Daunorubicin-induced variations in gene transcription: commitment to proliferation arrest, senescence and apoptosis

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We used a human cDNA macroarray containing various oncogenes and tumour suppressor genes to assess gene expression profiles in early-passage Jurkat T lymphocytes treated with clinically relevant concentrations of the antitumour antibiotic daunorubicin. Several oncogenes and tumour suppressor genes were either up- or down-regulated depending on the daunorubicin concentration used. The expression levels of some of these genes were confirmed by semi-quantitative reverse transcriptase–PCR. We also compared the changes in cell-cycle distribution and the apoptotic morphological characteristics of the cells treated with daunorubicin, using flow cytometry and fluorescence microscopy. Exposure to 182 nM daunorubicin (its IC₇₅ in Jurkat T cells: where IC₇₅ is the drug concentration that inhibits growth by 75%)

resulted in cell-cycle arrest in G₁ and almost immediate apoptosis. In contrast, decreasing the drug concentration to 91 nM (close to the IC₅₀) caused G₂ arrest and cell senescence-like growth arrest, whereas features of apoptosis and necrosis appeared only after longer incubation times. Gene expression profiles, cell-cycle distribution, the presence of DNA damage and the time-dependent response of Jurkat T cells to cell death were correlated clearly. The general behaviour of the genes suggests that cell-cycle arrest and cell death follow distinct pathways depending on drug concentration.

Key words: anthracycline, array, cell cycle, Jurkat T lymphocyte, transcriptome.

INTRODUCTION

In the presence of several DNA-binding drugs, tumour cells can suffer growth arrest and death [1–3]. The regulation of transcription plays a critical role in the control of growth and differentiation of normal and tumour cells (see [4,5] and references therein). Therefore there is a widespread interest in drugs, which can bind to DNA and affect the final destiny of tumour cells through the inhibition of gene transcription [6–10]. Changes in the transcription levels of certain genes, such as oncogenes or tumour-suppressor genes, may inhibit the development of tumours after drug treatment. Evidence is mounting that drug-induced cell death programmes can be either activated or suppressed depending on the cell type and the characteristics of antitumour agents [11–14].

Large-scale measurement of gene expression should provide new keys to gain more insights into cells treated with anticancer drugs [6,12,15]. DNA array technology permits the simultaneous measurement of the expression levels of thousands of genes. It can provide a comprehensive framework to determine the genes that are involved in changes in cell-cycle progression after treatment with DNA-binding drugs [6]. The patterns observed in genome-wide expression experiments may indicate the status of cellular processes and drug effects, although the correct interpretation of these results may be hindered by the interrelation between cell-cycle pathways, which makes it difficult to discriminate direct and indirect effects.

It is accepted widely that cell death after DNA damage by antitumour drugs occurs mainly due to programmed cell death (apoptosis). However, it has been observed that apoptosis is only the primary mediator of cell killing in some tumours [2]. The influence of the *p53* gene product, a key element in apoptotic response, has been characterized in depth [16,17]. The *p53* gene is a critical component of two distinct cellular responses to DNA damage, the induction of a reversible arrest at the G₁/S cell-

cycle checkpoint and the activation of apoptosis [2,16]. It is also involved in the halt in the G₂/M checkpoint [18]. Nonetheless, in some tumour cells, the final apoptotic response may depend on the transcription levels of other genes [2,19–22]. The elucidation of the factors that regulate different aspects of treatment-induced apoptosis and alternative ways of cell killing should improve the efficacy of anticancer therapy [2,11,23].

We aimed to study the correlation between the transcript profile of Jurkat T cells in the presence of a DNA-binding drug, and the drug activities through the resulting gene expression levels, with special emphasis on the link between the cell-cycle distribution and gene transcription profiles. We explored the sensitivity of gene expression in *p53*^{+/+} Jurkat T lymphocytes after treatment with daunorubicin. This is an anthracycline antibiotic used primarily in the treatment of acute myeloid leukaemia [7]. It recognizes (C + G)-rich DNA sequences specifically [24], and acts as both a transcription inhibitor [9,15] and a topoisomerase II inhibitor [25]. In the present paper, we compare experiments performed in parallel on the gene expression patterns of proliferating Jurkat T cells and daunorubicin-treated cells using macroarrays of cDNAs. Specific features of gene expression responses are identified, which are dependent on several key pathways. We also tested whether drug-induced quiescence and death in Jurkat T lymphocytes were associated with changes in the transcription profile of oncogenes and tumour suppressor genes, since several transcription regulators and components of signal-transduction pathways can regulate apoptosis [26].

MATERIALS AND METHODS

Cell line and culture conditions

Early-passage Jurkat T lymphocytes (A.T.C.C. TIB-152, clone E6-1, with no more than three serial subcultures after arrival)

Abbreviations used: BrdUrd, 5-bromo-2'-deoxyuridine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IC₇₅, drug concentration that inhibits growth by 75%; RT, reverse transcriptase; SA- β -gal, senescence-associated β -galactosidase.

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were maintained in RPMI 1640 medium (Gibco BRL, Invitrogen, Prat de Llobregat, Spain), supplemented with 10 % (v/v) foetal calf serum (Gibco BRL) and 2 mM L-glutamine (Gibco BRL), at 37 °C in a humidified atmosphere with 5 % CO₂. Viable cell number was determined using the exclusion of Trypan Blue dye (Fluka, Madrid, Spain) and a haemocytometer. Solutions containing 500 µM daunorubicin (Sigma) were prepared with sterile 150 mM NaCl, maintained at -20 °C, and brought to the final concentration with RPMI 1640 medium just before use.

Human array experimental design

The Human Oncogene/Tumour Suppressor Atlas Array (BD ClonTech, Palo Alto, CA, U.S.A.) was used to monitor changes in the levels of transcription of 199 genes in Jurkat T lymphocytes, with various concentrations of the anticancer antibiotic daunorubicin, compared with untreated cells. The annotated function for all these genes, as well as their accession numbers, can be found at the BD ClonTech homepage (<http://www.clontech.com/atlas>). Of these genes, 190 are commonly classified as oncogenes or tumour suppressor genes, whereas nine are regarded as housekeeping genes. Experiments were undertaken in triplicate and the results averaged. A database consisting of the average raw data for all genes in the different experiments and the processed (normalized) results, as supplementary material, is available as online data at <http://www.BiochemJ.org/bj372/bj3720703add.htm>

RNA extraction and cDNA synthesis

Total RNA was isolated, after 4 h, from control cells (those to which no drug was added) and from cells treated with 182 nM daunorubicin (its IC₇₅ in Jurkat T cells determined after 72 h continuous treatment [27]; where IC₇₅ is the drug concentration that inhibits cell growth by 75%) or 91 nM daunorubicin (close to the IC₅₀). The UltraspecRNA isolation reagent (Biotechx, Houston, TX, U.S.A.) was used following the procedure provided by the vendor. RNA was digested with RNase-free DNase I (BD ClonTech) in the presence of RNase inhibitors (RNasin; Promega), phenol-extracted and precipitated. The pellet was dissolved in RNase-free water, and the yield and the purity of total RNA were assessed spectrophotometrically.

Approx. 5 µg of RNA was used to convert total RNA into ³²P-labelled first-strand cDNA, using [α -³²P]ATP and the gene-specific CDS-primer mix for the Atlas array (BD ClonTech) following the method provided by the vendor. The labelled cDNA was purified from unincorporated [α -³²P]ATP using a NucleoSpin Column (BD ClonTech).

Human array hybridization

A set of Human Oncogene/Tumour Suppressor Atlas Arrays (BD ClonTech), containing cDNAs of 199 genes, were used for hybridization. Hybridization with the labelled probes was performed, in triplicate, using ExpressHyb solution (BD ClonTech) at 68 °C for 16 h. The arrays were rinsed with 2 × SSC (300 mM sodium chloride 30 mM trisodium citrate, pH 7.0), 1 % (w/v) SDS and washed using 0.1 × SSC, 0.5 % SDS at 68 °C for 30 min, and a final 5 min wash was performed in 200 ml of 2 × SSC at room temperature (25 °C). The Atlas Arrays were exposed to an Imaging Screen-K (Bio-Rad) and analysed using a Molecular Imager FX (Bio-Rad).

Expression profile analysis

For image processing, the intensity of the radioactive signals was analysed using Quantity One 4.1.1 software (Bio-Rad), and the peak areas were transferred into a standard spreadsheet program. To analyse the differential gene expression in the absence and in the presence of several concentrations of daunorubicin, the background in each array was subtracted from the spot values, and the average intensity of the housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), utilized widely as control in transcription experiments, was used to normalize the results and to adjust for differences in labelling and the quantities of RNA. The nine control genes in the array gave similar hybridization signals, including *GAPDH*, within a calculated 5 % error (see supplementary material).

After normalization, data for each gene were calculated as the log₂ of the expression ratio between 'daunorubicin-treated' and 'control' membranes. The J-Express software (MolMine AS, Bergen, Norway) was used in the comparison of the expression profiles. A post-normalization cut-off of 2.5-fold increase or decrease in the expression ratio was used. There is no firm theoretical basis for selecting a particular level of significance, but a cut-off of 2-fold increase or decrease is used generally. The number of genes analysed, which were clearly up- or down-regulated was almost the same using a much stringent 4-fold cut-off criteria (see the Results section and also the supplementary material available on the Biochemical Journal web site).

Semi-quantitative reverse transcriptase (RT)-PCR

Changes in gene expression were verified by semi-quantitative RT-PCR using *GAPDH* as an internal normalization standard. Total RNA was isolated, after 4 h, from control cells (those to which no drug was added) and the cells were treated with different concentrations of daunorubicin as indicated in the legends to Figures, and digested with DNase I as described above. Subsaturation RT-PCR conditions were adjusted to 10 ng of total RNA and 20–25 amplification cycles, with the annealing reactions performed at 50 °C for 1 min, using the OneStep PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The primers used (0.6 µM) are indicated in the legend to Figure 3. PCR samples were electrophoresed in a 2 % (w/v) agarose gel, stained with ethidium bromide and quantified using a Molecular Dynamics Densitometer.

Cell-cycle distribution and apoptosis

After treatment with daunorubicin for various periods of time, the cells were harvested and stained with propidium iodide (Sigma) as described elsewhere [28]. Nuclei were analysed with a Coulter Epics-XL flow cytometer (Coulter Corporation, Hialeah, FL, U.S.A.) at the 'Serveis Científico-Tècnics' (University of Barcelona, Spain) using the 488 nm line of an argon laser and standard optical emission filters. Percentages of cells at each phase of the cell cycle were estimated from their DNA content histograms after drug treatment. Apoptosis was quantified and distinguished from necrosis by using the Annexin-V-Fluos staining kit (Roche Diagnostics, Barcelona, Spain) and flow cytometry as described in [29].

For morphological observation, approx. 10⁴ daunorubicin-treated Jurkat cells were spun on to microscope slides, stained with 4,6-diamidino-2-phenylindole (Sigma) or with propidium iodide plus Annexin-V-Fluos (Roche Diagnostics) and analysed with a Carl Zeiss Axiophot fluorescence microscope. Apoptotic

cells were identified by the presence of cytoplasmic shrinking and nuclear condensation.

Quantification of cellular DNA fragmentation

Cell cultures were incubated for 16 h with 10 μ M 5-bromo-2'-deoxyuridine (BrdUrd) (Roche Diagnostics), harvested by centrifugation and redissolved in fresh RPMI 1640 medium (Gibco BRL). Thereafter, they were incubated with 92 or 182 nM daunorubicin for 4 or 10 h. Cells were lysed, and the DNA fragments were detected using anti-DNA antibody and anti-BrdUrd antibody conjugated to horseradish peroxidase by using the Cellular DNA fragmentation ELISA kit (Roche Diagnostics). Colour development was measured at 450 nm using a SPECTRAmax-250 microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

Senescence-associated β -galactosidase (SA- β -gal) staining and DNA synthesis assays to detect senescent cells

After treatment with daunorubicin, cells were fixed in 3% (v/v) formaldehyde and stained with fresh SA- β -gal stain solution as described elsewhere [30], using X-Gal (5-bromo-4-chloro-3-indolyl β -D-galactosidase) at pH 6.0. Senescence-like growth arrest was determined as the percentage of SA- β -gal+ cells appraised by phase-contrast microscopy, after scoring 50–100 cells for each control and daunorubicin-treated sample. DNA synthesis was assayed as described elsewhere [31], utilizing BrdUrd, anti-BrdUrd-POD (Roche Diagnostics) and a colorimetric reaction using 4-Nitro Blue Tetrazolium chloride (Roche Diagnostics), measured at 370 nm.

RESULTS

Analysis of changes in the transcriptome induced by daunorubicin

The Human Oncogene/Tumour Suppressor Atlas Array (BD ClonTech) was used, in experiments performed in triplicate, to monitor changes in the levels of transcription of 199 genes in Jurkat T lymphocytes, with various concentrations of the anticancer antibiotic daunorubicin, and compared with untreated cells (see the Materials and methods section).

Treatment of early-passage Jurkat T lymphocytes with either 182 nM daunorubicin (its IC_{75}) or 91 nM (close to the IC_{50} [9]) for 4 h altered gene transcription, with down-regulation of some genes and up-regulation of others. Figure 1 shows scatter plots of the corrected expression levels of the genes represented in the array. They correspond to the transcription levels of daunorubicin-treated Jurkat T cells versus untreated control cells. All the values were corrected for the array background, and normalized by the transcription of the housekeeping *GAPDH* gene. The graphic representations in Figure 1 suggest that cells treated with various doses of a DNA-binding drug stimulate or inhibit the expression of a distinct set of genes depending on the concentration. We would like to highlight that the daunorubicin concentrations used fall within clinically relevant values [3].

In listing of daunorubicin-responsive genes, only those with induction or repression rates equal or higher than 2.5-fold were selected (Figure 2, upper panel). For comparison purposes, the 190 genes were grouped according to whether they were up- or down-regulated after treatment with either drug concentration, by using a representation that clustered several sets of genes depending on their response to either drug dose (Figure 2, lower panel). In this panel, the outermost and innermost circles

Figure 1 Distribution of the daunorubicin-induced expression profiles in Jurkat T lymphocytes

Bivariate scatter plots represent, in logarithmic scales, the expression profiles of the untreated control versus the daunorubicin-induced transcription profiles. The values are corrected intensities in the Human Oncogene/Tumour Suppressor Atlas Array (BD ClonTech), representing transcription profile levels. (A) Cells treated with 91 nM daunorubicin (IC_{50}) or (B) cells treated with 182 nM daunorubicin (IC_{75}) for 4 h.

Table 1 A selection of differentially expressed genes in Jurkat T cells incubated with daunorubicin

The genes displayed have been implicated previously in checkpoint arrest and apoptosis

Genes	Daunorubicin (nM)	
	91	182
<i>ATM</i>	+ *	+
<i>p53</i>	+	+
<i>MDMX</i>	–	=
<i>BRCA1-associated ring domain protein 1</i>	–	+
<i>p21^{WAF1}</i>	+	=
<i>N-Ras</i>	+	=
<i>p16^{INK4}</i>	–	+
<i>p14^{INK4B}</i>	+	+
<i>Cyclin D3</i>	+	=
<i>Cyclin E</i>	+	–
<i>Cdc25A</i>	+	=
<i>Rb</i>	+ †	+
<i>130 kDa Rb-associated protein</i>	–	=
<i>RBQ-1</i>	=	+
<i>RBQ-3</i>	–	+
<i>E2F5</i>	+	+
<i>CDC-like kinase 2</i>	+	+

* +, up-regulated; –, down-regulated; =, no change.

† As inferred from the semi-quantitative RT-PCR analysis (Figure 3).

display genes that changed only at one of the two concentrations assayed. Among the 46 genes that changed with both treatments, 13 were up- or down-regulated depending on the daunorubicin concentration. In total, 59 of the 190 genes analysed showed opposite expression profiles at low and high drug concentrations (cf. upper and lower panels in Figure 2). This includes genes that are classified commonly as oncogenes or tumour suppressors, yet most of them may provide resistance to DNA damage, or be regarded as inducible genes involved potentially in repairing or preventing DNA damage [23,32,33]. From the bulk of genes that were either up- or down-regulated, Table 1 lists those whose annotated function is related to cell-cycle progression, arrest in G₁ or G₂ checkpoints, DNA damage and/or apoptosis [32,33]. The response of many genes depended on the drug concentration: some genes that were up-regulated at one dose were down-regulated at the other (Figure 2). This suggests

Figure 2 Analysis of gene expression using Human Oncogene/Tumour Suppressor Atlas Array

Histograms represent the distribution of $\log_2$ ratio values for the genes whose transcription profiles changed in any or both treatments with the IC_{50} and IC_{75} of daunorubicin. Genes were grouped in two sets depending on whether their transcription profiles were affected similarly by both treatments, or they showed differential response to the two daunorubicin concentrations analysed. Upper panel: open bars are corrected expression profiles in the presence of 91 nM daunorubicin and black bars correspond to corrected expression profiles in the presence of

that cell arrest and death follow a somewhat different pathway depending on drug concentration. The names of the genes, classified according to their response to daunorubicin, are indicated in the legend to Figure 2. Notably, several genes, listed in Table 1, presented expression levels clearly higher/lower than the 2.5-fold cut-off. For example, *p53*, a key gene in apoptosis, was up-regulated by approx. 14-fold in the presence of IC_{75} of daunorubicin, and approx. 20-fold in the presence of IC_{50} , whereas *cyclin E* was down-regulated 50 times (IC_{75}) but up-regulated in the presence of the IC_{50} . Data for all the genes analysed are provided, as supplementary material, at the Biochemical Journal web site. Given the relevance of several of these genes, including *p53* and *Rb*, to the development of the apoptotic response [16,17,33,34], we confirmed their response to daunorubicin by semi-quantitative RT-PCR. The transcription of *p53* was enhanced in both daunorubicin treatments (Figure 3 and Table 1). The p53 protein acts as a transcriptional regulator for many genes [16] and could stimulate the transcription of some genes involved in the apoptotic pathway.

Effects of daunorubicin on cell-cycle progression

Once we established that the profiles of gene expression in Jurkat T cells were diverse, we sought to verify whether the dose-dependent expressions of all the genes correlated with the changes observed in cell-cycle progression, growth arrest and cell death.

Cells treated with the two concentrations of daunorubicin were analysed by flow cytometry to test whether the dose-dependent expression of all the genes could correlate with the different changes in cell-cycle progression, growth arrest and cell death. Cell-cycle analysis of the 48 h treatment showed that the behaviour of Jurkat T lymphocytes varied depending on the daunorubicin concentration (Figure 4A). At 182 nM (IC_{75}), the drug increased abruptly the sub- G_0 , which reached a peak within the

182 nM daunorubicin. Numbers on the y-axis stand for the genes tested, according to the following list: 1, *TGF- β receptor III*; 2, *TGF- β 2*; 3, *p53*; 4, *heregulin- β 3*; 5, *guanine nucleotide-binding protein α 13 subunit*; 6, *pim-1*; 7, *Wilms' tumour protein*; 8, *ski-related oncogene* *snoN*; 9, *N-myc*; 10, *DCC*; 11, *CDC25 homologue*; 12, *FOS-related antigen 2*; 13, *ATM*; 14, *PI3 kinase*; 15, *CDC-like kinase 2*; 16, *c-yes*; 17, *APC*; 18, *ETS oncogene*; 19, *E2F5*; 20, *bcl-2*; 21, *semaphorin III*; 22, *p14-INK4B*; 23, *RAD50 homologue*; 24, *neogenin*; 25, *CD100 antigen*; 26, *TGF- β signalling protein*; 27, *tyrosine kinase tnk 1*; 28, *wingless-related MMTV integration site 2 protein*; 29, *junD*; 30, *jun-B*; 31, *APC*; 32, *v-Ki-RAS2B*; 33, *STAT2*, *STAT113*; 34, *erbB4*; 35, *mitochondrial transcription factor 1*; 36, *p16-INK4*; 37, *CBL-B*; 38, *5T4 oncofoetal antigen*; 39, *delta lactoferrin*; 40, *FKBP-rapamycin associated protein*; 41, *frizzled homologue 5*; 42, *BRCA1-associated ring domain protein 1*; 43, *RbQ3*; 44, *TATA sequence-binding protein*; 45, *β 1 catenin (CTNNB)*; 46, *cyclin E*; 47, *c-kit*; 48, *neuregulin 1*; 49, *erbB2*; 50, *tight junction protein 1*; 51, *Rb*; 52, *integrin-linked kinase*; 53, *RbQ1*; 54, *semaphorin III/F*; 55, *wingless-related MMTV integration site 8b protein*; 56, *neurofibromatosis protein type 1*; 57, *neural retin-specific leucine zipper protein*; 58, *MCC*; 59, *c-abl*; 60, *raf1*; 61, *prohibitin*; 62, *cyclin D3*; 63, *Cdc25A homologue*; 64, *ski*; 65, *TBP-associated factor 250 kDa subunit*; 66, *p21/WAF1*; 67, *guanine nucleotide regulatory protein tim 1*; 68, *nuclease-sensitive element DNA-binding protein*; 69, *c-fgr*; 70, *L-myc*; 71, *prefoldin 4*; 72, *c-kit ligand*; 73, *X-ray repair-complementing defective repair in Chinese hamster cells*; 74, *receptor interacting protein*; 75, *transducer of erbB2*; 76, *B-raf*; 77, *prefoldin 5*; 78, *smoothed homologue*; 79, *β -catenin*; 80, *ZAP70*; 81, *CDC-like kinase 3*; 82, *neurogenic locus notch protein homologue 1*; 83, *CDC2-related protein CHED*; 84, *N-Ras*; 85, *SAP102*; 86, *rel A*; 87, *130 kDa Rb-associated protein*; 88, *PCTAIRE 3*; 89, *mdmx*; 90, *frizzled-related FrzB*; 91, *ABL2*; 92, *tumour necrosis factor receptor superfamily member 10B*. The function for all these genes and their accession numbers can be found at: <http://www.clontech.com/atlas>. Bars labelled with arrows correspond to approximate ratios given the low transcript values obtained in one of the experiments. Lower panel: a diagram grouping together sets of genes according to their response to daunorubicin concentrations. In this panel, the outermost and innermost circles display genes that changed only at one of the daunorubicin concentrations assayed, but not at both of them. Genes represented in the other sectors changed with both drug concentrations. +, up-regulated gene expression; -, down-regulated gene expression; =, unchanged gene expression. In each set of two signs, the left sign represents the lower daunorubicin concentration, and the right sign corresponds to the higher one. Genes represented in the circles can be identified using the equivalent signs on the proximal part of the upper panel.

Figure 3 Differential expression of *p53* and *Rb* in the presence of 182 or 91 nM daunorubicin as observed by semi-quantitative RT-PCR

The housekeeping *GAPDH* gene was co-amplified as internal control. The specific primers used for RT-PCR amplification were: *GAPDH*dir, 5'-TCAGCCGCATCTTCTTTG-3'; *GAPDH*rev, 5'-TGATGGCATGGACTGTGGT-3'; *Rb*dir, 5'-AGAACAGGAGTGCACGGATAGC-3'; *Rb*rev, 5'-CGTGGTGTCTCTGTGTTCAA-3'; *p53*dir, 5'-TCAGCATCTTATCCGAGTGG-3'; *p53*rev, 5'-CCTGGGCATCCTTGAGTTC-3'.

first 10 h of treatment. During the 24–48 h period, cell-cycle distribution was impaired clearly and many cells were found in sub- G_0 . The cell-distribution profiles would suggest that cells found in the S-phase underwent apoptosis after drug treatment (Figure 4A). Continuous exposure to the IC_{50} daunorubicin revealed that after 4 h treatment, most cells showed the same distribution as controls, with a prominent G_1 peak. However, in the period up to 24 h, there was a progressive accumulation in G_2/M , followed by an undefined profile similar to the one determined for the higher daunorubicin concentration (Figure 4A).

Dependence of apoptosis, DNA fragmentation and senescence-like growth arrest on daunorubicin concentration

The presence of apoptotic Jurkat cells in the flow-cytometry assays was confirmed by double staining with propidium iodide and Annexin-V-fluos (Figure 4B). Whereas untreated controls maintained a relatively uniform distribution throughout the experiment, continuous treatment with 182 nM daunorubicin increased dramatically the number of apoptotic cells, starting after 4 h (positive staining with Annexin-V-fluos) and affecting almost 25 % of the cells after approx. 24 h, with another 20 % of necrotic cells. However, at the lower dose of daunorubicin, the number of apoptotic cells increased slightly at 24 h, from 5.2 to 7.9 %, and dramatically at 48 h, from 9.62 to 24.12 %. At the same time, there was a clear increase in the number of necrotic cells and/or secondary apoptosis (double staining with Annexin-V-fluos and propidium iodide in Figure 4B), which reached approx. 65 % of the cell population after 48 h.

After the 4 or 10 h continuous treatment with either IC_{50} or IC_{75} for daunorubicin, cellular DNA fragmentation was also analysed

Table 2 Quantification of intracellular DNA fragmentation in Jurkat T lymphocytes treated with various concentrations of daunorubicin

	Daunorubicin (nM)			
	91		182	
	4 h	10 h	4 h	10 h
% DNA damage*	7.4 ± 5.2	7.2 ± 6.0	26.3 ± 3.1	37.8 ± 4.4

* Values are expressed as the percentage of untreated controls and represent the means ± S.D. for three experiments.

Figure 4 Flow-cytometry analyses of Jurkat T cells incubated with daunorubicin

(A) Cell-cycle distribution determined at the time intervals indicated. Data indicate the percentage of cells in each phase. (B) Analysis of apoptotic cells by double staining with Annexin-V-fluos (y-axis) and propidium iodide (x-axis) at the time intervals indicated (R1, viable cells; R2, apoptotic cells; R3, necrotic plus secondary apoptotic cells).

by ELISA, as described in the Materials and methods section. Cells treated with 91 nM daunorubicin hardly showed DNA fragmentation, whereas in the presence of 182 nM daunorubicin, DNA fragmentation increased significantly (Table 2). Taken together, these data and the flow-cytometry results suggest that damage of DNA by daunorubicin accelerates the susceptibility of the cells to apoptosis. The DNA fragmentation analysed by ELISA does not discriminate the damage produced by stabilization of the topo II-cleavage complex and that induced by other mechanisms,

such as free-radical formation. However, the absence of DNA cleavage for the lower drug concentration is in keeping with the dose-dependent effects of anthracyclines on topoisomerase II cleavage [35].

Jurkat T cells that were halted in G₂ after treatment with 91 nM daunorubicin (Figure 4A) for 24 h showed phenotypic features of cell senescence, such as enlarged cells and granularity together with SA- β -gal staining [30,36] (cf. Figures 5A and 5B). The percentage of senescent cells was approx. 40% at 24 h, which is close to the percentage of cells that were stopped at the G₂ checkpoint. After 24 h of continuous treatment, these cells were unable to synthesize DNA, as demonstrated by the lack of BrdUrd incorporation.

Differences in the cell distribution and time-dependent apoptosis in cells treated with two clinically relevant daunorubicin concentrations prompted us to test whether cells that were halted in G₂ after the 48 h treatment with daunorubicin IC₅₀, and were suffering replicative-like senescence (Figures 5A and 5B), showed the morphological characteristics of apoptotic death after longer periods of treatment, as observed after the 24 h treatment. The morphology and the staining of Jurkat T cells studied by fluorescence microscopy indicated that the cells underwent delayed cell death in the presence of the lower daunorubicin concentration, which was mainly apoptotic after 48 h (Figure 5D), as in the presence of the higher drug concentration after the 24 h treatment (Figure 5E).

DISCUSSION

Jurkat T lymphocytes are an especially useful cell model, inasmuch as they are *p53*^{+/+} and can thus suffer the characteristic apoptosis observed in cells of lymphoid origin in the presence of external injuries [13]. We used this system to analyse the effects of clinically relevant concentrations of daunorubicin, which are well below those used prevalently

to study drug effects on topoisomerase activity [3,35]. Daunorubicin induced cell arrest and subsequent apoptotic death at both concentrations tested, yet the extent and the kinetics of the apoptotic response were dose-dependent. At the highest concentration tested, corresponding to IC₇₅, cells suffered apoptosis, in agreement with early observations of apoptosis in Jurkat T cells in the presence of various concentrations of anthracyclines [37], whereas at the lowest concentration, i.e. IC₅₀, cells underwent mitotic arrest and entered a senescence-like arrest process, and died eventually through an apoptotic pathway. These effects were corroborated by both flow-cytometry and standard tests for apoptosis and cell senescence (Figures 4 and 5), suggesting two complementary mechanisms of cell arrest induced by the antitumour antibiotic daunorubicin.

We have used a human cDNA expression array to determine the expression profiles of several genes classified commonly as tumour suppressors and/or oncogenes. Our results indicate that the fate of cells that suffered cell arrest and death after treatment with daunorubicin may be related to the differential expression of several key genes. Figure 6 presents a tentative explanation of the differential effects of high and low doses of daunorubicin by two pathways, in which the up-regulated *p53* plays a central role. These pathways are based on changes in the cell transcriptome, observed after treatment with daunorubicin (Figure 2). There is a fair agreement between the changes observed in the transcription of genes considered to participate in the halt of the cell cycle in G₁ or G₂ (Table 1) and those described to be involved in these checkpoints and cell arrest, senescence-like growth arrest and apoptosis [16–18,31,34,38,39]. The pro-apoptotic factor *p53*, as well as *ATM* and *Rb*, was up-regulated in both treatments, which is consistent with the fact that the cells ended up entering apoptosis regardless of the daunorubicin concentration. Notwithstanding, for the IC₅₀ daunorubicin concentration, double staining with Annexin-V-fluos and propidium iodide reflected an increase in the necrotic

Figure 5 Effects of daunorubicin on cell senescence-like growth arrest and apoptosis in Jurkat T lymphocytes

Untreated cells (A) and 91 nM daunorubicin-treated cells (B). After 24 h incubation, cells were fixed, analysed for SA- β -gal staining (a senescence marker) using a phase-contrast microscope and photographed, after 16 h, at $\times 400$ magnification. Approx. 35% of the treated cells can be scored as senescent cells, whereas control cells did not show this phenotype (approx. 1% cells stained with SA- β -gal). The morphological analysis was performed by fluorescence microscopy, using double staining with Annexin-V-fluos and propidium iodide. (C) Untreated cells showing no staining with propidium iodide plus Annexin-V-fluos, stained with 4,6-diamidino-2-phenylindole after 48 h. (D) Cells treated with 91 nM daunorubicin for 48 h and (E) with 182 nM daunorubicin for 24 h. Cells in (C–E) were photographed at $\times 400$ magnification, and they are shown at a final $\times 1300$ magnification to display, in detail, the morphological changes characteristic of apoptosis, which include the condensation of chromatin and the formation of apoptotic bodies.

Figure 6 Two tentative pathways used to explain how the transcription profiles of the genes analysed may induce the observed final fate of Jurkat T lymphocytes

Cells were treated with either IC₇₅ (left panel) or IC₅₀ (right panel) for daunorubicin. Both pathways were created considering the changes in the transcription profiles obtained by cDNA macroarray analysis (Figure 2) and the presence of bulk DNA damage (Table 2), together with the results available on the function of the corresponding gene products on the control of cell-cycle stops in G₁ or G₂/M, senescence-like growth arrest and apoptosis. Further details and references are provided in the main text. +, up-regulated; -, down-regulated. The presence of parentheses means that the response of these genes to daunorubicin was either outside the criteria of exclusion in the analysis of gene profiles (2.5-fold change) or not corroborated in our experiments because the genes were not present in the array.

population (Figure 4B), a result that is in keeping with the final destiny of senescent-human fibroblasts, which predominantly undergo necrotic death [40]. The main differences between the two proposed pathways are based on the control of *cyclin D3* and *cyclin E*, and their regulator *p16*. Treatment with IC₅₀ for daunorubicin up-regulated both cyclins (Figure 6, right panel), whereas the inhibitor *p16* was down-regulated. The gain of *cyclin D3/cyclin E* profiles together with the up-regulation of *p53*, *p21^{WAF1}*, *ATM*, *cdc25A* and *Rb* (Table 1) should induce cell arrest at the G₂/M checkpoint, as observed in the experiments (Figure 4). From this point onwards, the transcription profiles of *p53*, *Rb*, *p16*, *p21^{WAF1}* and *N-Ras*, whose gene expression was altered by daunorubicin (Figure 2 and Table 1), might be pivotal in the observed drug-induced accumulation in G₂, senescence-like growth arrest (Figure 5) and the control of the cell programme and subsequent apoptosis, in agreement with previous reports [14,18,31,38,39,41]. In contrast with IC₇₅ for daunorubicin (Figure 6, left panel), *cyclin E* was down-regulated, whereas *p16* was up-regulated (Table 1). The resulting loss of *cyclin D3/cyclin E* activity would induce cell arrest in the G₁ checkpoint, thereby causing immediate apoptosis. We propose that the main cause of this differential regulation is the dose-dependent DNA damage, which is known to induce cell-cycle arrest at the G₁ checkpoint [16,32,34]. DNA damage is known to stabilize the p53 protein [34], thus facilitating progress through apoptotic pathways [26]. In addition, destabilization of the p53 protein may induce cells under transient halt in the G₂ checkpoint to re-enter the cell cycle [18,22], committing themselves to apoptosis (Figure 5E).

Previous studies have suggested the sphingomyelin signal-transduction pathway in daunorubicin-induced apoptosis [42]. It

has been suggested that daunorubicin and TNF α induce ceramide accumulation [43]. However, these authors used cells treated with much higher concentrations than those employed here; thus, the alterations they observed in the sphingomyelin-signalling pathway [43] are not comparable directly. We noted that some of the transcription gene profiles of this pathway varied according to daunorubicin concentration (Figure 2), in agreement with the hypothesis that daunorubicin affects both apoptotic and survival pathways [42]. The *AP-1* gene that is involved in apoptosis is up-regulated at all drug concentrations, whereas a member of the NF- κ B family (*Rel A*), which belongs to a pathway that promotes survival, is only down-regulated at 91 nM daunorubicin (Figure 2).

There are experimental grounds to consider that other cell lines suffer non-apoptotic death and senescence in the presence of the anthracycline doxorubicin [44], and it has been suggested that the molecular signature of doxorubicin resistance is the expression of certain genes [12]. Daunorubicin, doxorubicin and other DNA-binding drugs are considered as topoisomerase II inhibitors [3,25,35]. Therefore the possible link between the changes in transcription after drug treatment and the introduction of DNA breaks should be further studied. DNA damage was evident only for the higher daunorubicin concentration (IC₇₅; Table 2). The higher DNA damage observed with 182 nM daunorubicin suggests that changes in transcription gene profiles, together with DNA damage, are fundamental to the rapid activation of the apoptotic response. Correlation between DNA damage, cell survival and drug exposure agrees with previous observations using doxorubicin [35]. Daunorubicin concentrations used here are, in general, lower than those used routinely to assay

topoisomerase II activity (see [3] and references therein). Results on induced DNA damage (Table 2) do not rule out that some topoisomerase-dependent gene-specific damage is involved in daunorubicin or doxorubicin action at low drug concentrations [25,45]. In any case, the intercalation of daunorubicin into DNA seems to be required for drug-inhibiting activity [45].

The elucidation of transcription profiles in tumour cells that undergo drug-induced apoptosis, or alternative cell death pathways, should provide potential strategies for diagnostics and therapeutic modulation of the antiproliferative response in cancer treatment. A known limitation of the study of the pharmacological effects of a drug using arrays is that the effects of a drug may not always be reflected at the transcription level [15]. However, the association found between daunorubicin concentration, differential gene transcription profiles, cell-cycle distribution and ultimately apoptotic death cannot be fortuitous. Up-regulation of *p53* may play a primary role in the apoptotic response to daunorubicin at any concentration. Daunorubicin might act by blocking the binding of some protein factors to DNA *in vivo*, as suggested using the yeast *Saccharomyces cerevisiae*, in which daunorubicin inhibits gene activation by transcription factors whose putative DNA-binding sites also contain drug-binding sites [46]. Mechanisms other than daunorubicin binding to DNA should be considered to understand the induced cell arrest and apoptosis, either direct, as in topoisomerase II-mediated cleavage of DNA, or indirect through changes in the genes that regulate the cellular checkpoints. Nevertheless, in a recent study aimed at detecting changes in transcription levels in the presence of several drugs [15], all the anthracyclines clustered together, although in a distinct position from other molecules classified usually as topoisomerase II inhibitors, suggesting that topoisomerase II is not the main target for low (clinically relevant) concentrations of anthracyclines.

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