drugs, such as VCR have been shown to induce a weak p53 protein accumulation [5–9]. Murine *p53*-deficient embryonic fibroblasts have also been described as able to trigger apoptosis after paclitaxel treatment [10]. Taken together, these results suggest that *p53*-independent pathways may be involved in the response to microtubule-active drugs. We therefore hypothesised that the new *p53* homologues, *p73* and *p63*, could act in these *p53*-independent responses.

The MCF7 and MDA-MB157 cell lines used in this study have been frequently described in the literature to be *p53*-proficient and *p53*-deficient, respectively, although the *p53* status of the MDA-MB157 cell line has not been clearly defined [36]. The 26 bp deletion detected in exon 4, resulting in a nonfunctional p53 protein, therefore clearly confirms that the MDA-MB157 cell line constitutes a valid model of *p53*-deficient cell line.

In these two cell lines, VCR treatment yielding cell toxicity of the same order of magnitude induced up-regulation of the *p53*-target genes, *p21* and *GADD45*, both at the transcript and protein levels (Fig. 2, Table 2). The constitutive mRNA level for *14-3-3σ*, a *p53*-inducible gene, was barely detectable in MDA-MB157 cells, but was up-regulated in response to VCR treatment (Fig. 3A, Table 2). Hypermethylation of the *14-3-3σ* locus, as reported by Ferguson *et al*. [40] in breast cancer cell lines and tissues, can explain such a low constitutive gene transcript level in MDA-MB157 cells. Up-regulation of the transcript level in response to VCR treatment may be due to *14-3-3σ* gene demethylation, but this remains to be demonstrated. However, 14-3-3σ protein could not be detected by immunoblotting in MDA-MB157 cells either before or after VCR treatment, probably due to its low basal level (Fig. 3B, Table 2). In *p53*-proficient MCF7 cells, constitutive *14-3-3σ* transcript and protein levels were found to be much higher than that of *p53*-deficient MDA-MB157 cells. The 14-3-3σ protein level significantly increased in response to VCR treatment (Fig. 3A and B, Table 2). Finally, our results show that, in response to VCR treatment, breast cancer cell lines over-expressed *p53*-inducible genes, independently of *p53* gene status. In agreement with our data, Gartenhaus *et al*. [41] described up-regulation of p21 protein in response to adriamycin in lymphoid cell lines, regardless of *p53* status; in contrast, Guillot *et al*. [35] found no p21 protein expression in *p53*-deficient breast cancer cell lines (including MDA-MB157) treated with adriamycin.

We observed a slight increase in *p53* mRNA in VCR-treated MCF7 cells, whereas no *p53* transcript could be detected in MDA-MB157 cells (Fig. 1A, Table 2). This result was confirmed by p53 protein analysis: p53 protein level was significantly increased in VCR-treated MCF7 cells (Fig. 1B, Table 2), while no p53 protein expression was detected in MDA-MB157 cells, reinforcing the idea that a *p53*-independent pathway is implicated, involving or not, the p53-homologues, proteins p63 and p73. Interestingly, the degree of induction of p53 expression was identical in MCF7 cells treated by VCR and DNA damaging agents (adriamycin and cisplatin). Our data concerning p53 up-regulation in MCF7 cells disagree with those reported by other authors [6,8], who failed to detect a high p53 accumulation in VCR-treated MCF7 cells. However, in these studies, MCF7 cells were treated for 16 or 18 hr, while MCF7 cells were studied herein after 24 hr of exposure to VCR. In line with our data, various studies have shown that the maximal effects of drugs on p53 protein induction were obtained after 24 hr of treatment [5,42]. Furthermore, Tishler *et al*. [43], clearly showed that vinblastine, a microtubule-active agent structurally and functionally analogous to VCR, increases p53 levels and activates the *p53*-dependent process in NIH-3T3 mouse fibroblasts treated for 24 hr. In our study, regarding mRNA and/or protein levels of *p53*-target genes, a slight but significant difference can be observed at low VCR doses in MCF7 *p53* wild type compared to MDA-MB157 *p53* negative (Fig. 2). This observation could reflect the involvement of two pathways in MCF7, one *p53*-dependent activated at low dose, the other, *p53*-independent activated at the low and high doses.

In untreated MDA-MB157 cells, we found a high level of p73 protein and a low level of p63 protein, compared to those found in MCF7 cells. Surprisingly, down-regulation of *p73* mRNA and protein levels was observed in both cell lines, indicating that *p73* does not replace *p53* after VCR treatment in the *p53*-deficient cell line. The down-regulation of *p73* expression might be explained by a decrease of ΔN species which are deprived of their transactivation domain and, when expressed, can therefore exert a dominant negative activity on *p53* and full-length *p73*. p73 Protein analysis revealed the presence of full-length p73, but a consistent lack of ΔNp73 protein in both cell lines, clearly indicating a decrease of the full-length p73 in response to VCR treatment. With respect to both the validity of the data and its reproducibility, it is important to note that the decrease of p73 expression in MCF7 and

MDA-MB157 cells appears to be specific to VCR treatment and would not be made biased by the representative amount of living cells. Indeed after treatment with other chemotherapeutic agents, the same cells exhibited—at a similar level of toxicity to that induced by VCR—not a down-regulation, but an up-regulation of p73 transcripts and proteins[1]. Moreover at this drug toxicity level, we observed that p53 as well as p53-target genes were up-regulated in MCF7 cells (Figs. 1 and 2).

Strikingly and noteworthy, the decrease of full-length p73 (mRNA and protein) has been observed in MCF7 and MDA-MB157 cells, after 24 hr of VCR treatment, at which time the cells rapidly became rounded and lost their monolayer organisation in the bottom of culture flasks, corresponding to disorganisation of the mitotic spindle. Whether the entire p73 protein acts as a constitutive cell morphogen likely to be altered by antimitotic agents has yet to be defined. Of note, following a 24-hr treatment, CDDP-treated cells at low doses were anchored to the plastic substrate, showed a normal morphology and an slight up-regulation of p73 expression (data not shown). Following treatment at very high doses, a p73 down-regulation expression was correlated with cell shrinking, they appeared round and then lost their substrate anchoring as observed in cells treated with VCR at low or/and high doses. Taken together these data reinforce the idea of a strong implication of *p73* in cell–cell interaction and probably in morphogenesis.

Recently, it has been described that *ΔNp73* and *Δexon2p73*, a *p73* isoform lacking exon 2, could be involved in carcinogenesis [44–46]. Our data revealed that *ΔNp73* was not expressed in either MCF7 or MDA-MB157 cells and that the *Δexon2p73* transcript level is down-regulated in the two cells lines under VCR treatment, in parallel with the decrease of the full-length p73 isoform expression (Fig. 6B). This suggest that the roles played by the *Δexon2p73* in carcinogenesis and in response to some drugs are different since an increase of *Δexon2p73* level has been described during carcinogenesis.

We studied p63 protein expression to determine whether *p63* could replace *p53* in MDA-MB157. No variation in p63α protein levels was detected in either MCF7 or MDA-MB157 cells after VCR treatment (Fig. 5B, Table 2). The fact that p63 protein is not modified in either MCF7 or MDA-MB157 cells in response to VCR treatment corroborates the hypothesis suggesting a classification of *p53*-family members into two groups: one group associated with response to genotoxic stress and/or morphogenic maintenance, including *p53* and *p73*, and another group involved in ectodermal development, represented by the *p63* gene, as previously proposed [25].

In conclusion, the existence of a fourth member of the *p53*-family genes or of another pathway involved in the induction of *p53*-responsive genes in VCR-treated *p53*-deficient cell lines, cannot be excluded. If *p53* and its homologue-genes possess the same structural organisation, the amino acid sequence homologies between p53, p63, and p73, are not very high, i.e. 22–63% according to the protein domain. This evidence explains the difficulty in finding the first *p53*-homologue, *p73*, given that this one was discovered 15 years after *p53*. We think that human genome sequencing and the publication of a complete sequence may be the key to finding out, more rapidly, the other homologues of important genes such as *p53*. However, we can not exclude the existence of another pathway different to that of the *p53*-family genes pathway in response to VCR. Indeed, VCR prevents the formation of the mitotic spindle; this mitotic disorganisation which is different to the cellular stress linked to DNA damage, could activate *p53* if present. In *p53*-negative cells such as MDA-MB157, it is possible that another pathway implicated in the control of the metaphasic plaque is involved. Genomic expression profiling could allow the genes implicated in this pathway to be found.

[1] Vayssade *et al.*, manuscript in preparation.

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