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Transcriptional profiling of MCF7 breast cancer cells in response to 5-Fluorouracil: Relationship with cell cycle changes and apoptosis, and identification of novel targets of p53

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The availability of oral precursors of 5-Fluorouracil (5-FU) and its favorable results in treating advanced breast cancer have renewed the interest in the molecular mechanisms underlying its cytotoxicity. We have compared the changes in cell cycle and cell death parameters induced by 2 different concentrations of 5-FU (IC50 and IC80) in the breast adenocarcinoma cell line MCF7. G1/S cell cycle arrest was associated with both concentrations, whereas cell death was mainly induced after IC80 5-FU. These changes were correlated with gene expression assessed by cDNA microarray analysis. Main findings included an overexpression of p53 target genes involved in cell cycle and apoptosis (*CDKN1A*/*p21*, *TP53INP*, *TNFRSF6*/*FAS* and *BBC3*/*PUMA*), and significant repression of *Myc*. High dose 5-FU also induced a higher regulation of the mitochondrial death genes *APAF1*, *BAK1* and *BCL2*, and induction of genes of the ID family. Furthermore, we establish a direct causal relationship between *p21*, *ID1* and *ID2* overexpression, increased acetylation of histones H3 and H4 and binding of p53 to their promoters as a result of 5-FU treatment. The relevance of these findings was further studied after interfering p53 expression in MCF7 cells (shp53 cells), showing a lower induction of both, *ID1* and *ID2* transcripts, after 5-FU when compared with MCF7 shGFP control cells. This molecular characterization of dose- and time-dependent modifications of gene expression after 5-FU treatment should provide a resource for future basic studies addressing the molecular mechanisms of chemotherapy in breast cancer.

Key words: 5-Fluorouracil; cDNA microarrays; p53; E2F1; ID proteins

Carcinogenesis is a multistep process involving genetic and epigenetic alterations that drive the progressive transformation of normal cells into malignant types. Deregulated processes involved in tumorigenesis, such as regulation of cell cycle progression, angiogenesis and apoptosis, provide rational targets for novel therapies. For this reason, there is now more emphasis on understanding the mechanisms of carcinogenesis and how these can be exploited when designing new therapeutic agents.[1] Capecitabine is an example of a rationally designed cytotoxic treatment. It is designed to generate 5-Fluorouracil (5-FU) preferentially in tumor cells, by exploiting the higher activity of the activating enzyme thymidine phosphorylase in tumors compared with healthy tissues. Tumor-specific activation has the potential to increase intratumor drug levels to enhance efficacy and minimize toxicity. Proof of this principle is provided by clinical trial results, showing that capecitabine is effective and has a favorable safety profile in the treatment of metastatic breast cancer.[1]

Capecitabine active metabolite, 5-FU, is one of the most widely used chemotherapy drugs for the treatment of solid tumors. However, despite its extensive use in clinical practice, only in recent years we have began to understand the molecular basis for 5-FU toxicity on cancer cells. As a pyrimidine analogue, it is transformed inside the cell into 3 different cytotoxic metabolites with effects at 3 different levels (see Longley *et al.*,[2] for a review): inhibition of thymidilate synthase (TS), incorporation into DNA and incorporation into RNA. It is thought that the major cytotoxic effects of 5-FU occur at the level of DNA synthesis as a competitive inhibitor of TS.[3] TS inhibition creates a dTTP/dUMP cellular pool imbalance with subsequent DNA damage, and cells die by a process called thymineless death.[4] However, a growing body of evidence suggests that additional mechanisms could be involved in the antiproliferative effects of 5-FU, including inhibition of RNA metabolism[2] and interference with polyamines metabolism.[5]

At the level of molecular effectors, p53 is considered as one of the main molecules involved in 5-FU cytotoxicity, as has been demonstrated by the higher resistance of p53-mutated cell lines[6,7] and studies showing the p53 dependence of Fas upregulation after thymineless death.[8] For this reason, some groups have focused their efforts in the search for potential mediators of p53 activity, involved in the induction of cell cycle arrest and apoptosis. These studies have brought the interest to new molecules such as BBC3/PUMA and FDXR,[9] although none of these targets has shown to be necessary for 5-FU cytotoxicity.

Since 5-FU is not administered as monotherapy, the precise percentage of response in breast cancer is not known, although in colon cancer it oscillates between 20 and 30%.[10] 5-FU cytotoxicity (and in consequence, clinical response) is influenced by the tissue concentration of the folate form of 5-FU, which in turn depends on the type of administration, whether oral prodrug, intravenous continuous infusion or bolus administration.[11,12] In fact, it has been postulated that the better response to the oral 5-FU prodrug 5DFUR is a consequence of the higher concentrations of intracellular 5-FU in neoplastic cells achieved after its administration.[13]

To elucidate the global mechanisms of 5-FU cytotoxicity in breast cancer cells, and the molecular pathways involved in its activity, we studied gene expression profiles by microarray analysis of the well-known MCF7 breast adenocarcinoma cell line after treatment with 2 concentrations of 5-FU. Gene expression was characterized in a dose-dependent manner and findings were correlated with cell cycle and cell death parameters.

Dose-dependent gene expression was associated with differences in cell cycle profile and cell death induction in MCF7 cells. Moreover, 2 genes of the ID family of transcription factors, *ID1* and *ID2*, are shown for the first time as actively transactivated by p53 in the context of 5-FU therapy. These and other well-known p53 targets were further validated by studying their expression

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after 5-FU treatment in an isogenic MCF7 cell line with attenuated expression of p53 protein. These findings are important for the understanding of the mechanism of action of 5-FU and other related chemotherapy drugs, and for unravelling new potential molecular targets of p53.

Material and methods

Cell culture and cell proliferation assays

Human breast carcinoma cell line with functional p53 protein, MCF7, was cultured in DMEM with Glutamax-1 (Gibco-BRL, Grand Island, NY) supplemented with 10% foetal bovine serum (Gibco-BRL), 1% penicillin-streptomycin (Gibco-BRL) and 0.4% fungizone (Gibco-BRL). Cells were grown and passaged by trypsinization.

5-FU (Sigma Chemicals, St. Louis, MO) was resuspended in DMEM culture medium and stored at 4°C for no more than 2 months. Inhibitory concentrations were defined as the concentrations that correspond to a reduction of cellular growth by a given percentage when compared with values of untreated cells. For this purpose, 4×10^4 cells were seeded in each well of 24-well plastic culture plates (Nunc, Brand Products, Rochester, NY). After 24 hr, the control point was washed with PBS, fixed 15 min in PBS + 1% glutaraldehyde (Sigma) and stored in PBS. For the remaining conditions, fresh medium was added with increasing concentrations of 5-FU (0–1 mM) and treated for 48 hr. After this period, sensitivity was evaluated using a crystal violet assay.[14] For this assay, each concentration (each time) was performed in triplicate wells, and in at least 3 independent experiments.

Cell cycle analysis and detection of DNA synthesis

Cell cycle distribution was measured with and without exposure to 5-FU. After culture at different time points, samples were harvested (including detached cells), suspended in PBS and fixed in 70% ethanol, and their DNA content was evaluated after propidium iodide staining. Fluorescence-activated cell sorting analysis was carried out using a FACScan flow cytometer (Beckton Dickinson, San Diego, CA) and CellQuest software. Cell debris and fixation artifacts were gated out.

The BD Pharmingen™ BrdU Flow Kit (BD Biosciences) was used to detect cells entering and progressing through the S (DNA synthesis) phase of the cell cycle. Bromodeoxyuridine (BrdU) was added 30 min before harvesting of cells in each time point, and incorporation to the cells was assessed according to manufacturer's recommendations. Simultaneous staining of DNA with 7-amino-actinomycin D (7-AAD) was used in combination with BrdU, followed by 2-color flow cytometric analysis.

Apoptosis detection

The Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) was used to detect apoptosis by flow cytometry. Cells (1×10^6 cells) were plated onto 75-cm^2 flasks and cultured with or without 5-FU. After 0–48 hr, cells were harvested (including detached cells), washed in PBS and pelleted by centrifugation. They were then resuspended at 10^6 cells/100 μl in a binding buffer, stained with 5 μl Annexin-V and 10 μl propidium iodide (50 μg/ml) and incubated in the dark for 15 min at room temperature. Then, 400 μl binding buffer was added and the cells were immediately processed with a FACScan flow cytometer.

RNA extraction, probe synthesis and hybridization on cDNA arrays

Cells (10^6) were plated in 150-cm cell culture flasks (Cultek SL, Madrid, Spain), and after 24 hr fresh culture medium was supplied and 5-FU was added. After 8, 24 and 48 hr, the medium was removed, cells were washed with PBS, trypsinized, and after centrifugation the pellet was frozen at −80°C. Total RNA was isolated using Rneasy Midi Kit (Qiagen, Hilden, Germany) as indicated by the manufacturer. Three microgram of total RNA from samples and Universal Human Reference RNA (Stratagene, La Jolla, CA), used as control, and T7-(deoxythymidine)24 oligo primer were used to amplify the double-strand cDNA by the Superscript Choice System (Life Technologies, Gaithersburg, MD). *In vitro* transcription was carried out with Megascript™ T7 (Ambion, Austin, TX). All of the samples were labeled with Cy5-dUTP fluorochrome (Amersham, Uppsala, Sweden) and the reference pool with Cy3-dUTP fluorochrome (Amersham) as described previously.[15] Each condition of treatment (time and concentration of 5-FU) was performed in duplicate arrays, with RNA obtained from independent experiments.

Hybridization was performed in 4× standard saline citrate, 1× bovine serum albumin, 2 μg/ml DNAs and 0.1% sodium dodecyl sulphate at 42°C for 15 hr. Slides were washed, dried and then scanned in a Scanarray 5000 XL scanner (GSI Lumonics Kanata, Ontario, Canada) at wavelengths of 635 and 532 nm for Cy5 and Cy3 dyes, respectively, to obtain 10-μm resolution images, which were quantified using the GenePix Pro 4.0 program (Axon Instruments, Union City, CA).

The cDNA array chip is a new version of the CNIO Oncochip[16] manufactured by the CNIO Genomic Unit (http://bioinfo.cnio.es/data/oncochip). This version contains 9,726 clones corresponding to 6,386 different genes. All of the microarray raw data tables have been deposited in the Gene Expression Omnibus under the accession number of GSE2584 (submitter H. Hernández-Vargas).

Data analysis

Fluorescence intensity measurements from each array element were compared with the median of local background in each channel and the elements with values less than this median were excluded. In addition, all spots smaller than 25 μm were manually deleted. The expression ratios of the duplicated spots on the array were averaged. For statistical analysis, we selected genes whose expression differed by a factor of at least 3-fold with respect to the reference pool. This ensures that the genes considered can be regarded as effectively repressed or induced. The SOTA and TreeView programs (http://gepas.bioinfo.cnio.es/cgi-bin/sotarray) were used for clustering analysis, assuming Euclidean distances between genes.

Quantitative and semiquantitative RT-PCR

Total RNA was extracted and purified as previously described. Semiquantitative PCR amplification was performed using primers for *FDXR*, *ID1*, *ID2*, *ID3*, *S100A10*, *TOPIIA*, *TP53INP1* and *TP53I3*/*PIG3* (sequences of primers are included in Table 2 of Supplementary Information). For semiquantitative analysis, RT-PCR was performed at low cycle numbers to avoid saturation, in triplicate samples, and quantified using the GelDoc software, using simultaneous amplification of GAPDH as a reference.

Quantitative real-time PCR (TaqMan) was performed with ABI PRISM 7700 Sequence Detection System Instrument and software (Applied Biosystems, Foster City, CA), using the manufacturer's recommended conditions. Each reaction was performed in triplicate from 2 cDNA dilutions. TaqMan reactions for target and internal control genes were performed in separate tubes. The comparative threshold cycle (C_t) method was used to calculate the amplification factor as specified by the manufacturer. The internal standard β2-microglobulin (Applied Biosystems) was used to normalize variations in RNA quality in the quantities of input cDNA. In preliminary work, this gene has been shown to be not regulated after several drug treatments in breast cancer cell lines. In addition, β2-microglobulin was included in the CNIO Oncochip, and no changes in expression were observed in any of the conditions evaluated in our study. The amount of target and endogenous reference was determined from a standard curve for each experimental sample. The sequence of oligonucleotides and TaqMan probes used for the analysis of *FAS*, *MIZ1*, *c-Myc*, *p21* and *Tp53INP1* were obtained using the Assays-by-Design (SM) File Builder program (Applied Biosystems).

Immunoblotting

Protein lysates were obtained from cells treated with 5-FU for up to 48 hr and untreated control cells, and total protein was measured using a colorimetric detection assay (BCA Protein Assay, Pierce, Rockford, IL). Equal amounts of protein lysates (20–30 μg) were separated by SDS-polyacrylamide gel electrophoresis on 10–12% gels, and electrotransferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA). Blots were blocked overnight at 4°C and then incubated with primary antibody for 1 hr at room temperature. Primary antibodies and dilutions included anti-p53 DO7 (Novocastra, Newcastle, UK), anti-p21 (Oncogene, Boston, MA), anti-E2F1 (Lab Vision/NeoMarkers, Fremont, CA), anti-DP1 (NeoMarkers), anti-BCL-2 (Dako, Glostrup, Denmark) and anti-α-tubulin (Sigma). Blots were rinsed with Tris-buffered saline/Tween 20 and incubated with peroxidase-conjugated secondary antibody (anti-mouse and anti-rabbit IgG-HRP; Dako) for 45 min at room temperature. Protein expression was detected using ECL Western blotting reagents (Amersham Biosciences).

Chromatin immunoprecipitation assay

To investigate the histone acetylation pattern, as well as the association of p53 to the promoter of selected genes, chromatin immunoprecipitation (ChIP) assays were performed as previously described.[17] Commercial DO7, anti-acetyl H3 and anti-acetyl H4 antibodies (Upstate Biotechnologies, Lake Placid, NY) were used. Chromatin was crosslinked by adding 1.5% formaldehyde directly to culture flasks. Chromatin was sheared to an average length of 0.25–1 kb for this analysis. Antibody-bound chromatin was brought down with protein A-Sepharose beads, followed by extensive washing and elution. Regions of homology for putative p53 binding sites were screened in selected genes showing high regulation after treatment with 5-FU (*H2AX*, *HDAC4*, *ID1*, *ID2*, *ID3*, *NR4A1*/*NUR77*, *c-Myc*, *p21*, *S100A10*, *SAT* and *TRAF4*), using the TRANSFAC database.[18] For correlations in the MATRIX analysis tool, a core similarity and a matrix similarity of at least 85% were used. PCR amplification was performed in 25 μl with specific primers for each of the promoters analyzed, including putative p53 binding sites. For each promoter, the sensitivity of PCR amplification was evaluated on serial dilutions of total DNA collected after sonication (input fraction). In PCR amplifications, the input DNA, ''unbound'' (wash) fraction of the no antibody control and, finally, ''unbound'' and ''bound'' fractions for each antibody were run in 2% agarose gels. Primer sequences are shown in Figure 4.

RNA interference to shut-down p53

The vectors expressing shRNA's against p53 (shp53) and GFP (shGFP, control) have been described previously.[19,20] Phoenix cells (from ATCC) carrying the amphotropic receptor were used to generate retroviral supernatants. The retrovirus infection protocol is as previously described.[21] Briefly, MCF7 cells were infected twice, in 2 rounds of 24 hr infection. Stably infected cell clones were selected in puromycin (2 μg/ml) (Sigma).

Results

5-FU sensitivity in MCF7 breast cancer cells

To test the sensitivity of MCF7 cells to 5-FU, we performed a dose/response assay as described in Materials and Methods. As shown in Figure 1*a*, MCF7 *p53*-wild type cell line has an IC50 of ~10 μM. For further experiments with MCF7 cells comparing the effect of 2 doses, we selected 10 and 500 μM 5-FU, inducing a growth inhibition after 48 hr of ~50 and 80%, respectively. From here, 10 and 500 μM concentrations of 5-FU in MCF7 cells will be referred to as IC50 and IC80, respectively. As shown in the time course experiment (Fig. 1*b*), both doses of 5-FU inhibit MCF7 cell growth after 24 hr.

Dose-dependent patterns of cell cycle arrest and cell death in MCF7 cells

Growth inhibition induced by most chemotherapy drugs is usually dependent on a delay in cell cycle, on the induction of cell death or both. Propidium iodide staining of DNA was used to test the effect of 2 concentrations of 5-FU in MCF7 cell cycle at several time points. Both doses induced a G1 phase block (Fig. 1*c*) after 8 hr of exposure. After 48 hr, cells accumulated in G0/G1 and G1/S phases with IC50 and IC80 concentrations, respectively. To have a more accurate view of DNA synthesis after 5-FU treatment, BrdU uptake was studied at the same time points (Fig. 1*d*). Cells treated with both concentrations of 5-FU showed an initial delay at the G1/S boundary. This delay was followed by a marked decrease of BrdU uptake (16% of BrdU positive cells) with the IC50 concentration after 48 hr. In contrast, with IC80 5-FU cells were still incorporating BrdU (69% of the cells were positive for BrdU) after 48 hr. As 5-FU depletes dTTP pools, the cells could incorporate the analogue (BrdU) in place of dTTP, and this can lead to progression from late G1 to early S-phase. However, according to previous studies, short incubations with BrdU are not enough to initiate new DNA synthesis,[22] and synchronization of cells in the G1/S boundary could instead be related to repair synthesis of DNA or programmed cell death initiation. In agreement with the latter possibility, a subG0 population of cells was only evident with IC80 5-FU (3.9% after 48 hr), indicating cell death (Fig. 1*d*). However, a subG0 population is usually difficult to evidence in MCF7 cells because of the lack of a functional Caspase-3 protein in this cell line, and the consequent lower DNA fragmentation associated with apoptosis.[23] In this sense, to further study this subG0 population, apoptosis induction was evaluated by Annexin-V staining that is not influenced by Caspase-3 deficiency. As seen in Figure 1*e*, apoptosis was highly induced after 48 hr of therapy with the IC80 dose of 5-FU. Low concentration of 5-FU also induced apoptosis after 48 hr, although to a lesser degree (17 *vs.* 43%).

Analysis of gene expression after 5-FU treatment of MCF7 cells

In an effort to understand the dose- and time-dependent mechanisms involved in 5-FU action, a global analysis of gene expression was performed with IC50 and IC80 concentrations of 5-FU in a time course experiment, and the main findings were correlated with the differences found in cell cycle and cell death parameters. Since we could see cell cycle changes after 8 hr of 5-FU exposure and apoptosis after 48 and 24 hr for IC50 and IC80 concentrations (Fig. 1, and data not shown), respectively, we evaluated gene expression profiles in MCF7 cells obtained at these same time points (*i.e.* 8, 24 and 48 hr). We used a threshold of 3-fold induction or repression for filtering significant genes regulated after 5-FU, relative to nontreated control cells. After normalization and filtering, we obtained a total of 120 genes modified with IC50 5-FU in any time point, 68 of which (57%) represented downregulated genes and 52 (43%) upregulated genes. In contrast, from the 221 genes regulated after IC80 5-FU, 122 (55%) were induced, 86 (39%) were repressed and a subset of 13 genes (6%) showed an initial upregulation, followed by repression after 48 hr of exposure.

When combining the data of both concentrations, a total of 300 genes were regulated in any time point. As shown in Figure 2, genes were grouped in 3 main clusters. The first cluster included 165 genes overexpressed in most conditions (55%), 57 genes (19%) in second cluster were downregulated with IC50 5-FU and upregulated with IC80 5-FU and third cluster included 78 genes (26%) downregulated mainly after exposure to IC80 5-FU. Some representative genes are shown for each cluster in Figure 2. As expected, the genes highly induced with both concentrations included known mediators of cell cycle arrest and apoptosis in a p53-wt context, *i.e. CDKN1A*/*p21*, *TNFRSF6*/*FAS*, *TP53INP1* and *BBC3*/*PUMA* (Fig. 2 and Table I). However, some relevant genes known to be involved in cell death and cell cycle showed a higher regulation with either of these 2 concentrations (Table I and Sup-

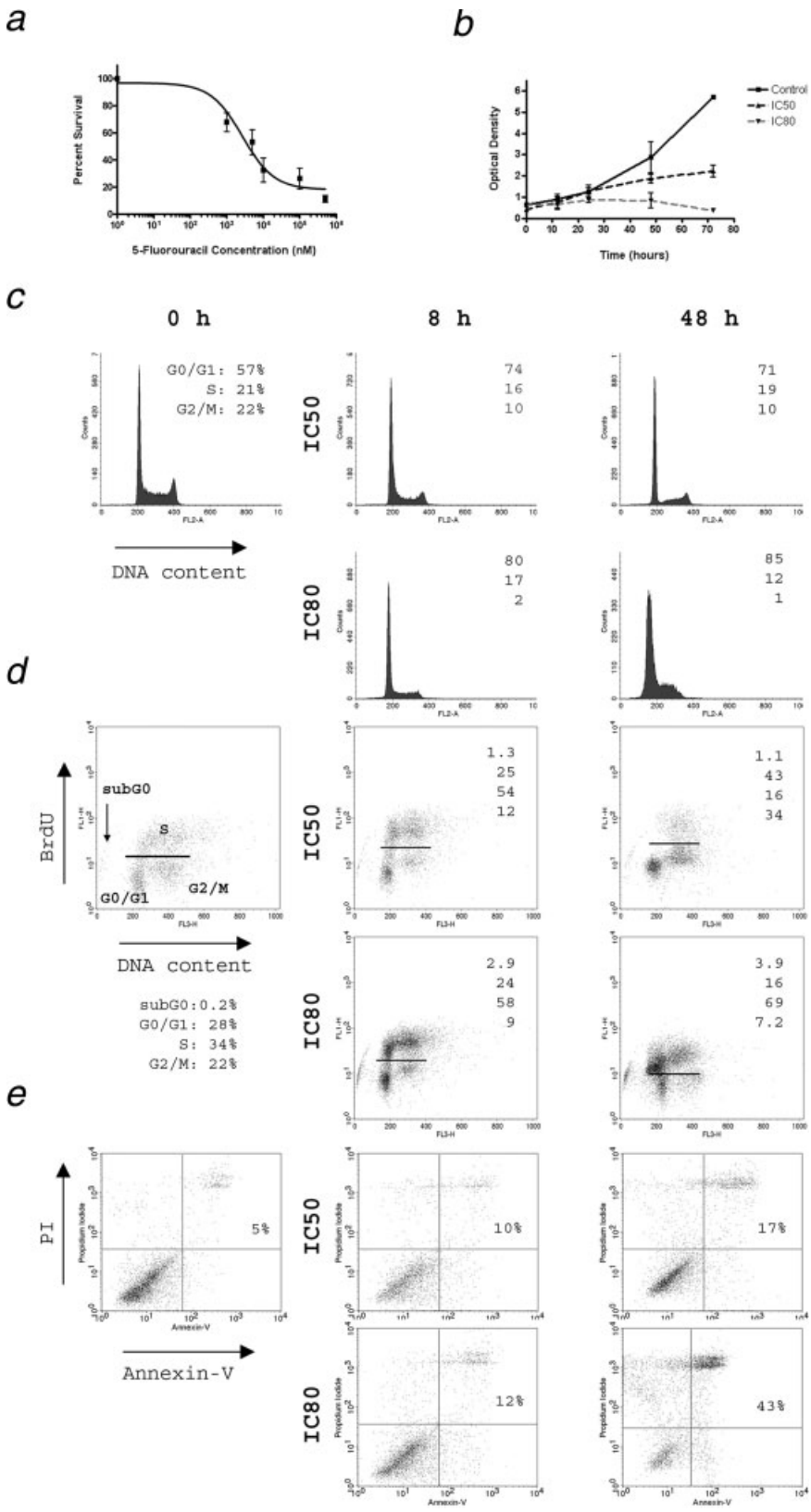

FIGURE 1 – Effects of 5-FU on cell viability, cell cycle and apoptosis. (*a*) Cell viability after treatment with 5-FU. MCF7 cells (4×10^4) were seeded in 24-well MTP. After 24 hr, different concentrations of 5-FU were added to triplicate wells. Percent of surviving cells was evaluated using a crystal violet assay, as described in Materials and Methods. (*b*) Time course after treatment of MCF7 cells with 10 μM (IC50) and 500 μM (IC80) 5-FU. Cell survival was evaluated as described for the viability assay, at different time points of treatment. (*c*) Effect of 5-FU on cell cycle. DNA content was measured on asynchronously growing cells using propidium iodide staining and fluorescence-activated cell sorter analysis at 8 and 48 hr following the start of treatment with IC50 and IC80 doses of 5-FU. The percentage of cells in each cell cycle phase is shown in the dot plot. (*d*) DNA synthesis after 5-FU treatment. BrdU uptake (Y-axis) *vs.* DNA content (X-axis) was assessed, as described in Materials and Methods, after 8 and 48 hr of treatment with IC50 and IC80 doses of 5-FU. S phase is considered as cells positive for BrdU staining. Gates for different cell cycle phases are shown in the control dot plot (0 hr), with the corresponding percentages shown below. (*e*) Apoptosis assay. Apoptosis was evaluated after treating cells with IC50 and IC80 5-FU, and staining with Annexin-V at different time points. Flow cytometry profile represents Annexin-V FITC staining in X-axis and propidium iodide in Y-axis. The number represents the percentage of double-positive cells in each condition (upper right quadrant).

plementary Data). Accordingly, regulation of *CCNG2*, *CCNB2*, *AURKA*/*STK15* and *TNFRSF11B* was higher with the IC50 dose. In contrast, regulation of *SFN*, *BAK1*, *TP53I3*/*PIG3*, *BCL2* and *APAF1* was higher with the IC80 dose (Table I).

Suppression of c-Myc *transcription after 5-FU treatment*

One of the genes more deeply suppressed after low and high doses of 5-FU was *c-Myc*, mainly after IC80 dose (Fig. 3*b*). For this reason, the expression of *c-Myc* and one of its most impor-

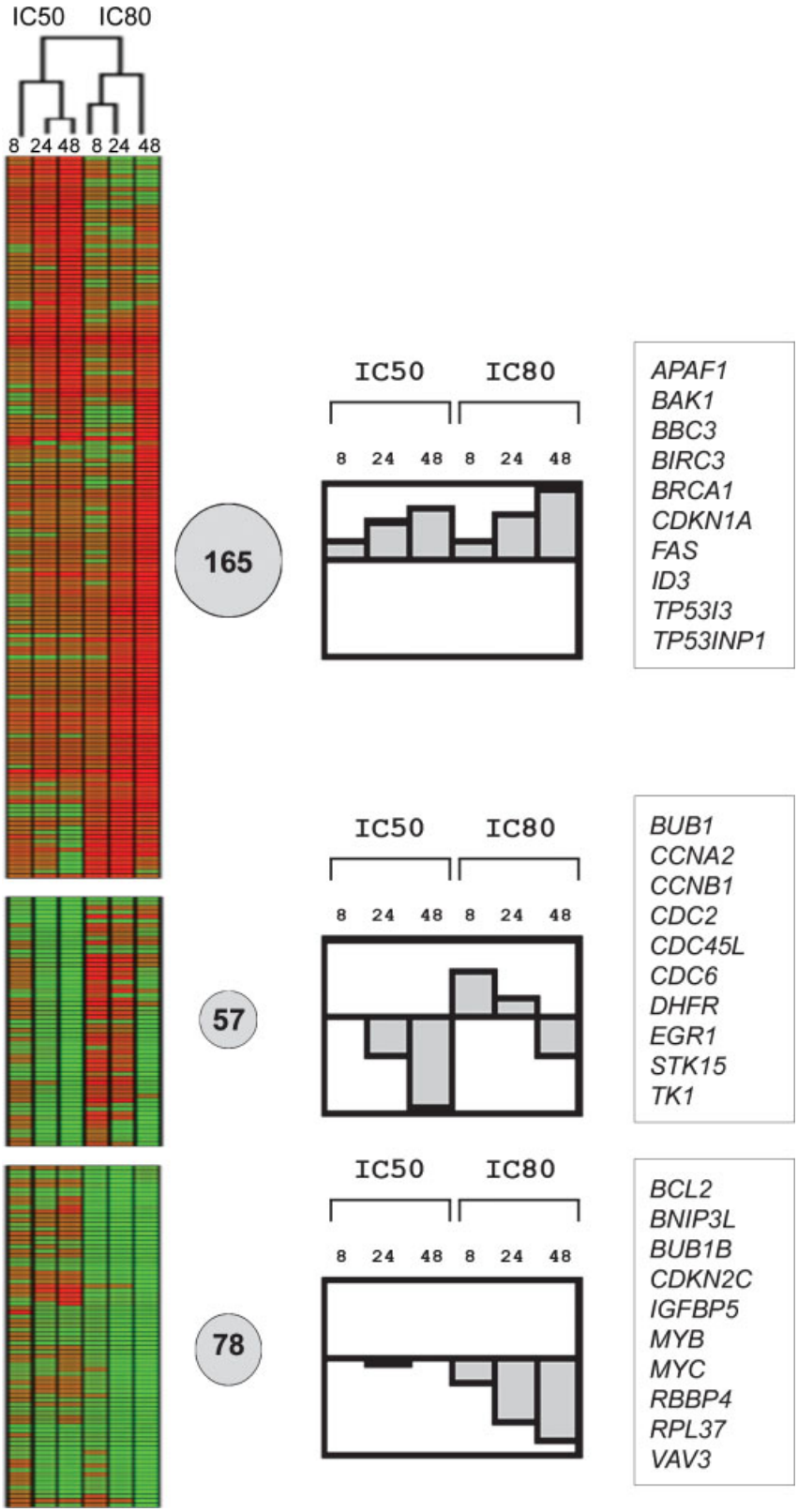

FIGURE 2 – SOTA-array and cluster analysis of genes regulated after treatment of MCF7 cells with IC50 (10 μM) and IC80 (500 μM) concentrations of 5-FU. RNA was extracted, amplified and hybridized at each time point against the Reference Pool, as described in Materials and Methods. The SOTA and TreeView programs (http://gepas.bioinfo.cnio.es/cgi-bin/sotarray) were used for clustering analysis. After Sotarray analysis, all selected genes were included in any of the 3 expression profiles represented in the bar graphic. The number of genes in each cluster, as well as some representative genes, is shown.

tant partners, *MIZ-1* (not included in the array), was tested by real-time quantitative RT-PCR after IC80 5-FU (Fig. 3*c*). Also in Figure 3*c*, relative expression of *CDKN1A*/*p21* and *c-Myc* is represented to test for concomitant changes in expression at longer time points, *i.e.* 72 hr, since it has been described an inverse correlation between c-Myc transcriptional activity and *p21* expression.[24,25] No significant changes in *MIZ-1* were seen, while *c-Myc* expression showed a trend to normalize at longer time points, and *p21* maintained an increasing trend.

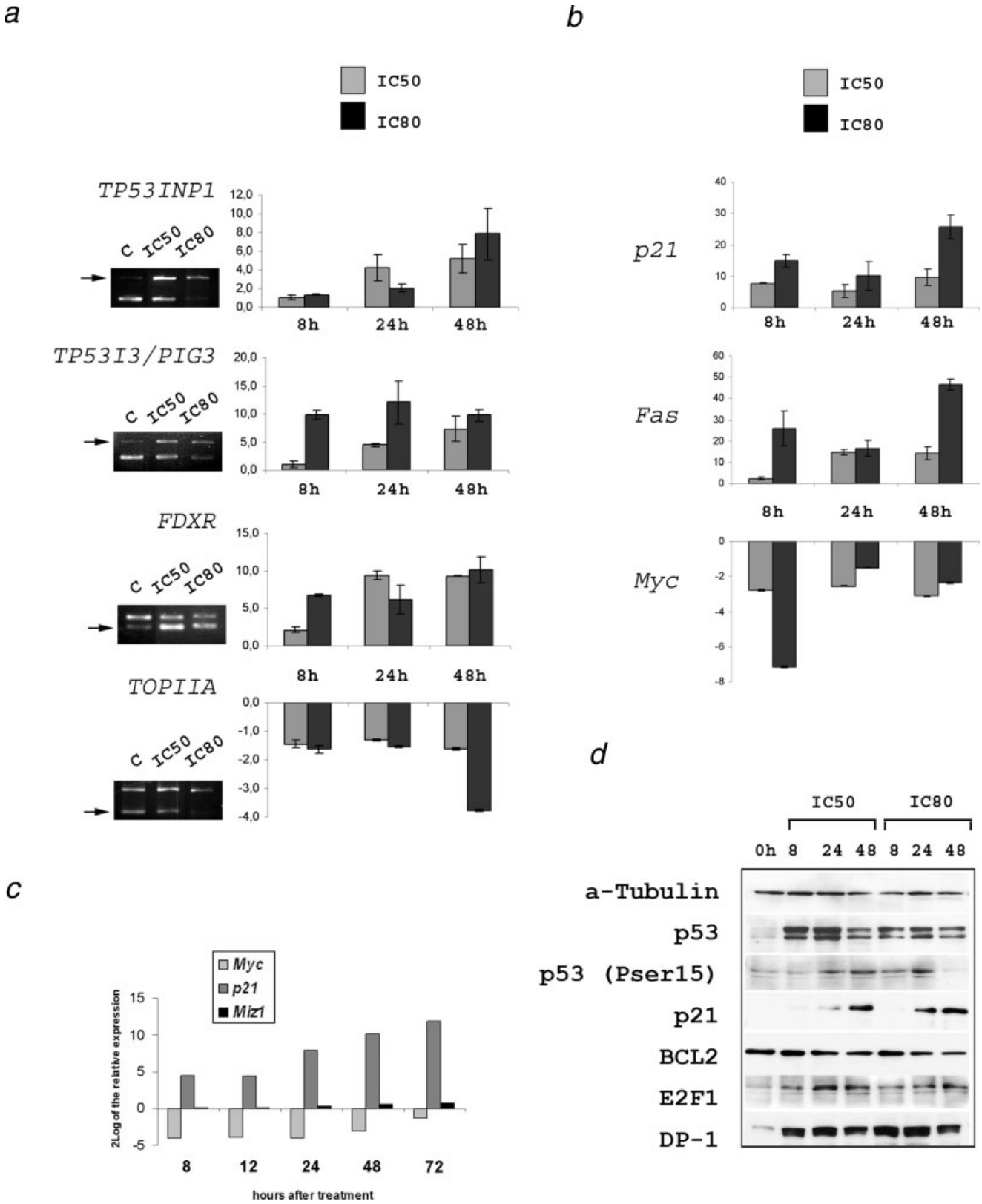

FIGURE 3 – Validation of microarray results. (*a*) Semiquantitative RT-PCR analysis of MCF7 cells treated with IC50 and IC80 5-FU. GADPH was used as internal control. Arrows indicate the gene of study. The corresponding quantification is shown at the right. Y-axis units are arbitrary units of gene expression related to nontreated MCF7 cells. Fold induction or repression is shown. (*b*) Quantitative real-time RT-PCR. Relative gene expression changes in MCF7 cells treated with IC50 and IC80 5-FU, measured by TaqMan real-time PCR. Samples were analyzed as described under Materials and Methods. Fold induction or repression is shown. (*c*) TaqMan real-time PCR in MCF7 cells showing expression of *Myc*, *p21* and *MIZ1* at longer time points. Fold induction is shown in logarithmic 2 scale. (*d*) Western blot analysis of protein expression in MCF7 cells after treatment with IC50 and IC80 5-FU. α-tubulin is included as a control.

TABLE I – INDUCED AND SUPPRESSED GENES IN MCF7 CELLS AFTER IC50 (10 μM) AND IC80 (500 μM) CONCENTRATIONS OF 5-FU

Gene name	IC50			IC80			p53	Description
	8 h	24 h	48 h	8 h	24 h	48 h		
Basic cellular function								
DHFR	1.23	−1.17	−1.99	3.34	2.27	−1.88		Dihydrofolate reductase
DUT	−1.15	−2.51	−3.51	−1.12	−1.88	−4.63		DUTP pyrophosphatase
FDXR	2.06	3.47	2.09	1.89	5.78	6.63	x	Ferredoxin reductase
HDAC4	−1.31	−1.55	−1.35	−1.98	−3.38	−3.03	x	Histone deacetylase 4
HSPB8	1.48	1.78	2.39	1.56	2.63	4.97		Heat shock 22 kDa protein 8
OAS2	1.33	3.15	6.84	1.08	1.23	1.42		2′-5′-oligoadenylate synthetase 2, 69/71 kDa
PANK1	1.28	1.16	1.02	1.66	1.98	3.42		Pantothenate kinase 1
SAT	−1.12	1.39	1.38	−1.18	1.99	6.90	x	Spermidine/spermine N1-acetyltransferase
Cell cycle								
BUB1B	−1.30	−1.85	−2.67	−1.90	−3.51	−6.25		BUB1 budding uninhibited by benzimidazoles 1 homolog β (yeast)
CCNA2	−1.28	−2.09	−8.07	2.97	2.58	−2.54	x	Cyclin A2
CCNB1	−1.91	−2.07	−3.61	−1.28	−1.29	−2.43		Cyclin B1
CCNB2	−1.35	−1.44	−3.98	1.37	1.18	−1.75	x	Glutamate decarboxylase 1 (GAD 1)
CCNG1	1.85	2.26	1.82	1.57	2.92	2.61	x	Cyclin G1
CCNG2	2.46	6.59	8.34	−1.40	−1.12	1.24	x	Cyclin G2
CDC2	1.02	−1.51	−3.73	1.81	1.72	−3.00	x	Cell division cycle 2, G1 to S and G2 to M
CDC45L	1.05	−1.58	−3.65	6.34	3.53	1.13		CDC45 cell division cycle 45-like (*S. cerevisiae*)
CDC6	1.21	−1.33	−4.40	1.77	1.30	−1.42	x	CDC6 cell division cycle 6 homolog (*S. cerevisiae*)
CDK2	1.23	−1.00	−1.72	3.43	4.33	−1.06		Cyclin-dependent kinase 2
CDKN1A	7.00	14.59	12.27	8.11	24.74	27.63	x	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CDKN2C	1.10	−1.19	−1.56	1.77	−1.50	−2.99		Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)
MAD2L1	−1.28	−2.40	−4.22	2.13	2.19	−2.26	x	MAD2 mitotic arrest deficient-like 1 (yeast)
SERTAD1	1.64	1.78	1.28	1.30	3.71	7.26		SERTA domain containing 1
SFN	1.40	1.85	1.09	2.17	3.63	3.25	x	Stratifin
STK15	−1.98	−2.26	−4.57	−1.25	−1.20	−1.59		Serine/threonine kinase 6
TK1	−1.03	−1.21	−2.33	2.66	3.12	1.06		Thymidine kinase 1, soluble
Cell death								
APAF1	1.48	1.56	1.28	1.46	2.70	3.16	x	Apoptotic protease activating factor
BAK1	1.14	1.19	1.07	1.51	3.60	6.31	x	BCL2-antagonist/killer 1
BBC3	4.52	9.16	8.86	3.22	6.56	7.88	x	BCL2 binding component 3
BCL2	1.01	−1.26	1.01	−1.18	−2.89	−3.89	x	B-cell CLL/lymphoma 2
BCL2L1	1.51	2.32	2.28	1.34	2.90	3.25		BCL2-like 1
BIRC3	−1.08	−1.17	1.20	1.22	1.52	3.15		Baculoviral IAP repeat-containing 3
DIABLO	−1.00	1.30	1.16	−1.03	2.06	4.62		Diablo homolog (Drosophila)
FAS	12.66	17.48	12.74	3.81	12.96	26.30	x	Fas (TNF receptor superfamily, member 6)
MDM2	1.24	2.14	2.14	2.25	6.42	6.03	x	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein
TNFAIP3	1.09	1.64	2.58	1.12	1.11	4.07		Tumor necrosis factor, α-induced protein 3
TNFRSF11B	1.75	2.41	3.98	1.48	1.43	1.72		Tumor necrosis factor receptor superfamily, member 11b
TNFRSF18	1.19	1.02	−1.95	2.63	4.17	1.19		Tumor necrosis factor receptor superfamily, member 18
TP53I3	−1.10	1.14	−1.07	1.14	5.23	5.46	x	Tumor protein p53 inducible protein 3
TP53INP1	7.28	22.01	25.51	4.76	17.91	32.67	x	Tumor protein p53 inducible nuclear protein 1
DNA damage/synthesis/repair								
BRCA1	1.39	1.06	−1.39	1.91	2.90	1.10	x	Breast cancer 1, early onset
DDIT4	2.00	3.83	4.01	2.94	9.81	22.36	x	DNA-damage-inducible transcript 4
FANCC	1.03	−1.20	1.06	−1.27	−2.97	−4.92		Fanconi anemia, complementation group C
JARID1A	−1.01	−1.17	−1.02	−1.25	−2.40	−3.10		Jumonji, AT rich interactive domain 1A (RBBP2-like)
KLF4	1.19	2.43	4.62	1.25	2.61	3.58		Kruppel-like factor 4 (gut)
MSH2	−1.09	−1.50	−1.99	1.02	−2.39	−4.65	x	MutS homolog 2, colon cancer, nonpolyposis type 1 (*E. coli*)
PCNA	1.76	1.19	−1.79	7.62	9.32	5.91	x	Proliferating cell nuclear antigen
PTTG1	−1.65	−1.96	−5.42	1.26	1.09	−1.27		Pituitary tumor-transforming 1
RAD54B	1.30	−1.19	−2.30	1.95	1.13	−2.92		RAD54 homolog B (*S. cerevisiae*)
RBBP4	−1.04	−1.17	1.04	−1.59	−3.02	−3.79	x	Retinoblastoma binding protein 4
RRM2	1.26	−2.12	−4.78	1.17	−1.31	−12.2		Ribonucleotide reductase M2 polypeptide
RRM2B	2.43	2.58	3.38	1.23	2.24	2.17	x	Ribonucleotide reductase M2 B (TP53 inducible)
Protein synthesis/trafficking/degradation								
CUL5	1.03	1.66	3.36	1.15	1.39	−1.05		Cullin 5
FBXO5	1.06	−1.90	−3.47	−1.12	−2.19	−4.81		F-box protein 5
G1P2	−1.27	1.75	6.00	1.39	2.47	7.33	x	Interferon, α-inducible protein (clone IFI-15K)
HSPC150	−1.09	−1.98	−3.65	2.37	1.32	−2.74		Ubiquitin-conjugating enzyme E2T (putative)
PLOD2	1.20	−1.48	−1.53	−1.18	−2.28	−3.82		Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2
PPP1R2	1.04	−1.06	1.78	−2.00	−6.57	−4.28		Protein phosphatase 1, regulatory (inhibitor) subunit 2
RPL37	−1.09	1.01	1.16	−2.90	−4.31	−2.43		Ribosomal protein L37
RPS27L	1.47	1.77	1.76	1.68	3.91	7.81		Ribosomal protein S27-like
SMURF1	1.18	1.45	1.30	−1.03	1.62	3.12		SMAD specific E3 ubiquitin protein ligase 1
UBE2C	−1.25	−1.18	−3.97	−1.04	1.60	−2.61		Ubiquitin-conjugating enzyme E2C

TABLE I – INDUCED AND SUPPRESSED GENES IN MCF7 CELLS AFTER IC50 (10 μM) AND IC80 (500 μM) CONCENTRATIONS OF 5-FU (CONTINUED)

Gene name	IC50			IC80			p53	Description
	8 h	24 h	48 h	8 h	24 h	48 h		
Signal transduction								
HRAS	1.43	1.68	1.17	1.67	2.54	3.72	x	V-Ha-ras Harvey rat sarcoma viral oncogene homolog
MAPKAP1	1.17	1.20	1.23	1.31	3.17	4.77		Mitogen-activated protein kinase associated protein 1
PHLDA3	1.69	2.09	1.44	2.08	4.68	6.67		Pleckstrin homology-like domain, family A, member 3
S100A10	1.58	2.27	2.06	2.31	2.86	4.29		S100 calcium binding protein A10 (annexin II ligand)
SOCS1	−1.09	1.40	1.61	1.42	2.23	3.76		Suppressor of cytokine signaling 1
STAT3	1.08	1.30	1.36	1.04	1.78	3.27		Signal transducer and activator of transcription 3
TGFB1	1.10	2.41	3.37	1.14	1.54	1.97		Transforming growth factor, β1 (Camurati-Engelmann disease)
TGFBR3	−1.66	−1.06	3.34	−1.25	−1.31	−1.45		Transforming growth factor, β-receptor III (betaglycan, 300 kDa)
Transcription related								
ATF3	1.87	2.81	2.62	−1.05	2.28	7.97	x	Activating transcription factor 3
CEBPB	1.11	1.49	1.28	−1.19	1.85	4.50		CCAAT/enhancer binding protein (C/EBP), β
EGR1	−2.20	−8.54	−8.93	8.89	2.98	2.73		Early growth response 1
EPAS1	−1.08	1.48	1.58	1.47	2.93	8.66		Endothelial PAS domain protein 1
ID1	−1.63	−1.37	−1.22	1.26	1.95	2.87		Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein
ID2	−1.24	−1.11	−1.02	1.61	2.00	3.08		Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein
ID3	1.22	1.88	2.17	3.58	13.92	26.59		Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein
JUN	1.29	1.24	1.26	1.74	1.85	4.20	x	V-jun sarcoma virus 17 oncogene homolog (avian)
JUNB	−1.00	1.26	1.37	1.81	3.09	4.97	x	Jun B proto-oncogene
MAF	1.37	1.86	2.37	1.11	1.34	3.92		V-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)
MYB	−1.39	−1.40	−1.24	−2.60	−3.62	−6.13	x	V-myb myeloblastosis viral oncogene homolog (avian)
MYBL2	1.04	−1.07	−1.69	3.22	2.20	−1.10	x	V-myb myeloblastosis viral oncogene homolog (avian)-like 2
MYC	−2.01	−3.30	−1.85	−1.79	−2.42	−2.54		V-myc myelocytomatosis viral oncogene homolog (avian)
NFIB	−1.16	−1.04	1.03	1.04	−3.41	−6.13		Nuclear factor I/B
NR4A1	−1.32	−1.48	−1.63	1.98	3.62	5.70		Nuclear receptor subfamily 4, group A, member 1
RBM14	2.53	1.27	−1.24	5.70	8.40	3.57		RNA binding motif protein 14

Relative mean gene expression is shown for each gene in a fold scale, grouped by ontogeny. p53-related genes are indicated in an independent column.

Differential regulation of p53 targets at low and high doses of 5-FU

To study the relevance of p53 activity in the expression profiles that we obtained, we crossed information on published p53 targets with our microarray data. As expected, a high number of known p53-related genes was regulated throughout the time course experiment with both, IC50 and IC80 doses of 5-FU (Table I). Although the percentage of direct p53-target genes was 15% with both concentrations of 5-FU, the absolute number of these genes was much higher with the IC80 dose (18 *vs.* 33), and interestingly, some of these genes were not previously reported in the context of 5-FU induced DNA damage (*i.e. BAK1*, *CCNG2*, *SFN* and *TP53I3*/*PIG3*). To validate these results, we performed immunoblots and RT-PCR assays for relevant p53 targets. As can be seen in Figure 3*d*, p53 protein was stabilized and phosphorylated in a similar way with both treatments (between 2.7- and 3.5-fold increase with both concentrations of 5-FU) and 3 of its most important targets (*CDKN1A*/*p21*, *TNFRSF6*/*FAS* and *TP53INP1*) were accordingly upregulated with both doses of 5-FU (Figs. 3*a*–3*c*). As shown, p21 transcription and protein expression were increased with both concentrations of 5-FU. Interestingly, some other known p53-targets, shown in Table I, were only regulated with IC50 5-FU (*CCNG2* and *RRM2B*) or IC80 5-FU (*i.e. TP53I3*/*PIG3*, *APAF1*, *SFN* and *BAK1*), highlighting possible dose-dependent mechanisms of p53 transactivation activity. The transcript for the antiapoptotic gene *BCL2* was markedly suppressed with IC80 5-FU (6.2-fold reduction), as well as its protein expression (2-fold reduction after 48 hr). A mild and transient reduction of BCL2 protein (1.3-fold reduction after 24 hr) was also observed with IC50 5-FU, although no change in microarray expression was detected (Fig. 3*a*, and Table I).

p53-Independent genes and pathways regulated in response to 5-FU

As mentioned earlier, less than 15% of the genes regulated after both concentrations of 5-FU is directly related with the p53 pathway. Remaining genes could be related either to indirect p53 activity, or to the cell response to the drug because of additional mechanisms of drug cytotoxicity or both. Remarkably, a set of genes belonging to the E2F pathway were regulated after 5-FU treatment. Some of these genes (Table I) were repressed with IC50 5-FU (*PTTG1*, *STK6*, *MAD2L1*, *CCNB2* and *BUB1*). In contrast, when analyzing gene expression in the time course experiment, some E2F genes were induced with IC80 5-FU and were grouped in the same cluster (cluster 2 in Fig. 3). This set of genes showed an initial upregulation at 8 and 24 hr with a subsequent normalization or downregulation at 48 hr. Because most of these genes are known to be differentially expressed during the G2/M phase of the cell cycle, it is possible to explain this repression by a decrease in the proportion of cells in this phase. However, many E2F targets were not modified or were even increased after IC80 5-FU (*APAF1*, *BAK1*, *EGR1*, *ID3*, *JUN*, *JUNB* and *PCNA*), even though very few cells in G2/M phase remained after 48 hr of treatment with the IC80 concentration (Figs. 1*b* and 1*c*). Because protein levels of E2F-1 were similarly increased with both concentrations of 5-FU (Fig. 3*d*) (at least 2-fold increase with both concentrations of 5-FU), there seems to be additional regulatory mechanisms involved in the activation of E2F-related targets with the IC80 concentration of 5-FU. For this reason, the E2F1 partner DP-1 was studied by immunoblotting. As shown in Figure 3*d*, protein levels of DP-1 were increased 2- and 3-fold after IC50 and IC80 5-FU therapy, respectively. DP-1 levels after IC80 5-FU remained high after 24 hr, and returned to basal levels after 48 hr, in accordance with microarray data (Cluster 2 in Fig. 2).

ChIP assays reveal novel p53 targets in MCF7 cells treated with 5-FU

Once the expression profile of MCF7 cells treated with 5-FU was defined, we undertook the task to elucidate the underlying mechanism involved in the upregulation of genes. Two possible scenarios exist: the overexpression of a particular gene is the result of direct association of p53 or, alternatively, indirect mechanisms of regulation can be invoked. It is, therefore, a key issue to distinguish between genes directly and indirectly regulated by p53. On the other hand, association of p53 (or other transcriptional factors) to the promoters that are overexpressed could be coupled to the recruitment of histone modifying activities or nucleosome remodelling machineries that alter either the chromatin structure (chromatin remodelling activities) or the posttranslational status of histones[26] at the promoter. The recruitment of histone modifying activities (such as histone acetyl transferases or histone deacetylases) should result in changes in the pattern of histone acetylation. To investigate these issues, we performed ChIP analysis.[17] This assay can determine whether a particular transcriptional factor (in this case p53) is bound to a specific promoter in its natural chromatin state (in this case the promoters of the overexpressed genes from our microarray analysis) or whether changes in the histone acetylation pattern occur. First, we performed ChIP assays with anti-acetyl H3 and anti-acetyl H4 antibodies to check whether overexpression of these genes was mediated through changes in the histone acetylation status. The promoters of 10 selected genes, showing regulation after treatment with 5-FU, were analyzed: *H2AX*, *HDAC4*, *ID1*, *ID2*, *ID3*, *NR4A1*/*NUR77*, *c-Myc*, *p21*, *S100A10*, *SAT* and *TRAF4*. In all of them, an increase in the acetylation of histones H3 and H4 was observed (Fig. 4*c*, and data not shown) in correlation with an increase in their transcript levels (Fig. 4*a*). This result suggests that the regulation of the expression of these genes is mediated through the modification of the histone acetylation pattern.

Also, we immunoprecipitated DNA from the control and 5-FU-treated MCF7 cells with anti-p53 antibodies to investigate the existence of direct association with the promoter of a few selected overexpressed genes. We had previously performed a search for putative p53-binding sites in the promoter regions of the aforementioned genes using the TRANSFAC tool. After finding some potential binding sites for p53, we had designed primers including these regions (Fig. 4*b*), as described in Materials and Methods. The results obtained with the anti-p53 antibody showed that *p21* is a target for p53 in MCF7 cells treated with 5-FU, and it is transactivated in a time- and dose-dependent manner (Fig. 4*d*). Most interestingly, ChIP revealed the existence of novel p53 targets after 5-FU treatment. In particular, 2 genes related with TGF-β signaling, inhibitors of DNA binding 1 (*ID1*) and 2 (*ID2*), show p53 binding to their promoters (Fig. 4*c*). This result appeared to be reproducible in 3 independent ChIP assays and provides a mechanistic connection between p21, ID1 and ID2 overexpression and p53 regulation.

Expression of 5-FU response genes in p53 depleted MCF7 cells

To further confirm that p53 does indeed play a role in the regulation of ID genes, as suggested by ChIP experiments, we have generated by RNA interference technique MCF7 cells stably expressing a shRNA to shut-down p53 expression (shp53). These cells and the corresponding control (shGFP) were used as an isogenic tool to study p53-dependent responses and to confirm some of the findings reported here. As shown in Figures 5*a* and 5*b*, there is a marked repression of p53 protein expression in MCF7 shp53 cells, and almost no p53 accumulation even after exposure to a high concentration of 5-FU. To validate the use of these cells, we analyzed the expression of 2 main targets of p53, *p21* and *Tp53INP1*. As shown in Figure 5*c*, there is a much lower induction of both transcripts after 5-FU treatment in shp53 cells when compared with control shGFP MCF7 cells. After verifying the nonfunctional state of p53 in shp53 MCF7 cells, *ID1* and *ID2* gene expression was also studied. As expected after ChIP experiments, both genes showed no detectable induction after 5-FU therapy in shp53, in contrast to more than 2-fold induction in control shGFP cells, giving more support for a role of p53 in the transactivation of these genes in response to 5-FU.

In addition, to test the connection between c-Myc and p53 in this context, we analyzed *c-Myc* expression in shp53 cells. Interestingly, as shown in Figure 5*c*, *c-Myc* repression was significantly lower in shp53 MCF7 cells when compared with the shGFP control cells. This finding supports a role for p53 in the high repression of *c-Myc* transcription observed after 5-FU exposure.

Discussion

The underlying molecular mechanisms of toxicity of 5-FU and other antineoplastic drugs are only partially known, in spite of its extended clinical use. There is compelling evidence that one of its main effectors is p53, known as an important mediator in the action of many other drugs and DNA damaging agents.[27,28] In fact, previous reports have demonstrated a correlation between 5-FU treatment and changes in p53 and p53 target molecules.[9,29] Therefore, *p53*-status could be involved in the significant variability of clinical response to chemotherapy. In addition to *p53*-status, the clinical response to 5-FU is known to be influenced by plasma and tissue concentrations of the drug, which in turn are known to be affected by several factors, including timing and type of administration (*e.g.* intravenous continuous infusion *versus* bolus treatment). Moreover, the differences in tissue concentrations of 5-FU could result in quantitative and qualitative differences in the molecular mechanisms of action of this drug.[30] Together with these considerations, the availability of new precursors of 5-FU and its potential use for the treatment of breast cancer prompted us to study dose- and time-dependent modifications of gene expression after exposure of MCF7 breast adenocarcinoma cells to 5-FU.

As expected, many p53 target genes were regulated upon treatment with 5-FU. However, although p53 can be considered as the main player under all conditions evaluated in our study, important differences could be appreciated between low and high doses of 5-FU (IC50: 10 μM *vs.* IC80: 500 μM). Overall, cell cycle arrest was a main feature of low doses and apoptosis was mainly induced with high doses of 5-FU. A balance between both activities seems to influence the final outcome. However, what determines the choice between cytostasis and apoptosis is not clear. It has been suggested that the transcription factor c-Myc is a principal determinant of this choice in colon cancer cells treated with DNA damaging agents.[24] c-Myc is directly recruited to the *p21* promoter by the DNA-binding protein Miz-1 and this interaction blocks *p21* induction by p53 and other activators.[24,25] As a result, c-Myc switches from cytostatic to apoptotic the p53-dependent response of cancer cells to DNA damage. In this sense, we found, besides a high *p21* induction, a marked repression of the *c-Myc* transcript. To gain further insights into the relationships between p21, c-Myc and MIZ, we analyzed the expression pattern of the transcripts in a time course experiment from 8 to 72 hr. No significant changes in *MIZ-1* were seen, while *c-Myc* expression showed a higher repression at 8 hr and a trend to normalize at longer time points. In contrast, *p21* maintained an increasing trend of expression. Our data indicate that, although transcriptional repression of *c-Myc* in response to 5-FU might facilitate the induction of p21 and cytostasis at 8 hr, a decrease in the levels of p21 is not involved in the switching from cell cycle arrest to apoptosis. Interestingly, when studying *c-Myc* expression in an isogenic shp53 MCF7 cell line, we found that *c-Myc* repression was lower. These results are in agreement with a recent report by Ho *et al*., demonstrating that *c-Myc* is directly repressed by p53 in a number of mouse and human cell lines, and that this repression occurs independently of p21 transactivation.[31]

According to microarray findings, several genes could be involved in the partial release of cells from cell cycle arrest observed with IC80 concentrations of 5-FU. One possible explana-

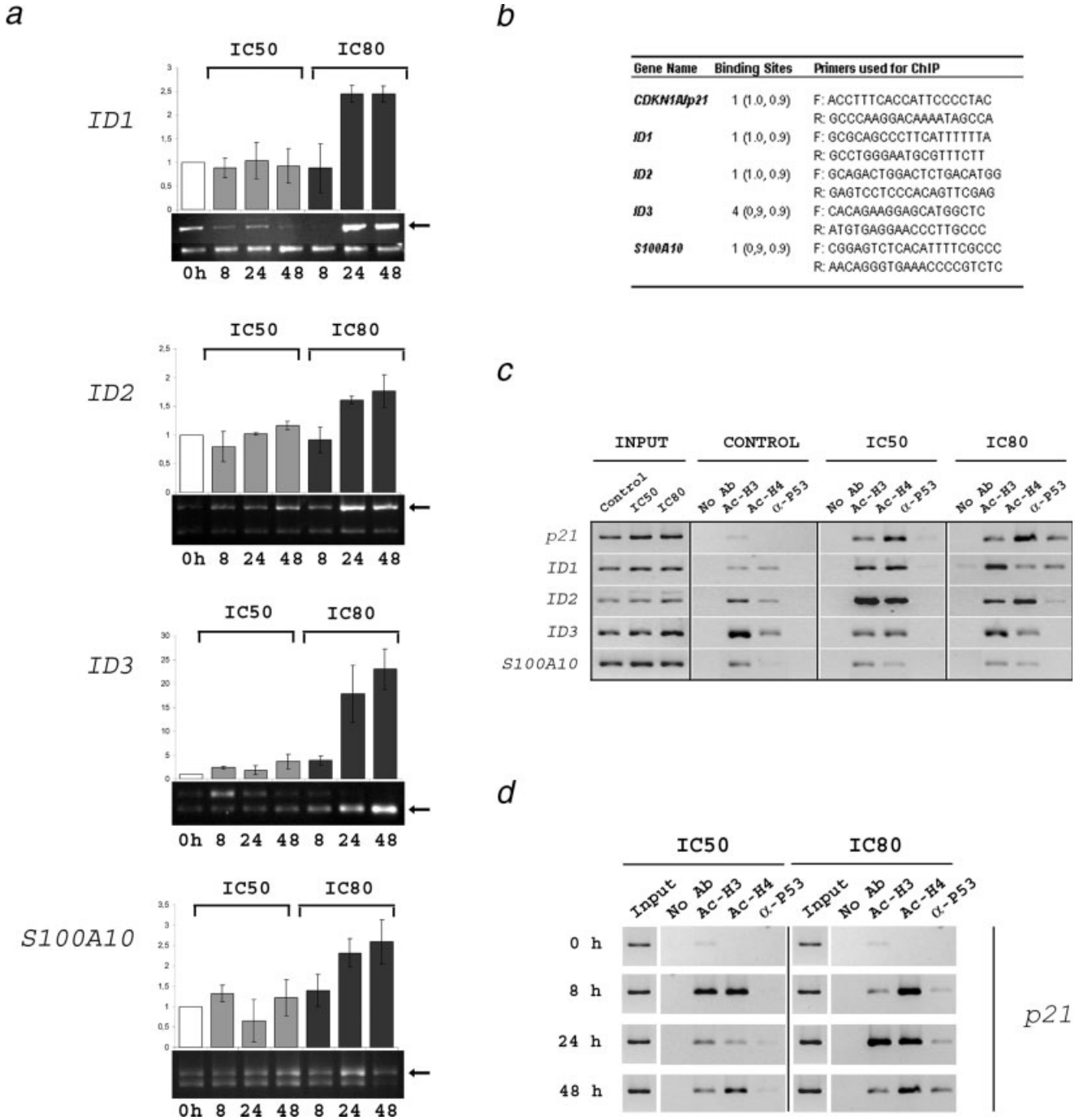

FIGURE 4 – ChIP assay. A, semiquantitative PCR analysis of MCF7 cells treated with IC50 and IC80 5-FU. GADPH was used as internal control. Arrows indicate the gene of study. Expression relative to the internal control is shown in the bar graph. (*b*) Analysis of putative p53-binding elements. Promoters of selected genes were evaluated with the Match tool of the TRANSFAC database. Number of putative p53 binding sites is shown, with the corresponding correlation coefficient for core and matrix sequences (first and second number in parenthesis, respectively). Sequences used for ChIP assay are also shown. (*c*) Histone acetylation and p53 ChIP after 5-FU treatment. MCF7 cells were left untreated (*c*) or treated with IC50 and IC80 5-FU. ChIPs were performed with the indicated antibodies, and PCR was performed for the specific promoters included in (*b*). (*d*) Time course p53 ChIP for p21 promoter. MCF7 cells were left untreated (−) or treated with IC50 and IC80 5-FU for 8, 24 and 48 hr. ChIP was performed with DO7 anti-p53 antibody, and PCR was performed with primers included (*b*). Histone acetylation for histones H3 and H4 is also shown.

tion for this bypassing effect is the induction of the E2F pathway, known to be necessary for the G1/S transition.[32] E2F comprises a group of transcription factors typically associated with DP proteins (1 and 2) and regulated by the retinoblastoma family. By microarray analysis, E2F1 targets showed a trend to be repressed with IC50 5-FU and to be induced or not modified with IC80 concentrations of 5-FU. In fact, one cluster of E2F-related genes shared the same pattern of early induction, followed by a progressive downregulation at 48 hr, only after treatment with IC80 5-FU. Besides, although protein levels of E2F1 were equally increased

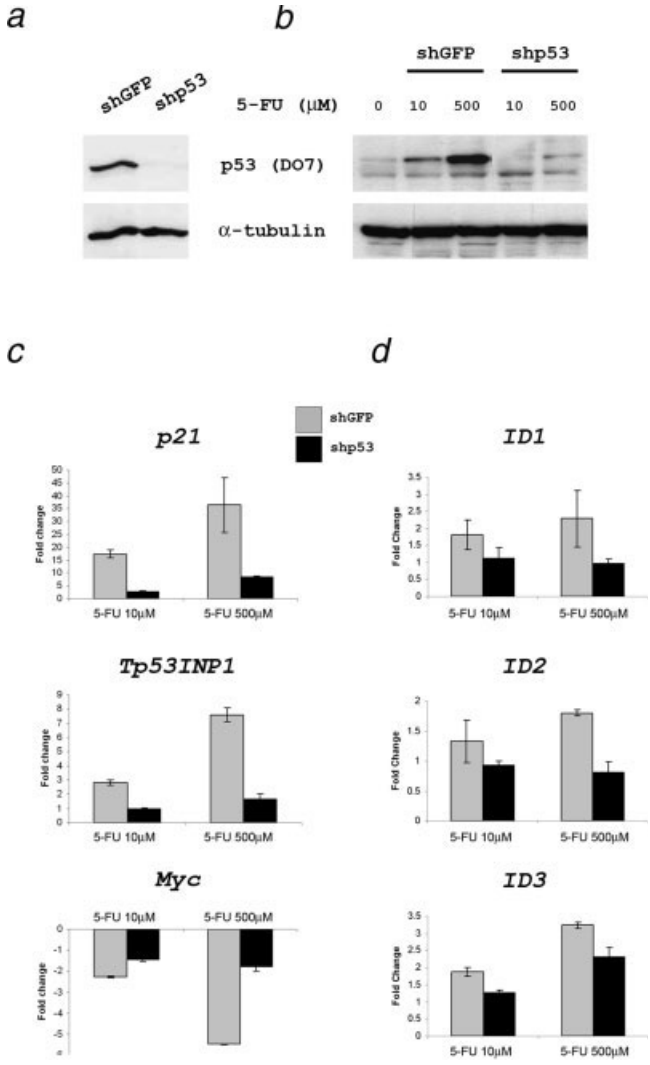

FIGURE 5 – Response to 5-FU in shp53 MCF7 cells. (*a*) Western blot analysis of p53 protein expression in shp53 MCF7 cells *versus* control shGFP MCF7 cells. α-tubulin is included as a control. (*b*) Western blot analysis showing accumulation of p53 in response to IC50 and IC80 5-FU in shp53 MCF7 cells. (*c*) Quantitative real-time RT-PCR. TaqMan real-time PCR in shp53 *versus* shGFP MCF7 cells showing expression of *p21*, *Tp53INP* and *Myc* after treatment with 5-FU. Fold induction or repression is shown relative to control cells. (*d*) Semiquantitative PCR analysis of ID genes in shGFP *versus* shp53 MCF7 cells treated with IC50 and IC80 5-FU. GADPH was used as internal control. Fold induction or repression is shown relative to the internal control.

after both concentrations of 5-FU, the induction of the E2F1 partner DP-1 was higher with IC80 5-FU. Although essential for G1/S transition, the E2F pathway has also been implicated in DNA-damage response, and apoptosis through p53-dependent and p53-independent mechanisms.[33] Moreover, according to Wu and Levine, it has been shown to cooperate with p53 to mediate apoptosis.[34] In this sense, the activity of E2F1 and its partner DP-1 overcome the p53-induced growth arrest, and the conflicting signals result in activation of an apoptotic pathway as has been suggested in several studies.[34,35] According to our data, this mechanism could be activated above a certain level of DNA damage induced by 5-FU, as a requirement to overcome the G1/S checkpoint.

With respect to genes involved in apoptosis, an important role for the death receptor FAS has been given in the thymineless death induced by 5-FU in a p53-dependent manner,[4] also confirmed by our data in the shp53 MCF7 cell line. In addition, the *SAT* gene was upregulated after 5-FU exposure in a dose- and time-dependent manner. The product of this gene is involved in polyamine metabolism and induction of caspase-dependent apoptosis, and was recently described as a 5-FU-inducible transcriptional target.[36] Finally, a group of several genes involved in apoptosis, some of them specifically involved in the mitochondrial pathway of apoptosis, were regulated with higher concentrations of 5-FU, including *APAF1*, *BAK1*, *BBC3*/*PUMA*, *BCL2*, *JUN*, *JUNB* and *TP53I3*/*PIG3*. These results suggest that a higher regulation or the activation of mitochondrial pathway genes could be related with the higher induction of cell death observed with IC80 5-FU in MCF7 cells.

Most importantly, this model of chemotherapy-induced DNA damage permitted the assessment of the mechanisms involved in the induction of gene expression by 5-FU. Some of the most upregulated genes included *S100A10* and genes of the ID family, *ID1*, *ID2* and *ID3*. In particular, an increase in the levels of acetylation of histone H4 to these promoters was observed, indicating that 5-FU exposure induces active transcription through the modification of the histone acetylation pattern. Moreover, recruitment of p53 to the promoters of *p21*, *ID1* and *ID2* genes could be demonstrated by ChIP in our study. p21 had been previously demonstrated to be a p53 target under different conditions, and our results indicate that p21 is also activated through direct binding of p53 as a response to 5-FU treatment. For further validation of these findings, we shut down *p53* expression by RNA interference in MCF7 cells. As expected, induction of *ID1* and *ID2* transcripts was significantly lower in response to 5-FU when compared with control shGFP MCF7 cells. In this manner ID1 and ID2 are revealed as novel targets of direct activation through p53 interaction with their respective promoters. It is possible to speculate about the potential involvement of ID proteins in drug-induced cytotoxicity under these conditions. In fact, ID proteins have been implicated in cell growth, differentiation and tumorigenesis, and are integrated at multiple points in G1 cell cycle control required for progression through the S phase of the cell cycle.[37] Specifically, by binding to proteins of the Rb family, ID2 is involved in the positive regulation of the E2F pathway.[37] Therefore, the ID2 induction observed with higher concentrations of 5-FU would be in agreement with an activation of the E2F pathway. However, functional studies would be necessary to assess the relevance of ID proteins in response to chemotherapy in this context.

As the differences in gene expression observed with both concentrations of 5-FU are not only quantitative but also qualitative, this could be explained by dose-dependent changes in the mechanism of action of this drug. In this sense, cytotoxicity induced by 5-FU can be dependent on TS inhibition or incorporation of metabolites into nucleic acids. Although previous reports have shown that 10μM 5-FU is largely independent of TS inhibition,[38] further studies are required to rule out this possibility at higher concentrations of the drug. It should be noted, however, that dialyzed foetal calf serum was not used in our experiments, and this could have an influence on cell sensitivity as thymidine kinase-mediated rescue of TS inhibition was not prevented.

Summarizing, we described a comprehensive molecular characterization of dose- and time-dependent modifications of gene expression in the *p53*-wild type breast cancer cell line MCF7 after 5-FU treatment. Several reports of gene microarray analyses of MCF7 cells exposed to 5-FU have recently been published, using low (10 μM)[36] or high doses (300 μM)[39] of 5-FU. However, the 2 doses had not been compared in a single study before, correlating gene expression with cell cycle and cell death parameters in several time points. Our study confirms several findings of these previous reports and identifies new molecular changes associated with 5-FU cytotoxicity, and new targets of p53 in this setting. Future experiments using the p53-depleted cell lines will be necessary to understand the p53-mediated

responses to chemotherapy drugs. These data should provide a valuable resource for future basic studies addressing the molecular mechanisms of chemotherapy in breast cancer, and the search for potential new p53-dependent and independent molecular targets.

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