ANEXO II

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Occurrence of DNA Sequences Specifically Recognized by Drugs in Human Promoters

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Abstract

Several DNA-binding drugs are being developed to create tailored molecules which can discriminate among the different sequences of the whole genome. By discriminating among specific sites in DNA, these molecules may provide optimal drug therapy. The complete sequencing of the human genome offers a wealth of DNA targets to be analyzed as potential drug-binding sites. To increase our understanding of DNA-drug interactions and their selectivity, we have studied the relative and absolute occurrence of CG-rich sequences, of various lengths, in human gene promoters. In several promoters, including those of oncogenes, cell cycle regulation factors, tumor suppressors and housekeeping genes, the presence of potential binding sites containing CpG steps (in which many drugs are known to intercalate) is variable, but in many cases these sites are not randomly distributed. Sequences 6-7 base pairs in length, like CGCCCCG or CGCCCC, occur only once in some promoters, thus they may be potentially specific therapeutic targets.

Introduction

The design of small molecules that recognize specific sequences in DNA is considered a reasonable approach to drug development (1-5). DNA-binding compounds can inhibit the binding of several protein factors to DNA and DNA-associated enzymes. Therefore, they may have a therapeutic effect including anti-cancer activity. With these considerations in mind, several groups have devised high-affinity binding ligands (5-8).

Small molecules do not provide ideal DNA-binding drugs for targeting a unique sequence in the genome (9). However, despite their low specificity, many of these ligands are effective antitumor drugs, and they are currently used in chemotherapy (10). There is a clear need to develop molecules that can permeate cells and bind selectively to extended sequences of DNA, thereby ideally regulating the transcriptional activity of the intended genes (11-13). We should consider better to target the DNA responsible for the disease (the ‘pathogenic genes’) for example by controlling their expression at the transcription level, and not the downstream effects.

The sequencing of the human genome has brought with it a wealth of information about potential DNA targets. In living organisms the recognition of the sequence information on DNA involves the binding of proteins to specific control regions in genes. Therefore, any drug aimed to disrupt a DNA-protein complex must compete to bind to DNA in order to exert its therapeutic effects. Consequently, the control of gene expression at the transcription level is a promising field of research and drug development (3, 11, 12).

Some experimental classes of drugs are currently being investigated to ideally cre-
ate tailored molecules that can bind to specific sites in the whole genome, with both high affinity and sequence discrimination. However, the number of drugs that recognize more than about four base pairs is still limited (6). Many approaches to targeting DNA involve the extension of molecular interactions in the minor groove, and have produced a new class of DNA binding compounds, the lexitropsins or polyamide dimers (14–16). It has recently been shown that some unfused aromatic dications can also recognize a DNA target in a strong-highly-cooperative mode and permit both GC and AT base pairs in the binding site (4).

A different, yet complementary approach known as “Modular Design Approach” is based on the incorporation of distinct classes of ligands into a single molecule. This process involves identifying existing motifs that bind to DNA, assessing them for sequence-selectivity, and assembling them with other created motifs to construct tight-binding ligands (17). This approach, considers not only the structures of related complexes/molecules, but also the thermodynamics and kinetics of complex formation. Following this design strategy, and using monomeric anthracyclines, bisintercalating molecules that occupied a greater number of base pairs have been designed and synthesized, thus attaining a stringent recognition relative to the monomer (2, 18). A major advantage of the modular design, as made for some bistintercalating anthracyclines, is the simplification of structure-based design process. For example, this strategy produced a DNA-binding drug, bisanthracycline WP631, which displays a very tight binding affinity, and it has a binding constant close to that of many DNA-binding protein factors (2, 19).

At first glance, it could be argued that the development of intercalating drugs to target single sites in the whole genome is hampered by the fact that their chromophores only interact with two base pairs (9). Although there is a clear preference of almost all known intercalating moieties for CpG steps in DNA (20, 21), the well-described intercalating moieties can be used to design drugs to bind more favorably to GC-rich sequences in gene promoters.

Drugs that bind to CpG-containing tracts in DNA can be used to gain additional discriminatory properties for the intended therapeutic drug. Because intercalating chromophores are ‘exquisitely’ limited in selecting location, the binding to erroneous sequences should be negligible. In this way, the synthesis of polyintercalators is advantageous because they reduce the number of wrongly recognized sequences, and could create molecules of sufficient length to target unique sites in the human genome. Studies of bisintercalator and polyintercalator DNA-binding ligands has provided us with some promising results (2, 6, 20, 22). There are grounds to consider that variation in the transcript level of several genes may be related to the mechanisms of drug sensitivity (23). Therefore, targeting the promoter regions of genes involved in the development of cancer can be regarded as axiomatic (1).

Here, we analyzed the occurrence of CG-rich sequences expanding from two to up seven-eight base pair length in the binding site. We have sought to verify the frequency with which potential drug-binding sites, in which intercalation would take place, are found in human genome.

Given the sequence preference binding observed for these molecules, we analyzed CpG-containing DNA sequences in 26 human gene promoters. We also defined whether increasing the length in the reading frame provided unique targets. Conceptually, our study could be used to provide guidelines for future efforts to develop sequence-specific CG-binding agents with improved biological activity.
Materials and Methods

Human proximal promoter sequences.

The proximal regions of 26 promoters were used to analyze the availability of potential binding sites containing the CpG step. Intercalation of DNA-binding drugs mainly occurs in CpG-containing tracts (6, 20).

To avoid bias in the selection of promoters, we used two complementary approaches. We selected genes that participate in distinct aspects of cell behavior, and that may be relevant in cancer control and development. We considered promoters of genes related to: apoptosis, cell cycle regulation, tumor suppression, plus a housekeeping gene, (glyceraldehyde 3-phosphate dehydrogenase (GAPDH)), commonly used as a control in gene expression analyses. We also analyzed an unrelated satellite DNA (24) and a 1000-bp random DNA sequence. We did not choose the promoters beforehand, but considered some of those which correspond to the genes available as components of the Atlas-Nylon Human Trial Array (Clontech Laboratories, Palo Alto, CA). The promoters analyzed, and the function of their gene related products, are indicated in Table I.

Table I

<table>
<thead>
<tr>
<th>PROMOTER</th>
<th>GENE FUNCTION</th>
<th>ACC. NUMBER</th>
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</thead>
<tbody>
<tr>
<td>c-myc P1</td>
<td>oncogene</td>
<td>EP11146</td>
</tr>
<tr>
<td>c-myc P2</td>
<td>oncogene</td>
<td>EP11148</td>
</tr>
<tr>
<td>abl-1</td>
<td>oncogene</td>
<td>EP11148</td>
</tr>
<tr>
<td>BRC2</td>
<td>oncogene</td>
<td>EP11148</td>
</tr>
<tr>
<td>erb-b3</td>
<td>oncogene</td>
<td>EP11148</td>
</tr>
<tr>
<td>jun</td>
<td>oncogene</td>
<td>EP11148</td>
</tr>
<tr>
<td>p53</td>
<td>tumor suppressor</td>
<td>EP11148</td>
</tr>
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<td>Rho1</td>
<td>tumor suppressor</td>
<td>EP11148</td>
</tr>
<tr>
<td>bcl-2 P2</td>
<td>apoptosis</td>
<td>EP12007</td>
</tr>
<tr>
<td>bax</td>
<td>apoptosis</td>
<td>EP12007</td>
</tr>
<tr>
<td>bhk</td>
<td>apoptosis</td>
<td>EP12007</td>
</tr>
<tr>
<td>bcl-x</td>
<td>apoptosis</td>
<td>EP12007</td>
</tr>
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<td>caspase 3</td>
<td>EP12007</td>
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<tr>
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<td>cell cycle regulation</td>
<td>EP12007</td>
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<td>cyclin G1</td>
<td>cell cycle regulation</td>
<td>EP12007</td>
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<td>cyclin A</td>
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<td>EP12007</td>
</tr>
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<td>CDK10</td>
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<td>EP12007</td>
</tr>
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<td>cdc25A</td>
<td>cell cycle regulation</td>
<td>EP12007</td>
</tr>
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<td>cell cycle regulation</td>
<td>EP12007</td>
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<tr>
<td>JAK1</td>
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<td>EP12007</td>
</tr>
<tr>
<td>TGF</td>
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<td>EP12007</td>
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<tr>
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<td>EP12007</td>
</tr>
<tr>
<td>DHFR</td>
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<td>EP12007</td>
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<tr>
<td>GAPDH</td>
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</tr>
<tr>
<td>Psp. s.</td>
<td>satellite DNA</td>
<td>EP12007</td>
</tr>
</tbody>
</table>

(a) Accession number corresponding to the different promoter/gene banks, as described in Materials and Methods.
(b) An unrelated A+T-rich satellite DNA of the beetle Pimelia sparsa sparsa used as a control.

Promoter sequences were retrieved, when available, from the eukaryotic promoter database (25), which contains an annotated collection of RNA polymerase II promoters (accessible at: http://www.epd.isb-sib.ch); while other promoters were retrieved from GenBank (http://www.ncbi.nlm.nih.gov). The remaining promoters were obtained from the Human Genome Server (Project Esembl, the Sanger Center; http://www.ensembl.org), from which the desired promoter regions were hand-copied directly. The lengths of the regions analyzed varied because of the inherent difficulties in accessing sequences of longer upstream fragments, or because of ambiguities (i.e., partial sequence availability for some promoters in the databases). Table I shows the accession numbers for all promoters.

Analysis of base content and frequency of the different sequences.

The promoter sequences were saved in plain text format using Microsoft Word 98
Calculations were made on the frequency of occurrence of the different sequences in DNA in actual human gene promoters. In this way, we determined the occurrence of certain sequences without assuming that the overall sequence may be treated as chemically random, as classically undertaken in DNA-protein interactions (26, 27). We studied the four types of base-pairs in DNA, which could be read by a drug through the minor groove, and thus a particular tract can be recognized directly (28).

Nonetheless, for the sake of comparison, we also calculated the theoretical length of DNA that could be recognized as a unique sequence of a human cell. A random distribution of base pairs was assumed in the promoter (26, 27, 29) and the expected frequency calculated.

If we consider that the overall base composition is random (A, C, T and G equally represented) the probability of a particular base occurring at a certain position (Pt) can be defined as:

\[ Pt = Pn \times N \]

where \( Pn \) is \((0.25)^y\), in which \( y \) corresponds to the number of bases in the binding site. In the human genome \( N \) is around \( 3 \times 10^9 \); or \( N \) takes the value of the length, in nucleotides, of any of the promoters studied.

When the two central bases were unambiguously defined, as, for example, in CGCCCG, this involves the following calculation:

\[ Pt = Pn \times N = (0.25)^6 \times N \]

(for \( N = 968 \) Pt is approx. 0.2; for \( N = 501 \) Pt is approx. 0.1 — i.e., twice in about 10 000 bases —; and for the whole genome it should occur around 7.3 x \( 10^5 \) times).

However, for a random distribution, the probability of occurrence of the hexanucleotide CGNNCG (in which \( N \) can be any of the four bases) was defined as:

\[ Pt = Pn \times N = (0.25)^4 \times N \]

in which \( y = 4 \), because the probability of \( N \) (any base) equals 1.

(for \( N = 968 \) Pt is approx. 4; for \( N = 501 \) Pt is approx. 2; and for the whole genome the sequence should occur \( 3.5 \times 10^7 \) times).

Hence, a binding site of 15-16 bases would be required for any sequence to be randomly distributed, because:

\[ Pt = Pn \times N = (0.25)^{16} \times 3 \times 10^9 \approx 1 \]

These calculations were used to easy comparisons with the frequencies observed (see below). Therefore, no correction was performed on the fact that in high eukaryotic cells the A+T fraction represents about 60% of base composition (29). (Indeed, since a sequence can occur in either DNA strand, the probability of finding it should be multiplied by a factor of 2. However, since our theoretical approach was mainly on the presence of inverted repeats like CGNNCG, we only analyzed one of the strands, because these sites are equivalent for both strands).
Although, our calculations of the expected frequencies of any tract can be considered somewhat simplified (27, 29), it was a useful approach for comparisons with the actual sequences observed in the distinct promoters. Here we took specificity to be the occurrence of a number and composition of certain bases (for example the presence of CpG steps) required to define a unique binding site in gene promoters.

We also searched for potential binding sites for the Sp1 transcription factor, which recognizes a CG-rich sequence in promoters (30), using ‘TESS: Transcription element search software on the www’ available at: http://www.cbil.upenn.edu/tess (Match filters: 10% maximum allowable mismatch, 10 minimum element length and 12.0 minimum lg-likelihood).

Results

Table II displays the frequency of occurrence of CG-containing DNA tracts of different lengths in several promoters. Among them there is the housekeeping gene GAPDH, commonly used as a control in gene transcription experiments in vivo. For the sake of comparison, this table also presents a 1000-bp random DNA sequence, and the satellite DNA unit of Pimelia sparsa-sparsa (24) as a model of an unrelated-AT-rich DNA sequence.

Table II

<table>
<thead>
<tr>
<th>PROMOTER</th>
<th>length analyzed (bp)</th>
<th>% CG</th>
<th>CG</th>
<th>CGNNCG</th>
<th>CGCCC</th>
<th>CGGGCC</th>
<th>CGCGCG</th>
<th>CGGGGCG</th>
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<td>0</td>
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<td>0</td>
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<td>1</td>
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<td>9</td>
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<td>cyclin D1</td>
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<td>37</td>
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<tr>
<td>GM-CSF</td>
<td>501</td>
<td>54.0</td>
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<td>0</td>
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<td>Pimelia sparsa sp. satellite DNA</td>
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</table>

The frequency of the sequences in the promoters was not uniform. Figure 1 shows eight panels corresponding to the frequencies with which some of the analyzed CG-rich tracts occurred. The promoters were arranged in panels mainly according to their functions, as described in Table I. All promoters contained one or more CpG steps, in which an intercalator can be accommodated (20). Frequencies were compared with those expected for a random distribution (see Materials and Methods). Panels A, C, E and G show the clustering of promoters according to their basic function in a living cell (Table I). Panels B, D, F and H are equivalent to the other four panels but show the occurrence of a CGNNCG in one strand of the deoxyribonucleic acid. We calculated whether the frequencies observed were deviated from the random distribution. We also analyzed the cases in which the defined
Figure 1: Column graphs showing the frequency of occurrence of the CpG step (panels A, C, E and G) or the CGNNCG tract (panels B, D, F and H) in the proximal region of several human promoters. (□): observed frequency, (■): calculated frequency, performed as described in Materials and Methods. A and B: oncogene promoters plus GADPH, and a random DNA sequence. C to F: genes involved in signal transduction plus an unrelated satellite DNA from Pimelia sparsa sparsa. G and H: gene tumor suppressors and genes involved in apoptosis. Further details on the different promoters are indicated in Table I.
sequences were unique for a determined promoter, as well as the likely presence of such sites in the complete genome.

As expected (9, 27, 29), the observed frequencies of the different GC-rich tracts did not show an average distribution of the four nucleotides. Furthermore, in several promoters the 6 bp-long sequence CGNNCG, which contains an ambiguous/undefined NN, was present once or twice only, or it was even absent (see, for example, the BRCA2 (Figure 2A), GM-CSF and cdc25A promoters (Table II)). In contrast, the promoter of jun oncogene (31) contained the sequence eleven times (Figure 1B). Another example of clear deviation between observed and expected frequencies in CG-rich tracts was provided by the MAPK4 promoter (Figures 1E and F). This is the promoter of a kinase that is activated in response to the stimulation of cell growth differentiation, among other stimuli (32). In the random sequence, the frequency of CGNNCG was slightly higher than the expected random distribution, whereas in the AT-rich satellite of P. sparsa the frequency of CpG was significantly lower, as expected from the low percentage of cytosines plus guanines (Table II). It is worth mentioning that these results reveal that neither the random distribution, nor any general mathematical model (26), can reliably reflect the occurrence of CpG-containing sites in promoters.

Our results also allowed us to compare the effect of a more concrete definition of the nucleotides in the central part of CGNNCG. In this context, Table II shows the frequencies observed for the CGCCCC and CGGGCG tracts. This major accuracy in the study of the occurrence of different sequences in the center of the binding site provided a clear example of how a mismatch in the reading frame (i.e., the absence of the ability in a drug binding to DNA to discriminate between the four bases) reduced the sequence selectively, as it may also reduce the total affinity of the drug (33). In this respect, an outstanding observation was that the eleven 6 bp-long sites in the jun promoter (Table II and Figure 1) were reduced to two when a central GG was considered. The eight CGNNCC sites found in the erb-b3 promoter, whose product is involved in the pathogenesis of some malignancies in breast cells (34), became only one CGCCCC, because a better recognition-specificity was achieved for the intermediate nucleotides (Table I and Figure 1).

An illustrative example of the possibilities offered by a bisintercalating molecule that expands about six base pairs, is provided by the cyclin D1 promoter (35). The 37 occurrences of a CpG step (Table II and Figure 1C) became 3 CGNNCG tracts (Figure 1D), but a better definition of the central nucleotides in the binding site within the proximal 500-bases of its promoter built up a unique CGGACG, and CGGGCG was not present, while the longer CGGGGCG occurred once.

Another example of the extent to which the definition of the central two bases can be fundamental in discriminating ability was provided by the bax promoter. This is the promoter of a gene that is involved in tumor development because its overexpression reduces apoptotic death (36). In the bax promoter CGCCCC and CGGGCG occurred once. Indeed, a larger CGGGGCG tract was still found once. The well-characterized breast cancer susceptibility gene BRCA2 (37) is another example of a promoter with a unique CGTGCG (Figure 1F and Table II). Additional examples of how CpG intercalating drugs that recognize a 6-bp sequence might discriminate for unique sites in promoters are shown in Table II. The oncogene abl-1, which is involved in the development of chronic myelogenous leukemia (38), contained six CGNNCG sequences in the 1000 bp, upstream from initiation site. If the sequence were represented randomly, we would expect to find it four times (Figure 1B); however, only one CGCGCG was observed.

In living cells, many promoters contain Sp1 binding sites (25, 30). This protein recognizes a CG-rich DNA sequence and is involved in the transactivated transcription of some genes (39). The bisintercalating anthracycline WP631 has provided
previous experimental evidence that a drug binding about 6 bp can strongly inhibit the Sp1-activated transcription in vitro (12), by direct interaction with the Sp1-binding site. Targeting of Sp1-binding sites could be of potential therapeutic interest (19). Some of the genes studied which contain CGNNCG sites in their promoters present an Sp1-responsive element (determined as described in Materials and Methods). This is the case for the abl-1, BRCA2, erb-b3, cdc25A and jun promoters. Figure 2 shows the sequence of the proximal region of three of these promoters, in which some prominent CG-tracts, and putative Sp1 binding sites, are indicated.

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- 934  CGAA  TCCTAAAGAAT  GCAAAAGATG  GGCTGAGTG  GTGCTTGCTA  GCTCTGAAATC  -881
- 880  CGAGGGCGG  GGGAGAGACG  CAATGGGAG  -801
- 800  CGGTCTCTCT  TTAATATG  AAAGTGACG  ATGCAGGTG  CGGGATGCT  -721
- 720  CGAGGGGAC  AGCCGCTAC  -641
- 640  CGGGCGG  ATGAGCTG  -561
- 560  TTGAGGAG  TGCCACAGT  GAAGAGGT  -481
- 480  ACTGGAAC  ATAGTTCTAG  ATGGAGCTAG  AGGGAGTTT  TAAGGATG  -401
- 400  CCATCTAG  TTTTGGTAT  ATAGGGTCT  TGTTGAGT  GAGACACTCC  -321
- 320  TTATCTAG  GCCTAGGTC  GTCTCCGGAG  GGGGCCCACC  CAAGAGCTG  -241
- 240  GAGTGGACG  TAGGGGCT  CCCCACAGT  -161
- 160  GCTCCGG  TCTCTTTCTC  TAAGGATG  TGCCACAG  CACCCCAAGG  -81
- 80  CGGGGCT  CCCAGGCTCT  CCGGGTCGT  GTGGTCCGTG  AGAGGAGCT  -1

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- 949  CGGGGGGG  GCCCTGGCTG  -481
- 899  CGGTGGGAG  AGCTAGAGG  CGGGAGGAG  TCAGCGCTG  -401
- 840  GAGGGCCCT  CGGGGCT  TCTCTCTCT  TGCTGAGGG  -321
- 720  CCCCTGGCTG  GGGGAGGAG  TCAGCGCTG  -241
- 560  CGGGGCT  CCCCTGGCTG  GCCCTGGCTG  -81
- 80  TTGAGGAG  TTGGGAGG  GAGAGGAGGG  -1

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- 934  CAAT  TGCCGGCAT  ATAAAGACGTG  CCCCCTGGCTG  GCAAGAGCTG  -881
- 880  CCAGGGGG  GGAGGGAGG  GTGCTTGCTA  GCAATACAAA  -801
- 800  TTTCCTCTCG  TTCTCTGCTG  GCCTAGGAGG  TAGGGGAGG  -721
- 720  CCAGCTAGG  TGCAGCTG  GGGGGGAGG  -641
- 640  AGAAGAGCG  AGGAGGAGG  -561
- 560  CGGGGAGG  AGCCGCTAC  -481
- 480  CGGGGAGG  AGGAGGAGG  -401
- 400  GGGGAGG  AGGAGGAGG  -321
- 320  GCCTGGGAG  GGGGAGG  -241
- 240  AGAAGAGG  ATAGGAGG  TGCCGGCTG  -1

Mansilla & Portugal

Figure 2: An example of sequences of the proximal region of the human gene promoters analyzed, showing some of the CG-rich sequences, including the putative Sp1 binding sites. (A): BRCA 2 promoter, (B): cyclin D1 promoter, (C): jun promoter. Potential Sp1-binding sites are indicated by bold letters, CGCCCG and CGGGGCG are double underlined and CGGGGCGG is dotted underlined. Other CGNNCG tracts are single underlined. More details on all the promoters analyzed are presented in Table II.
Anti-cancer drugs that bind to DNA are considered to fall short of the ideal of identifying a single target in a genome. However, it seems possible to increase both the binding affinity and the specificity of a DNA binding ligand (5, 6). In the near future it will also be possible to ensure the target accuracy by enhancing the interaction at specific sites (3, 9, 17, 33).

We present the first detailed analysis of the occurrence of drug-binding sites in a variety of promoters, which are known to be involved in cell cycle control and oncogenesis (Table I and Figure 1). Our finding that DNA sequences of 6-7 bp in length can be targeted by specific inhibitors of gene transcription (because of their effect of RNA polymerase readout of a potentially unique promoter) could contribute to the pursuit of novel DNA-binding drugs. This finding is in keeping with other studies that have shown that recognition of about 6-7 base pair DNA tracts may suffice for gene-specific regulation in vivo (13, 40).

For a randomly distributed sequence, around 15-17 base pairs may be required to get a unique binding site (29) (see the suitable calculations in Materials and Methods). Our results show that targeting unique sequences might not always require long DNA-binding ligands. However, it is clear that the chance of any drug to discriminate among DNA tracts is proportional to the length of the sequence. By way of illustration, if a sequence like CGCCCG is found only once in a promoter (Figure 1 and Table II), it should be considered a potential target for therapeutic intervention. However, it is beyond reasonable doubt that this sequence might be represented more times in the genome, so longer sequences may be necessary to bind to a single site in a whole genome (9, 33). Given that the human genome contains around $3 \times 10^9$ base pairs, a CGCCCG tract may occur many more times. Table II shows that it occurs thirteen times in the twenty-six promoters analyzed, yet the frequency varies between the promoters. Moreover, this and other potential binding sites can be buried inside the cell nucleus (9).

Despite that increasing the length of the reading frame should theoretically produce drugs with improved sequence selectivity (29), the achievement of a perfect exactness of binding might not be realistic (26, 33) and longer sequences may not always be better, since accumulated mismatch errors could occur due to difficulties in discriminating between some DNA bases in the minor groove (15, 28, 41, 42). Drug targeting of DNA sequences containing CpG steps has to be useful because of the subtle preference of intercalators for CG-sites, thus avoiding mismatch reading. This exquisite preference could be enhanced by improvement of the minor groove linkers (4, 14, 15, 17), which could be used to link intercalating moieties to produce polyintercalation (22). Nevertheless, other factors such as the structure of DNA at these sequences, and the thermodynamics of the interaction, may be crucial in the recognition (6, 26). The occurrence of CG-rich sequences observed (Table II) is consistent with the possibility that ligands which expand about six-eight base pairs could be enough to target a unique site in a promoter (13, 40). Evidently, the effect may be specific enough, but some pleiotropic effects may still take place.

It seems that optimum targeting to short DNA sequences might be attained both in vivo and in vitro (3, 11, 12, 19, 40). It has been shown that an eight-ring polyamide directly inhibited the expression of a 5S RNA gene in cultured fibroblasts (13). However, there are no reports of the effects on other genes that may contain the binding sequence. Given the size of a genome (about $3 \times 10^9$ bp in humans), it probably contains more than one occurrence of a rather short DNA sequence.

Some bisanthracyclines have been synthesized following the “Modular Design Approach” (17). These drugs show that not only the CpG binding chromophores,
but changes in the linker, might lead to new drugs able to bind tightly to 6-8 bp DNA sequences, with binding constants close to those of some protein factors (2). Further changes in linker composition could avoid some mismatches, and thus increase specificity. For instance, bisantracycline WP631 (2, 18), which binds very tightly to DNA, is likely to follow a sequence preference of CGTACG ≈ CGATCG >> CGGCCG ≈ CGCCCG (42). It is worth noting that the sequence CGA/TT/ACG was not found in any of the promoter regions analyzed in this study.

It is possible to create linkers that can extend the interaction in the minor groove, thereby avoiding the reduction of the binding magnitude, compared to the ideal sequence recognition. The strong capacity of WP631 to inhibit Sp1-activated transcription better than basal transcription supports this assumption (12).

When designing bis- or poly-intercalators to recognize CpG-containing promoters, it must be taken into account that, CG-rich sequences are less likely to be found in human DNA, since CpG steps are underrepresented in eukaryotic organisms (9, 43). This situation therefore favors the use of CG-sites as targets for potential drug treatments. Besides, CG-rich sequences are very unevenly distributed in genomes, including the called CG islands that mark the 5’-end of transcription units (43), and surround the promoters of housekeeping genes.

Improved DNA targeting may also be obtained by building polyintercalators not only using chromophores involved in the classical intercalation at CpG (6, 20), but also assembling different intercalating motifs, such as the chromophore of cryptolepine, which has been found to intercalate into CpC/GpG steps (21). Moreover, improved linkers should be able to expand binding to longer DNA fragments in the minor-groove, while keeping the isohelical nature of the ligand that is required to fit large drugs inside the DNA grooves (7). This could be done in shorter sequences without the need to recognize DNA tracts of about 15-17 base pairs in length.

In summary, our results on CG-rich sequences in several human promoters indicate the possibility to tailor intercalating drugs to fit sequences involved in gene regulation. Sequences of 6-7 base pairs in length, like CGCCCG or CGCCCCG, occur only once in some promoters, and may be specific therapeutic targets. However, generalization of sequence-selective binding to the complete genome must be interpreted with care. Targeting the promoter regions of determined genes may be a useful strategy for optimal drug therapy, since variations at the transcription level of particular genes affect the mechanisms of drug sensitivity (19, 23).

**Acknowledgments**

This work was financed by a grant from the Spanish DGESIC (PB98-0469), and the Centre de Referencia en Biotecnologia de la Generalitat de Catalunya.

**References and Footnotes**

Drug Recognition Sequences in Human Promoters

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Induction of G2/M arrest and inhibition of c-myc and p53 transcription by WP631 in Jurkat T lymphocytes

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Abstract

WP631, a new DNA-binding drug that bisintercalates into DNA with high affinity, seems to be highly cytotoxic against Jurkat T lymphocytes. The purpose of this study was to gain new insights into the mechanisms by which WP631 halts proliferation in this cell type. Treating Jurkat cells with nanomolar concentrations of WP631 produced G2/M arrest, inhibited the transcription of c-myc and p53 genes, and induced limited apoptosis during the duration of treatment. Suppression of c-myc and p53 expression, and time-dependent decline in c-Myc and p53 protein levels, was associated with growth arrest. A weak interdependence was also found between the potent antiproliferative activity and the apoptotic response; treatment with WP631 for 24–36 hr produced arrest in G2/M and allowed for partial DNA repair. Longer treatments with WP631 allowed some repaired cells to re-enter the cell cycle, but produced aneuploidy or apoptosis in others. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: DNA binding; G\textsubscript{2} phase; Jurkat cells; c-myc; p53; Polyploidy

1. Introduction

Small molecules that bind with high affinity to DNA and recognize extended sequences are being explored as potential antitumor agents [1–4]. Recent studies on targeting small molecules to specific sequences in DNA have led to the design of novel DNA-binding bisintercalating agents with significantly increased DNA-binding affinity [2,5] and high antitumor activity. One such molecule, WP631 (Fig. 1), was designed on the basis of the structure of DNA–daunorubicin complexes [2]. WP631 shows an ultratight binding affinity for a six base-pair DNA sequence, which is of the same range of several DNA-binding proteins [2,4,6,7]. At nanomolar concentrations, WP631 displaces the Sp1 transcription factor from its binding site in promoters, thereby interfering strongly with the eukaryotic transcription machinery \textit{in vitro} [8]. This is accompanied by a remarkable biological activity. Preliminary studies have established that WP631 is more active than some monoanthracyclines against the breast carcinoma MCF-7/VP-16 cell line, in which it overcomes a specific form of multidrug resistance [2]. It is also more potent than daunorubicin or doxorubicin in inhibiting the growth of Jurkat T lymphocytes [9].

In the present study, we explored the mechanisms that account for the cytotoxicity of WP631. Jurkat cells treated with nanomolar concentrations of WP631 showed accumulation in the G2/M phase and limited apoptotic death over a 72 hr interval. Since susceptibility to drug-induced apoptosis in leukemia cells seems to be regulated by c-myc and p53, among other genes [10], the levels of their mRNAs and proteins were also analyzed to study the possible relationship between WP631, the expression of these genes and cell growth arrest.

2. Materials and methods

2.1. Cell line and culture conditions

Jurkat T lymphocytes were obtained from the cell culture facilities at the Department of Biochemistry of the University of Barcelona, Spain. Cells were maintained in RPMI
with 30 mM HCl in 2-propanol. Absorbance was determined at 570 nm using a SPECTRAMax-250 microplate spectrofluorometer (Molecular Devices). Viable cell number was determined based on the exclusion of Trypan Blue (Fluka) as described elsewhere [12].

2.3. Morphological examination

The morphology of Jurkat cells after drug treatment was analyzed by light microscopy. Smears were prepared by spreading a cell suspension with the edge of a coverslip. The smears were air-dried, stained with 20% Wright-Giemsa and coverslipped. Apoptotic cells were identified by the presence of cytoplasmic shrinking and nuclear condensation.

2.4. Flow cytometry

After treatment with WP631 for various periods, the cells were harvested and stained with propidium iodide (Sigma) as described elsewhere [13]. Nuclei were analyzed with an Epics Elite flow cytometer (Coulter Corporation) at the ‘Serveis Cientifico-Tecnics de la Universitat de Barcelona,’ using the 488 nm line of an argon laser and standard optical emission filters. Cell percentages 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) and flow cytometry in accordance with described procedures [14] using 488 nm excitation and 515 nm bandpass filter for fluorescence detection.

2.5. Analysis of internucleosomal DNA damage

Qualitative analysis of DNA fragments resulting from internucleosomal cleavage in cells undergoing apoptosis was carried out by following procedures described elsewhere [15], with minor modifications. Briefly, 2 × 10⁶ Jurkat T cells treated with WP631—or with daunorubicin (Sigma) for comparison—were lysed in 40 µL of a buffer consisting of 10 mM Tris–HCl (pH 7.4), 10 mM NaCl, 1 mM EDTA, 0.5% SDS and 0.5 mg/mL proteinase K for 1 hr at 50°C. Thereafter, 1 µL of 1 mg/mL RNAse A was added and incubated for 2 hr at 50°C. The high molecular weight DNA was precipitated by addition of NaCl up to 1 M. After centrifugation, at 500 g for 30 min, the supernatants were collected by ethanol precipitation and the pellets resuspended in 50% glycerol containing 0.02% bromophenol blue. Samples were analyzed by electrophoresis in 1.8% agarose gels, stained with 0.5 µg/mL ethidium bromide, and photographed under UV light.

2.6. Northern blot analysis

Total RNA was isolated from WP631-treated and control cells (those to which no drug was added) by using the

Fig. 1. Chemical structure of WP631.

1640 medium (GibcoBRL); supplemented with 10% fetal calf serum (GibcoBRL) and 2 mM l-glutamine (GibcoBRL), at 37°C in a humidified atmosphere with 5% CO₂.

A 500 µM stock solution of WP631 was prepared with sterile 150 mM NaCl, maintained −20°C, and brought to the final concentration with RPMI 1640 medium just before use.

2.2. Growth arrest and cell death assays

The capacity of WP631 to interfere with the growth of Jurkat cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye assay [11] in 96-well microtiter plates (Coming Costar) with flat-bottomed wells, in a total volume of 100 µL. Cells sub-cultured at a density of 5 × 10⁴ cells/mL were incubated with various concentrations of WP631 at 37°C for 72 hr. Upon completion of the incubation, MTT (Sigma) was added to each culture (15 µL per well). The dark-colored crystals that were produced by viable cells were solubilized
UltraspecRNA isolation reagent (Biotexc) according to the procedure provided by the vendor. RNA samples were denatured in 0.02 M 3-(N-morpholino)propanesulfonic acid (pH 7.0) containing 2.2 M formaldehyde and 50% formamide, and transferred to a Hybond-N+ membrane (Amersham Biosciences). Radiolabeled probes for human c-myc exon 2, p53 (kindly provided by Evelyne May) and GAPDH c-DNA were prepared by using Ready-to-Go Labeling Beads (Amersham Biosciences) and [32P]dCTP. The blots were hybridized with the labeled probes and washed under stringent conditions before autoradiography [16]. Signal intensities were quantified in a Molecular Dynamics densitometer and normalized using the GAPDH probe as reference.

2.7. Western blot analysis

Protein was extracted from WP631-treated and control cells, at the indicated times, with a lysis buffer consisting of 50 mM Tris–HCl (pH 8), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.1 mM phenylmethylsulphonyl fluoride, containing protease inhibitors. Total protein was quantified by the Bradford assay (Bio-Rad). Denatured proteins (30–50 µg per sample) were subjected to electrophoresis on SDS-polyacrylamide gels (12% for c-Myc and p53 and 10% for actin), blotted onto Optitran BA-S85 membranes (Schleicher & Schuell), analyzed with antibodies (purchased from Sigma and Oncogene), and detected by chemiluminescence using luminol (Sigma). Signal intensities were quantified in a Molecular Dynamics densitometer and normalized using actin as reference.

3. Results

Over the course of a 72 hr drug treatment, WP631 produced a concentration-dependent decrease in cell growth. Its IC50 (drug concentration required to inhibit cell growth by 50%), determined from dose–response curves, was 18 nM, and its IC75 (drug concentration required to inhibit cell growth by 75%) was 60 nM, in agreement with our previous findings with Jurkat cells [9]. We thus aimed to gain new insights into the mechanisms followed by WP631 to halt cell proliferation. Morphological changes observed after that Jurkat cells were exposed to 60 nM WP631 for 72 hr (Fig. 2) included chromatin condensation and nuclear fragmentation corresponding to apoptotic cells. Although these effects were pronounced, they affected only a limited number of cells (Fig. 2A).

Cells treated with WP631 were also analyzed by flow cytometry and double staining with propidium iodide and Annexin-V-fluorescein (Fig. 3) to distinguish necrotic cells (propidium iodide staining) from the translocation of phosphatidylserine to the outer layer of the plasma membrane (Annexin-V) [14]. The total amount of apoptotic plus necrotic cells was rather small, especially in light of the low WP631 concentration required to stop cell growth. They represented, through the total period of treatment, about 5–10% of cells. The number of apoptotic cells was between 4% at 24 hr (Fig. 3B) and around 8% at 72 hr. Continuous exposure to WP631 did not produce a significant change in the number of cells suffering necrosis or apoptosis, compared to the control. However, after 72 hr treatment the double staining (propidium iodide plus Annexin-V) uncovered the presence of an additional 2% of apoptotic cells, which were not evident by using the less sensitive flow cytometry analysis with propidium iodide staining only.

Ethidium bromide-stained agarose gels (Fig. 4) showed internucleosomal DNA fragmentation, a hallmark of apoptosis, in cells treated with WP631 or daunorubicin. Therefore, from a qualitative point of view, WP631 did induce some apoptotic cell death in Jurkat cells. However, the results presented in Fig. 3 suggest that apoptosis was infrequent in the total population of quiescent cells after WP631 treatment.

Flow cytometry was used to evaluate the effect of WP631 on the cell cycle distribution. In the absence of WP631 (Fig. 5A), the average distribution of the lymphocytes corresponded to 37.1% G0/G1, 28.3% S phase, and 33.5% G2/M, with a 1.1% sub-G1 peak. After 32 hr of drug treatment (Fig. 5B) the distribution was 29.7% G0/G1, 17.7% S phase, and 42.6% G2/M, with a 10% sub-G1 peak. These values are similar to those previously obtained at 36 hr [9]. The effects of treatment with WP631 for up to 36 hr on G2 accumulation are shown in Fig. 6A. Thereafter, the proportion of cells in G2 became similar to that of the

Fig. 2. Effect of WP631 on the morphology of Jurkat T lymphocytes. (A) A 20% Wright-Giemsa stained preparation. Morphological changes include condensation of chromatin, nuclear fragmentation, membrane blabbing, and formation of apoptotic bodies; (B) a magnified cell (2.5×) showing details of the apoptotic bodies after 72 hr treatment with 60 nM WP631 (IC75).
untreated control cells. Fig. 6B shows the influence of WP631 on the viability of Jurkat cells, measured by Trypan Blue exclusion, at different times, together with the analysis of cell proliferation using MTT assays, which measures the antiproliferative effect of WP631 (i.e. cells which are not metabolically active). These experiments showed that the IC50 of WP631 produced low growth inhibition over 32–36 hr treatment, followed by a decline in the number of proliferating cells (empty bars in Fig. 6B) reaching a 25% at 72 hr. However, the same time-dependent proportion number of non-viable Trypan-stained cells did no decrease much (filled bars in Fig. 6B). Treatment with WP631 for 62–72 hr-induced polyploidy (Fig. 7) whereas untreated Jurkat cells maintained a nearly uniform distribution of cells in each phase of the cell cycle. The number of apoptotic and necrotic cells at 72 hr could not be quantified in this experiment directly. In fact, apoptotic G2 phase cells exhibit a reduced DNA content, which could overlap the content of G1 cells. Nevertheless, they were calculated as about 12% by double staining with propidium iodide and Annexin-V-fluorescein.

We treated exponentially growing Jurkat cells with WP631 and analyzed the expression of the oncogene c-myc because the c-Myc protein targets genes are involved in cell growth, apoptosis, and in arrest at both the G1 and G2 checkpoints [17,18]. We also analyzed the transcription of the p53 gene, because the p53 protein is essential in the apoptotic response to many drugs [19], and mediates apoptosis by preventing the proliferation induced by oncogene activation [20]. Northern blot analyses (Fig. 8)
showed that the exposure of Jurkat T lymphocytes to WP631 produced a time-dependent decrease in both c-myc and p53 mRNA levels by about 80% after 3 hr of treatment (Fig. 8B), whereas the expression of the housekeeping GAPDH remained almost unaltered. Reduction in c-myc and p53 RNA levels can prevent apoptosis [20]. The inhibition of p53 mRNA synthesis may also influence G1/S and G2/M checkpoints [21,22] and participate in the appearance of aneuploidy (Fig. 7 and [23]).

Fig. 9 shows the time-dependent reduction on the levels of the c-myc and p53 proteins in the presence of WP631. After about 4 hr of continuous treatment with the bisanthracycline, both protein levels decreased in parallel, although, after 24 hr, the amount of c-Myc was clearly lower than p53 (Fig. 9B). The presence of some c-Myc protein during the first hours of treatment may be required to abate G1 arrest, allowing cells to go to phase S. The rapid decrease in p53 after about 32 hr (slope of the curve in Fig. 9B), and the concomitant materialization of polyploidy, would indicate that p53 played an important role in maintaining the G2 arrest shown in Fig. 6A. These results agree with p53 involvement in G2 arrest [24].

The experiments presented in Figs. 8 and 9 strongly suggest that Jurkat T cells, which were in a transient G2-arrest after treatment with WP631 (Fig. 6A), might

![Fig. 7. Flow cytometry analysis of Jurkat T cells in the presence of 60 nM WP631, after 62 hr of continuous treatment. A peak corresponding to aneuploid cells is indicated by the arrow.](image)

![Fig. 8. (A) A representative Northern blot showing time-dependent reduction in c-myc and p53 expression, but not in the housekeeping GAPDH gene exposed to 60 nM WP631 (c753). (B) Quantitative representation of the time-dependent suppression of gene expression by WP631 in Jurkat cells: c-myc (▲), p53 ( ○ ) and GAPDH ( □ ). Data are the means ± SD for three experiments, normalized for GAPDH expression.](image)
Damage in DNA might be enhanced through the collision between trapped topoisomerase II cleavage complexes and the replication fork, or transcription complexes [26]. Some cell death should appear if cells with unrepaired damage attempt mitosis, thus explaining the marginal presence of apoptotic cells (Fig. 3). The accumulation of Jurkat cells in G₂ until about 36 hr treatment (Fig. 6) might also be connected with the capacity of p53 to maintain growth arrest in G₂.

We observed the presence of aneuploid cell changes that occurred during still longer treatments. Notably, daunorubicin causes higher apoptotic cell death in Jurkat T cells in similar experimental conditions [27]. G₂ arrest is considered an effect of DNA-binding agents because cells engaged in cell division are particularly susceptible to DNA damage [15,28,29]. This arrest would demand p53 activity [29]. Protein levels decreased in a time-dependent manner, and, after about 32–36 hr (Fig. 9), the cells that elicited G₂ block without fully restored DNA integrity would result in the observed polyploidy (Fig. 7). In the present study, we report a time-dependent decrease in p53 protein, which was followed by polyploidy at longer periods of treatment. A result that agrees with the correlation that has been established between loss of p53 and genetic instability [30]. These findings are consistent with other reports that low levels of p53 render cells more susceptible to gene amplification and the development of aneuploidy [23,31]. Prolonging the G₂/M transition would permit some cells to repair DNA and complete their replication [32]. Our results substantiate that several cytostatic drugs perturb the cell cycle distribution and produce changes in DNA content, which lead to a remarkable increase in cell mass and ploidy [29,30].

Reduced c-myc expression occurs in response to drugs that produce mainly G₂ arrest, but it is the inhibition of p53 gene that mainly determines the extent of growth arrest and apoptosis [21,22]. WP631 clearly decreased the levels of p53 mRNA. The decrease in the levels of c-Myc protein after treatment with WP631 possibly affected the G₁ checkpoint, a result that is in keeping with the low apoptosis detected (Fig. 3). Jurkat cells failed to arrest in G₁. The absence of G₁ arrest in Jurkat cells, despite the inhibition of c-myc transcription after 2–4 hr treatment, can be explained by the presence of previously synthesized c-Myc protein (cf Figs. 8 and 9). Notwithstanding, the absence of G₁ arrest can also be due to other defects in checkpoint function, as it has been described in other cell lines in the presence of certain drugs [33].

The refractoriness of MCF-7 cells to apoptosis induced by doxorubicin [12,34] or WP631 [9] and the limited apoptosis observed in Jurkat T cells after 72 hr treatment agrees with finding that the transcription of c-myc was highly sensitive to nanomolar concentrations of WP631 (Fig. 8). There is a known association between the effects of some drugs on c-myc expression and the response to DNA damage in MCF-7 cells [12,34]. We recently reported

Fig. 9. (A) A representative Western blot showing time-dependent reduction in c-Myc and p53 levels, but not in the actin levels, in Jurkat T cells exposed to 60 nM WP631. (B) Quantitative representation of the time-dependent decrease in protein levels by WP631, as percent of protein at time 0, c-Myc ( ), p53 ( ). Data are the means ± SD for two independent experiments, normalized for the levels of actin.

overcome the G₂ checkpoint when p53 levels clearly decreased after about 32 hr. This resulted in polyploidy (Fig. 5), with only about 5% death cells, but 75% of quiescent cells measured using the MTT proliferation assay (Fig. 6B).

4. Discussion

Strong transcription inhibitors like WP631 [8,9] can induce quiescence in growing Jurkat cells at rather low concentrations and can inhibit the transcription of several genes such as p53 and c-myc, which are associated with cellular proliferation and are also important in apoptosis.

WP631 is a strong growth inhibitor of some tumor cells in culture [2,9]. The apparent cell growth inhibition in our study, measured by MTT assay, resulted from several factors. Not all Jurkat T cells underwent apoptosis after treatment with WP631 over a 62 hr period, but rather underwent either immediate or delayed growth cessation, which was accompanied by an increase in the DNA content per cell. The cytotoxic effects of WP631 in Jurkat cells could be accompanied by other additional events like DNA damage, which is frequently linked to G₂/M arrest [12,25].
a diminution in c-myc RNA levels, without apoptosis, in MCF-7 cells as a result of WP631 treatment [9]. However, internucleosomal DNA fragmentation was observed in Jurkat cells (Fig. 4). Since the synthesis of p53 mRNA was also strongly inhibited by WP631 in a time-dependent manner, the limited apoptosis in Jurkat cells during the first 12–24 hr of treatment could have resulted from storage of the protein in the cytoplasm, as detected by Western blot analysis (Fig. 9), or through a different mechanism independent of p53. Hence, WP631 should directly produce cell cycle arrest in G2 (quantified in Fig. 6). An effect that may be enhanced by the indirect effect of p53 on the down-regulation of c-myc. The presence of limited apoptosis during the 72 hr of continuous exposure to the drug is not at variance with the presence of active p53-independent apoptotic pathways in Jurkat cells [35].

In summary, we have found an apparent interdependence between the low c-myc and p53 RNA levels observed after drug treatment, the time-dependent decrease in c-Myc and p53, and cell cycle arrest. From a quantitative point of view, the main effect of WP631 on Jurkat T lymphocytes is cell arrest at the G2 checkpoint, at least during the first 36–48 hr of treatment, which was followed by polyploidy. These results support, but do not prove, that in Jurkat T lymphocytes, altered c-myc expression by WP631 is directly linked to cell pathways leading to growth arrest. A similar correlation has been described for DNA-binding agents in other cell lines [9,34,36]. Transcription inhibition seems to be more complex in a cell system than in vitro, in which WP631 was a strong inhibitor in basal and Sp1-activated transcription [8]. Since the human c-myc contains a Sp1-responsible promoter ([37], and references therein) cell distribution in the different phases of the cell cycle may be associated with the extend of transcription of the oncogene. Nevertheless, a WP631 effect on the binding of Sp1 to the c-myc promoter in vivo does not imply specificity, since p53 was also inhibited, even though it lacks functional Sp1-binding sites in the proximal promoter [38]. Nevertheless, the inhibition of p53 transcription could be an indirect consequence of the absence of the c-Myc trans-activation of the p53 promoter [39]. Additional genes may be altered in vivo. The use of DNA arrays, together with the determination of the levels of different proteins other than p53 and c-Myc, should provide us with further evidences about the genes ultimately responsible of the antiproliferative effects of WP631. Experiments along these aspects are being undertaken in our laboratory.

Acknowledgments

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References


Promoter-specific inhibition of transcription by daunorubicin in Saccharomyces cerevisiae

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Several anti-tumour drugs exert some of their cytotoxic effects by direct binding to DNA, thus inhibiting the transcription of certain genes. We analysed the influence of the anti-tumour antibiotic daunorubicin on the transcription of different genes in vivo using the budding yeast Saccharomyces cerevisiae. Daunorubicin only affected wild-type yeast strains at very high concentrations; however, erg6 mutant strains (but not pdr1, pdr3 or pdr5 strains) were sensitive to daunorubicin at low micromolar concentrations. In Aerg6 strains, daunorubicin inhibited the galactose-induced transcription by Gal4p in a specific manner, since the transcription of identical reporters driven by other activators (either constitutive or inducible) was not inhibited. The drug concentrations at which Gal4p function was inhibited did not affect cell growth or viability. Furthermore, daunorubicin inhibited the growth in galactose and the transcriptional induction of resident Gal4p-driven genes upon galactose addition, two processes absolutely dependent on Gal4p function. We propose that daunorubicin and some transcription factors compete for DNA sequences encompassing CpG steps, and that this is the main determinant of the effects of the drug on transcription in vivo. Our approach may foster the development of anti-tumour drugs with more specific mechanisms of action.

Key words: anthracycline, anti-cancer treatment, DNA-intercalating drug, ERG6, semi-quantitative reverse transcriptase (RT) PCR.

INTRODUCTION

The anthracycline antibiotic daunorubicin (daunomycin) is widely used in the treatment of cancer [1]. It accumulates in the nuclei of living cells and intercalates into DNA quantitatively [2–4], a property associated with some of the most relevant effects of the drug: inhibition of DNA replication and gene transcription both in vivo and in vitro [5], displacement of protein factors from the transcription complex in vitro [6] and topoisomerase II poisoning [7]. Daunorubicin binds preferentially (but not exclusively) to 5′-WCG-3′ tracts [8,9]; such DNA sequence specificity has led to the suggestion that daunorubicin binding to DNA may interfere with the binding of transcription factors to overlapping recognition sites. This model would explain several effects of daunorubicin, such as inhibition of RNA polymerase II in vitro [10], inhibition of both DNA and RNA synthesis in HeLa cells [11] and suppression of the coordinate initiation of DNA replication in Xenopus oocyte extracts [12]. A similar explanation would apply to the specific blocking of transcriptional activation by other DNA-binding drugs, such as distamycin A [13] or the bisanthracycline WP631 [6].

In this paper we dissect daunorubicin cytotoxicity in a cell system amenable to rigorous genetic analysis, the yeast Saccharomyces cerevisiae, which shares many common regulatory mechanisms with vertebrates, ranging from cell cycle to transcriptional regulation [14]. The main setback to the use of S. cerevisiae cells in pharmacological studies is their resistance to anti-tumour drugs [14,15], which is due to the strict permeability barrier formed by the yeast cellular membrane. Here we tested two genetic approaches to overcome this barrier. First, we used yeast mutants deficient in the multidrug-resistance complex (termed PDR in yeast), which expels a variety of chemical compounds from the cell by a complex system of active membrane transporters [16]. Secondly, we assayed a deletion of the ERG6 gene, which codes for an enzyme essential to the synthesis of ergosterol and that renders cells sensitive to the topoisomerase I inhibitor camptothecin and other drugs [15]. Our data showed that daunorubicin was much more effective in Aerg6 mutants than in wild-type or PDR-deficient yeast strains.

DNA recognition sequences for some yeast transcription factors contain CpG steps (where CpG refers specifically to the sequence 5′-CG-3′), which constitute preferential binding sites for daunorubicin. One of these factors is Gal4p, which recognizes the interrupted palindromic sequence CGGN_p104CCG, known as UASgal [the upstream activation sequence (UAS) of the GAL1-10 gene promoter] [17], which includes two CpG steps. Gal4p is a key yeast transcription factor of several genes involved in galactose utilization, the GAL genes [18]. Expression of Gal4p is repressed by glucose and constitutive when cells grow on other carbon sources, like raffinose. In non-repressing conditions, GAL genes become strongly activated upon galactose addition, in a process that does not require de novo synthesis of Gal4p and that depends on the functional suppression of a specific repressor, Gal80p [18]. The control of GAL genes is a unique system to analyse specific effects on transcriptional activation, since the expression of the main regulator (Gal4p) and its ability to activate transcription can be manipulated separately. Our finding that daunorubicin specifically impaired Gal4p-driven promoters (but not promoters that do not contain CpG steps) may explain the specific inhibitory effects of daunorubicin on the transcription of certain genes in vivo. To our knowledge, this is the first report of a drug with a DNA-sequence-recognition site of only 3 bp that discriminates the cognate DNA sequences of distinct transcription factors.

Abbreviations used: Rap1p, repression/activation protein I; RT, reverse transcriptase; UAS, upstream activation sequence; UASgal, UAS of the GAL1-10 gene promoter.

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MATERIALS AND METHODS

Yeast strains and plasmids

The yeast strain BY4741 (MATa ura3∆0 leu2∆0 his3∆1 met15∆0) and its Δpdr1, Δpdr2, Δpdr5 and Δerg6 derivatives were obtained from EUROSCARF, Frankfurt, Germany. All reporter plasmids are derivatives of pSFLA-178K, a multi-copy 2 µm yeast plasmid that encompasses the CYC1 minimal promoter driving the Escherichia coli β-galactosidase gene [19]. Sequences of the UASs used in this study are shown in Table 1 and the resulting plasmids are described elsewhere [19,20]. In all cases, oligonucleotides encompassing the relevant sequences were inserted into the unique KpnI site of pSFLA-178K. The expression plasmid pH5HE0 contains the human oestrogen hormone receptor HE0 [21] cloned into the constitutive yeast expression vector pAAH5 [22].

Yeast transformation

The wild-type strain BY4741 was transformed following the lithium acetate method [23]. The erg6 mutant, which does not transform easily with this method, was transformed by electroporation, as described elsewhere [24].

Drug treatments

Daunorubicin was purchased from Sigma (St. Louis, MO, U.S.A.). It was freshly prepared as a 500 µM stock solution in sterile 150 mM NaCl, and diluted to the final concentrations before use. Exponential growing yeasts were then exposed to the different drug concentrations and incubated in the dark at 30 °C for the times indicated.

β-Galactosidase assays

Cells carrying the plasmids were grown in selective media until the late logarithmic phase. A suitable number of cells was then transferred to microfuge tubes in 250 µl of YPD (5 g/l yeast extract, 10 g/l peptone and 20 g/l glucose; all from Pronadisa, Madrid, Spain), and the corresponding concentration of daunorubicin was added. After incubation at 30 °C in a roller for the indicated times, β-galactosidase assays were performed using yeast protein extracts obtained with Y-PER (Pierce, Rockford, IL, U.S.A.), as described elsewhere [25]. For galactose induction, Δerg6 cells transformed with plasmid pGAL were grown overnight in YEP medium (identical to YPD but without glucose) plus 2 % raffinose. After adjusting the D600 to 0.1–0.2, 1 % galactose was added together with daunorubicin. For oestrogen induction, Δerg6 cells transformed with pH5HE0 and pVITB2x were grown in selective medium (6.7 g/l yeast nitrogen base without amino acids (Difco, Basel, Switzerland) and 20 g/l glucose, supplemented with 0.1 g/l prototrophic markers as required) and then transferred to YPD plus 1 nM oestradiol and the indicated concentrations of daunorubicin.

RNA extraction and semi-quantitative reverse transcriptase (RT) PCR

Total RNA was extracted from 50 ml yeast cultures by the hot phenol method, as described elsewhere [26]. The samples were treated as described in the Results section. RT-PCR was performed using the OneStep PCR kit from Qiagen (Hilden, Germany) following the manufacturer’s instructions. Sub-saturating RT-PCR conditions were adjusted to 10 ng of total RNA per reaction and to 25 amplification cycles. Tubulin (TUB1) was used as a reference gene. Primers used were as follows: TUB1 upper primer, 5’-AAGGGTTCTTTGTTTACC-3’; TUB1 lower, 5’-GCCATGTATTTCATTCT-3’; GAL1 upper, 5’-CCAAGACCATTAGGCGAAA-3’; GAL1 lower, 5’-GACGGGCAGAAGCATCA-3’; GAL3 upper, 5’-GGCCATAGATCCGTTGTGT-3’; GAL3 lower, 5’-CTAGTGTCGCGGCGAACT-3’; GAL7 upper, 5’-GGTCAACAGAGGCTGCTT-3’; GAL7 lower, 5’-CGAGCCTAAGGCAGCATA-3’.

Sequence analysis

The sequence of yeast gene promoters and the identification of the relevant regulatory sequences were obtained from the Saccharomyces Genome Database (http://genome-www.stanford.edu/Saccharomyces/). The last visit made was on 23rd April 2002.

RESULTS

Sensitivity of yeast mutants to daunorubicin

Daunorubicin, like other DNA-intercalating drugs, affects wild-type yeast strains at millimolar concentrations [27], which are at least three orders of magnitude above pharmacological levels

Table 1: Activating sequences and transcription properties of the plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Activating sequence</th>
<th>Binding factor</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGAL</td>
<td>TCGGAGGAGGATCTTCGAGAT</td>
<td>Gal4p</td>
<td>I</td>
</tr>
<tr>
<td>pRPS2d</td>
<td>TCAGACCGATACAATAACAGCTAATT</td>
<td>Rap1p</td>
<td>C</td>
</tr>
<tr>
<td>pVITB2x</td>
<td>AGTCGCTTGAGC</td>
<td>Oestrogen receptor</td>
<td>I</td>
</tr>
</tbody>
</table>

![Figure 1: Effect of daunorubicin on the growth of yeast cells with different genetic backgrounds](http://example.com/figure1.png)

Yeast BY4741 cells (■) and Δerg6 (●), Δpdr1 (○), Δpdr3 (Δ) and Δpdr5 (◇) derivatives were treated for 16 h with various concentrations of daunorubicin. Drug effects on growth are represented as the percentage of D600 reached by the treated culture relative to the untreated control for each strain. Values represent means from two independent experiments, with similar results.
Table 2: Effect of daunorubicin concentration on yeast cell viability after 6 and 24 h of treatment

Viability is expressed as a percentage of total cells (means ± S.D. from four independent experiments).

<table>
<thead>
<tr>
<th>Daunorubicin (µM)</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>74.9 ± 8.1</td>
</tr>
<tr>
<td>10</td>
<td>54.0 ± 13.4</td>
</tr>
<tr>
<td>25</td>
<td>48.0 ± 4.1</td>
</tr>
<tr>
<td>50</td>
<td>36.2 ± 7.1</td>
</tr>
</tbody>
</table>

Figure 2: Effects of daunorubicin on the transcription of reporter plasmids

The yeast Δerg6 strain were transfected with the plasmids pGAL, pRPG2d, or co-transfected with the plasmids pVITB2x and pHSH60. Transformed cells were treated with various daunorubicin concentrations for 6 h; for inducible systems, appropriate inducers (galactose for the pGAL-transformed strain and oestradiol for the pVITB2x/pHSH60 co-transfected strain) were added simultaneously. Note that pGAL-transformed cells were grown in raffinose prior to galactose addition to overcome glucose repression. Values represent β-galactosidase activities relative to the untreated control. Values represent means from two independent experiments, with similar results.

[28]. We explored the sensitivity to this drug of yeast mutants with deficiencies in cell membrane permeability. The growth of the wild-type strain was not inhibited at any of the daunorubicin concentrations tested (up to 40 µM; Figure 1), whereas deletion of the ERG6 gene (and thus lack of ergosterol in the yeast membrane) increased the sensitivity to daunorubicin at low micromolar concentrations (Figure 1). In contrast, deletion of three key regulators of the yeast multidrug-resistance system (Pdr1p, Pdr3p and Pdr5p) had no effect on the sensitivity of the mutated strains to daunorubicin, even at concentrations up to 40 µM (Figure 1). As multidrug resistance in yeast requires the function of the three PDR genes analysed [16], resistance to daunorubicin in yeast cells should be mediated by exclusion of the drug by the cell membrane rather than by excretion of the drug to the medium through the multidrug-resistance system.

Growth inhibition of erg6 mutants by daunorubicin was paralleled by a decrease in the proportion of viable cells in the culture. Table 2 shows the proportion of viable cells in erg6 cultures treated with different daunorubicin concentrations for 6 or 24 h. Survival was about 50% at 10 µM daunorubicin in both treatments. At higher drug concentrations, cell viability decreased dramatically during the 24 h-exposure treatment. Hence, we performed treatments for 6 h or less in further functional analyses to reduce the influence of cell growth and cell viability on the results.

Transcriptional inhibition by daunorubicin

The DNA-binding sequence for Gal4p (UASgal [17]) contains two palindromic CGG repeats, which are also binding sites for daunorubicin (Table 1). We thus explored the effect of the drug on the ability of Gal4p to activate transcription. A synthetic UASgal site was cloned in front of the minimal CYC1 promoter fused to the lacZ gene coding sequence (plasmid pGAL; Table 1). This construct is silent in the presence of glucose and active when cells grow in galactose, owing to the binding of Gal4p to the UASgal. The transcription of pGAL in the presence of galactose was highly sensitive to the presence of daunorubicin, with an IC50 around 2 µM (Figure 2).

A major concern about the effects of DNA-intercalating drugs on transcription is their pleiotropic nature. To show that the effect of daunorubicin was neither due to a general malfunction of the yeast cell nor to a non-specific decrease in β-galactosidase activity, we tested the effect of daunorubicin on the constitutive plasmid pRPG2d. This plasmid is identical to pGAL, except for the replacement of the UASgal by two DNA-binding sites for the ubiquitous factor repression/activation protein I (Rap1p; pRPG2d, see Table 1 [19]). Daunorubicin did not affect the transcription of the pRPG2d construct at concentrations up to 50 µM (Figure 2). This demonstrates that daunorubicin did not affect β-galactosidase measurements or the enzymatic activity of β-galactosidase by itself.

However, a constitutive reporter may not be an adequate control for an inducible promoter like UASgal. Therefore, we took advantage of the ability of the mammalian oestrogen receptor to activate transcription in yeast in an oestrogen-inducible manner [29]. Unlike UASgal, the DNA-recognition sequence for the oestrogen receptor, the oestrogen-responsive element, does not contain CpG steps, the preferred DNA-binding sites for daunorubicin [21]. Plasmid pVITB2x was identical to pGAL, except for the substitution of the UASgal by a canonical oestrogen-responsive element (Table 1). This reporter construct is essentially silent in the absence of oestrogen, even when an expression plasmid for the oestrogen receptor is co-transfected into the same strain, but is strongly activated when oestradiol is added to the culture [20]. The activation of pVITB2x by oestradiol was not prevented by the simultaneous addition of daunorubicin (Figure 2); therefore we conclude that the effects of daunorubicin on the pGAL reporter transcription were specific of the UASgal.

Specific inhibition of GAL genes by daunorubicin

A direct prediction from our reporter data is that daunorubicin should prevent the activation of resident genes under Gal4p control, the GAL genes. As these are essential for galactose utilization, daunorubicin-treated cells should not utilize galactose as a carbon source. Figure 3(A) shows that daunorubicin concentrations as low as 200 mM prevented growth in a medium in which galactose was the only carbon source, whereas growth in glucose was inhibited only at daunorubicin concentrations over 10 µM (see also Figure 1). These results indicate that daunorubicin affected galactose utilization specifically.
The direct effects of daunorubicin on the transcription of several GAL genes are shown in Figure 3(B). A single culture of the Δerg6 strain grown in raffinose was divided into three identical cultures, which were incubated for 2 h either in 2% raffinose, in a mixture of 2% raffinose and 1% galactose or in the same raffinose/galactose mixture with the addition of 3 µM daunorubicin. The three cultures grew at similar rates, as daunorubicin had no effect on growth in raffinose at these concentrations (results not shown). Semi-quantitative RT-PCR corresponding to TUB1, GAL1, GAL3, and GAL7 promoters revealed the virtual absence of GAL1 and GAL7 mRNAs, and a low level of GAL3 mRNA in the raffinose-grown culture (Figure 3B, left-hand lane). The addition of galactose strongly induced the transcription of the three GAL genes (Figure 3B, middle lane), which is consistent with the current model of regulation of GAL genes [18]. However, the induction of these genes by galactose was reduced when daunorubicin was added simultaneously (Figure 3B, right-hand lane), especially for GAL1 and GAL7. Quantification of the bands in Figure 3(B) indicated that daunorubicin reduced transcription of GAL1 and GAL7 to 39 and 29% of the untreated control value respectively. This effect was somewhat lower than that observed for pGAL at the same drug concentration (28%; Figure 2). The effect on GAL3 was clearly lower, down to 69% of the untreated control value. Transcription of the housekeeping TUB1 gene was identical in all three cultures. Figure 3(C) shows the Gal4p-DNA-binding sites in the GAL1, GAL3 and GAL7 promoters, as well as high-affinity sites for daunorubicin over the same sequences. As for the pGAL reporter plasmid (Table 1), there is a clear overlap between the DNA-recognition sequences for the transcription factor and for the drug. The transcriptional activation of GAL genes by galactose depends entirely on the binding of Gal4p to their promoters [18]; therefore, these results sustain the proposed specific inhibition of the Gal4p function by daunorubicin.

DISCUSSION

Identification of the appropriate targets of potential drugs to improve the efficacy of anti-tumour targets on mammalian cells in culture is complicated by the presence of multiple alterations in these cells. These ill-characterized alterations include gene mutations, chromosomal aberrations, unpaired metabolism and failures of various aspects of apoptotic pathways. Northern blot experiments have revealed the effects of several anti-tumour drugs on the steady-state levels of several mRNAs in cultured mammalian cells [10,28]. However, the inherently complexity of transcriptional regulation in higher eukaryotes makes it difficult to differentiate the direct effects on the promoters from indirect phenomena affecting the general metabolism of the cell. Therefore, we used the yeast S. cerevisiae to study the determinants of the cell response to a particular cytotoxic effect, the inhibition of transcription [14].

Standard laboratory yeast strains proved fairly insensitive to daunorubicin at concentrations up to 50 µM, in agreement with published data on the cytotoxicity of several drugs in yeast [27]. We found that only Δerg6 strains, but not strains lacking the PDR function, were sensitive to daunorubicin concentrations in the therapeutic range. Δerg6 strains are characterized by a severe distortion of the cell membrane composition, due to the lack of ergosterol. This suggests that the main factor protecting yeast cells from intercalating drugs is their exclusion by the cell membrane rather than the ability of the multidrug-resistance system to expel the drugs from the cells. A similar conclusion was reported for the topoisomerase I inhibitor camptothecin [15], whereas multidrug-resistance systems seem to be the main determinant of the resistance to anthracyclines in mammalian cells [30]. Daunorubicin slightly affected yeast mitochondrial DNA, as judged by the low rate of appearance of petite colonies (results not shown), as described elsewhere [31].

Low concentrations (1–3 µM) of daunorubicin specifically affected several processes related to galactose utilization and Gal4p function in sensitive Δerg6 yeast strains. For example, transcriptional activation of the UASgal-based reporter pGAL was very sensitive to daunorubicin, whereas transcription of the related reporter plasmids pRPG2d and pVITB2x was unaffected.
Promoter inhibition by daunorubicin in yeast

by drug concentrations up to 50 µM. The minimal differences in base sequence between these three constructs (short tracts of only 20–40 bp) make it unlikely that the different responses to daunorubicin were due to differential plasmid stability or to general effects on β-galactosidase transcription, translation or enzymic activity. Our experimental design ensured that the relevant transcription factors were present on daunorubicin addition. Rap1p and the oestrogen receptor from pH5HE0 are expressed constitutively [19,22], whereas Gal4p expression is maximal in yeast cells growing in raffinose [18]. Therefore it is highly improbable that the differential responses to the presence of daunorubicin reported here are due to anything but a specific effect of the drug on the respective UASs and on their recognition by the relevant transcription factors.

Our data show the specific inhibition of Gal4p function at different levels. Relatively low concentrations (1–3 µM) of daunorubicin prevented the growth of yeast cells in galactose, which requires Gal4p function, whereas they did not affect growth in other carbon sources, such as glucose or raffinose, whose utilization is independent of Gal4p [18]. Similar low concentrations of daunorubicin reduced the response of GAL1, GAL3 and GAL7 to galactose, which requires Gal4p binding to UASgal sites present in their promoters [18]. The slight effect of daunorubicin on the GAL3 response to galactose may be due to the low induction of this gene by galactose (4-fold) when compared with the very strong induction of either GAL1 or GAL7 [18].

Intercalating drugs can alter cell growth not only by producing lesions on DNA, but also by preventing the binding of essential transcription factors to DNA, as they may compete with the factors for binding to DNA, provided their cognate sequences coincide. We propose such a mechanism for the inhibition of Gal4p-driven transcription by daunorubicin, since the drug and Gal4p might compete for two crucial CpG steps in the UASgal [17]. A similar mechanism has been proposed to explain the inhibition of Sp1-activated transcription upon addition of anthracyclines and bisanthracines in vitro and in vitro [6,32] or the inhibition of myogenic differentiation in cell cultures by other drugs [13]. The lack of sensitivity of pVITB2x and pRPG2d to daunorubicin is also consistent with this model, since the DNA-recognition sites for the corresponding transcription factors (the oestrogen receptor and Rap1p) do not contain CpG steps (Table 1).

The finding that daunorubicin can discriminate between UASs to inhibit gene transcription by RNA polymerase II is remarkable, since it recognizes DNA tracts of only 3 bp in length. To our knowledge, such a discriminatory capacity has been unambiguously reported in vivo only for RNA polymerase III genes using a ligand whose DNA-binding site is about 6 bp [33]. The development of small molecules with the ability to interfere with the expression of specific genes in living cells may improve the current therapies for human diseases [34,35], since they may block specific pathways necessary for cell growth. This approach may foster the development of new anti-cancer drugs with alternative mechanisms of action.

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A comparative analysis of the time-dependent antiproliferative effects of daunorubicin and WP631

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Jurkat T lymphocytes were treated with daunorubicin and WP631, a daunorubicin-based DNA binding agent, in experiments aimed to analyze cellular uptake of these drugs and their effect on cell viability. WP631 was taken up more slowly than daunorubicin, but laser confocal microscopy and spectrofluorometric quantification showed that the drug accumulated in the cells. Despite the slow uptake rate, the antiproliferative capacity of WP631 (measured as IC50 after a 72-h continuous treatment) was greater than that of daunorubicin. The propensities of daunorubicin and WP631 to promote apoptosis were compared. Our results indicate that the major effect of WP631 was a G2/M arrest followed, after about 72 h of treatment, by polyplody and mitotic (reproductive) death. In contrast, daunorubicin induced a rapid response with classic features of apoptosis.

Keywords: anthracyclines; p53; cell-cycle; mitotic catastrophe; Jurkat T lymphocytes.

Anthracyclines are among the most potent and clinically useful drugs in cancer treatment [1]. Anthracycline antibiotics are DNA intercalators [2,3], and the antitumor activity of daunorubicin, a prominent member of this group of antibiotics, may be associated with its binding to DNA, although several mechanisms have been proposed to fully explain the cytotoxic actions of these antitumor molecules [1,4,5].

Detailed information on the structural and thermodynamic basis of daunorubicin binding to DNA [2,3,6] has provided the foundation upon which to design WP631, a new bisanthracycline (Fig. 1) resulting from a ‘Modular Design Approach’ [7]. WP631 bisintercalates into DNA, and displays enhanced binding affinity and sequence selectivity over monomeric daunorubicin [8]. These characteristics make WP631 a more effective antitumor drug against some cell lines, including a multidrug-resistant one [8,9]. Moreover, there are grounds for considering that WP631 is a potent inhibitor of transcription through direct competition with transcription factors [9–11].

Anthracyclines induce apoptosis, although this might be the final cell response to other events such as unpairing of DNA replication or inhibition of transcription and topoisomerase activity [4,12]. Interaction of anthracyclines with DNA-topoisomerase II complexes may trigger apoptosis. In Jurkat T lymphocytes, daunorubicin, and the related drug doxorubicin, are considered inducers of apoptosis [13]. Nevertheless, this effect may only be true for some cell types or drugs, as the onset of apoptosis appears to depend on the cell line [4,14]. Alternatively, G2 arrest by anthracyclines may result from the disruption of some cell cycle activities [15,16], and thus in some cases the rapid induction of apoptosis may not be the main mechanism leading to cell death [17].

Despite the potent effect of WP631 on the viability of Jurkat cells [9], continuous treatment over 72 h produces only marginal apoptosis. Arrest in G2 after treatment, which depends on the levels of p53 protein [16], suggests that the extent of p53-dependent apoptosis is not a critical factor in the sensitivity to WP631 [16]. Although it is widely accepted that the sensitivity of cells to damaging agents, including anthracyclines, might reflect cell death by apoptosis [18], the relationship between the efficacy of drug treatment and the induction of apoptosis is still an open issue [19,20]. Here we show that low concentrations of WP631 produce nonapoptotic cell death, in contrast with monomeric anthracyclines that can produce nonapoptotic tumor cell death only at high (supraclinical) concentrations [4]. Genomic site damage may explain the differences in drug efficacy between the mono-intercalating anthracyclines and the more sequence-selective bisanthracyline WP631. To gain further insight into the causes of the distinct behavior of daunorubicin and WP631, we compared the intracellular accumulation of these compounds in Jurkat T cells overtime. We also examined the rate and overall level of cell killing by either drug by apoptosis or mitotic, reproductive, death after G2 arrest.

Materials and methods

Daunorubicin and WP631

Solutions containing 500 μM daunorubicin (Sigma) or WP631 were prepared with sterile 150 mM NaCl, maintained at −20 °C, and brought to the final concentration with RPMI 1640 medium just before use.
Cell culture

Jurkat T lymphocytes were obtained from the cell culture facilities at the Department of Biochemistry of the University of Barcelona, Spain. Cells were maintained in RPMI 1640 medium (GibcoBRL, Life Technologies, Spain) supplemented with 10% fetal bovine serum (GibcoBRL), penicillin (100 U·mL⁻¹), streptomycin (100 µg·mL⁻¹) and 2 mM L-glutamine (GibcoBRL), at 37 °C in a humidified atmosphere with 5% CO₂.

Drug treatments and cytotoxicity assays

The effect of WP631 on Jurkat cells growth was determined by using the MTT dye assay [21] in 96-well microtiter plates with flat-bottomed wells (Corning Costar, Corning, NY, USA) in a total volume of 100 µL. Cells subcultured at a density of 5 × 10⁴ cells·mL⁻¹ were incubated with various concentrations of daunorubicin or WP631 at 37 °C for 24 or 72 h. After incubation, MTT (Sigma) was added to each culture (50 µg, 15 µL per well). The dark-colored crystals produced by viable cells were solubilized with 30 mM HCl in 2-propanol. Absorbance was determined at 570 nm using a SPECTRAMax 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Flow cytometry

After treatment with either 182 nm daunorubicin or 60 nm WP631 (that is, their respective IC₇₅ at 72 h, see below) for various periods of time, the cells were harvested and stained with propidium iodide (Sigma) as described elsewhere [22]. Nuclei were analyzed with a Coulter Epics-XL flow cytometer (Coulter Corporation; Hialeah, FL, USA) at the ‘Serveis Científico-Tecnis’ of the University of Barcelona, 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 according to procedures described in [23].

The capacity of daunorubicin and WP631 to produce cell death was determined by monitoring the decline in the number of cells originally cultured. Cell viability was assessed by exclusion of Trypan blue dye (Fluka, Buchs, Switzerland) using a hemocytometer.

Spectrofluorimetric quantification of intracellular drug accumulation

Cellular accumulation of daunorubicin or WP631 was quantified as described elsewhere [24], with minor modifications, using cultures of about 10⁷ cells. Cells were incubated with either 182 nm daunorubicin or 60 nm WP631 [their IC₇₅ measured at 72 h (Fig. 2)] for 2, 24, or 72 h. The cells were then rinsed three times with ice-cold...
RPMI 1640 medium, and the drugs were extracted from the cells using 2 mL of 80 mm HCl in 2-propanol for 16 h at 4 °C. The concentrations of the two drugs were measured using a Shimadzu RF-1501 spectrofluorophotometer (Shimadzu, Columbia, MD) with an excitation wavelength of 480 nm and an emission wavelength of 555 nm. The fluorescence intensity emitted was translated into concentrations of drug using a daunorubicin or WP631 standard curve, and expressed as ng drug per 10^7 viable cells, assessed before and after treatment by exclusion of Trypan blue dye.

Confocal microscopy

Cells in exponential growth phase were harvested after treatment with 60 nm WP631 for various periods, rinsed three times with an ice-cold buffer consisting of 20 mm Hepes (pH 7.4) containing 130 mm NaCl, 6 mm KCl and 1 mm glucose, and resuspended in 200 μL of the same buffer. Confocal laser microscopy was performed with a Leica confocal TCS-4D microscope system (Leica Microsystems; Heidelberg, Germany) using the fluorescence of the bisantrachrysine WP631 as unique fluorophore.

Detection of p53 protein levels by Western blot

Total protein was extracted from WP631-treated and control cells, at different times, with a lysis buffer consisting of 50 mm Tris/HCl (pH 8), 150 mm NaCl, 5 mm EDTA, 0.5% Nonidet P-40, 0.1 mm phenylmethanesulfonil fluoride, containing protease inhibitors, and quantified by the Bradford assay (Bio-Rad, Hercules, CA, USA). About 30 μg of denatured proteins were subjected to electrophoresis on SDS-polyacrylamide gels (12% for p53 and 10% for actin), blotted onto Optitran BA-S85 membranes (Schleicher & Schuell, Dassel, Germany), analyzed with antibodies (Sigma), and detected by chemiluminescence. Signal intensities were quantified in a Molecular Dynamics densitometer and normalized using actin as reference.

Cytological analysis of multinucleated cells

For morphological observation of multinucleated cells, a Compucyte Laser Scanning Cytometer (Compucyte; Cambridge, MA, USA) was used at the 'Serveis Cientifico-Tecnics' of the University of Barcelona. The presence of multinucleated cells was assessed on microscope slides containing samples prepared as described above for flow cytometry. After establishing a scan area, the slides were analyzed using a 40 × objective and 5 mW of Argon laser power. The entire cell preparation was examined. A cell gallery was created by relocation of cells from each of the major peaks in the histogram of integrated red fluorescence. The presence of polyploid cells was determined by setting an appropriate histogram gate, and the morphology was established under the microscope.

Alternatively, about 10^4 WP631-treated cells were spun onto microscope slides, stained with DAPI (Sigma) and analyzed with a Carl-Zeiss Axiophot fluorescence microscope.

Enlarged cells that contained multiple evenly stained nuclear fragments (polyploid micronucleated cells) were deemed to undergo mitotic death [14,25].

**Dilution (clonogenic) survival assay**

Because Jurkat T lymphocytes grow in suspension without forming colonies, a limiting dilution assay [26] was used to determine clonogenic cell survival. In brief, after 72-h of continuous treatment with 60 nm WP631, cells were harvested and resuspended in fresh medium. Then, 1, 10, 100, or 1000 cells were seeded into each six wells of a 96-well microtiter plate (Corning Costar). The wells in which cell growth occurred were identified after 3 days by Trypan blue exclusion. The number of cells needed to achieve growth in 50% of the wells (TD_{50}) was obtained by fitting the experimental data to a logistic regression. The surviving fraction of cells (SF) was calculated as: TD_{50} untreated/ TD_{50} WP631-treated cells.

**Results**

Proliferation of Jurkat cells treated with a range of concentrations of daunorubicin or WP631 is illustrated in Fig. 2. Data were obtained after 24 h (panels A and B) and 72 h (panels C and D). No significant effects on cell proliferation were observed after 24-h treatment with bisintercalator WP631 (Fig. 2B), when about 93% viable cells were detected at 60 nm WP631 concentration (this concentration of WP631 is equivalent to the IC_{75} measured after 72-h continuous treatment in Fig. 2D). Even at higher drug concentrations, about 90% of cells were still viable at 24 h (panel B). However, at 72 h (panel D) the viability had declined insofar as the IC_{50} was as low as 17.7 nm WP631. In contrast, an IC_{50} for daunorubicin could be determined at either 24 h and 72 h. The daunorubicin concentrations required to decrease cell survival by 50% were clearly higher at 24 h than at 72 h. After 24 h of treatment with around 200 nm daunorubicin, cell viability was about 50%, whereas at 72 h it was only about 15% (cf. Fig. 2A,C). Quantification of viable cells, at various times, in the presence of 60 nm WP631 (the IC_{75} of which was measured at 72 h) is shown in Fig. 2E. It illustrates the ability of the cells to exclude Trypan blue for up to 140 h. However, by 72–80 h of incubation the number of viable cells dropped considerably, and most of them died during the following days.

Flow cytometry analyses of Jurkat T cells treated with daunorubicin showed more than 60% apoptotic cells after 24-h continuous treatment (Fig. 3B,D), while WP631 produced marginal apoptosis (Fig. 3C,E). Therefore, the two drugs may use distinct mechanisms to halt cell growth and promote death in drug-treated cells, which will be analyzed below.

We studied whether the time-dependent survival curves in presence of the drugs (Fig. 2) were merely due to a slower absorption of WP631. Two approaches were used, which take advantage of the fluorescence of anthracyclines. The absorbed daunorubicin or WP631 were quantified by spectrofluorometry of cell lysates of cells treated with either drug. Daunorubicin was captured by the cells more rapidly, and in a greater amount, than WP631 at any time analyzed between 2 and 72 h (Fig. 4). For WP631 only, the differences in the time-dependent uptake, between 2 and either 24 or 72 h, was statistically significant (P < 0.05, Student’s t-test). Nonetheless, there was no significant differences in the uptake of this bisintercalator between 24
microscopy analysis of cells treated with WP631 was performed at different times. For the sake of comparison, Fig. 5 shows both phase contrast and fluorescence photographs of selected field of cells obtained under the same magnification and contrast acquisition characteristics, and using the autofluorescence of the anthracycline as unique fluorophore in the microscopic assay. WP631 accumulated progressively in the cell, and the nuclei encircled by the fluorescence of WP631 was observed (Fig 5) indicating nuclear accumulation. We aimed to verify that the relatively low uptake of WP631 was at the origin of the low apoptosis, and whether longer periods of treatment could cause significant apoptosis in the presence of this bisintercalator.

Flow cytometry analysis revealed that cells transiently arrested in G2 by WP631 overcame the G2/M checkpoint to produce polyploid cells, 72 h after drug administration, over a period that lasted about 78 h. Figure 6A shows that some of the treated cells briefly accumulated with a DNA content higher than 4n (polyploidy), while a progressive increase in the sub-G0 peak was apparent. Some cells that were G2/M arrested seemed to re-enter unrepaired into the cell cycle. Moreover, some cells arrested in G2 became polyploid (Fig. 6A) and may be condemned to mitotic failure. At 72 h, 5.9% cells were polyploid, while this percentage changed to 9.7% after 75 h treatment and to 3.5% after 78 h. The levels of p53 in cells continuously treated with WP631 decreased, which was consistent with the suppression of the arrest in G2/M, the production of polyploidy, and the final death, independent of p53, by mitotic catastrophe (Fig. 6). Indeed, the p53 protein was present in about 25–50% of that in control, untreated, cells up to about 72-h continuous treatment (Fig. 6B).

The generation of mitotic death by WP631 after G2 arrest was inferred from the presence of enlarged cells containing multiple evenly stained micronuclei. Figure 7 shows that cell cultures continuously treated with 60 nM WP631 (i.e. at its IC50) presented, after a 72-h treatment, multinucleated cells characteristic of mitotic catastrophe [14]. Transient arrest in G2 did not rescue cells from death in response to WP631, as judged by a decrease in the number of viable cells measured by Trypan blue exclusion (Fig. 2E), or the sub-G0 peak seen after about 96 h of treatment (Fig. 6A). Likewise, a clonogenic survival assay, see Materials and methods, showed a surviving factor (SF) of 0.032, which indicates that only about 3% of the cells that were quiescent after 72-h treatment did not suffer clonogenic cell death. The appearance of multinucleated cells (Fig. 7), together with the low clonogenic survival after about 96 h, clearly point to mitotic (reproductive) death induced by WP631.

**Discussion**

We have previously described that Jurkat T cells treated with WP631 suffered only marginal apoptosis, but mainly arrest in G2/M [16]. However, these cells overcame the halt in G2/M when the levels of p53 were reduced significantly in a time-dependent manner [16]. Here, we show that under these circumstances (Fig. 7) the cells that were able to overcome G2/M arrest underwent endoreduplication, become multinucleated, and ultimately died by mitotic (reproductive) death [14,25]. The extent of cell survival monitored by a clonogenic assay indicated that cells that did
not die by apoptosis in the presence of WP631, were actually sensitive to the treatment. In fact, they died within 3 days after the end of continuous treatment (Figs 6 and 7). Most of the cells showed polyploidy and multinucleation instead of displaying signs of ‘classical’ apoptosis as nuclear condensation or DNA fragmentation.

The differences in the kinetics of daunorubicin and WP631 uptake are not due to a resistance of Jurkat cells to the drug because WP631 produced strong inhibition of transcription of various genes after 2-h treatment [16]. The WP631 concentrations that inhibited p53 transcription were in the low nanomolar range (i.e. only by the drug already absorbed by the cells). It is noteworthy that the comparatively low drug concentrations of the bisintercalator reached inside the cells, compared to daunorubicin, should suffice to cause the specific effects on transcription in Jurkat T cells, as described elsewhere [16]. We have previously shown that WP631 is a strong inhibitor of Sp1-activated transcription in vitro, at nanomolar range concentrations [10]. The observation of WP631 accumulation inside the nuclei is consistent with the location prevalently observed with other anthracyclines [27,28]. The bisanthracycline accumulated slower than daunorubicin, but was absorbed freely.

We previously suggested that the improved antitumor efficacy of WP631 on some cell lines [8,9] might be the result of its strong effect on the transcription of some key genes, such as p53 and c-myf [16]. The inhibition of p53 activity should produce that cells attesting to divide would undergo G2/M arrest [16] and mitotic catastrophe, in agreement with the requirement for p53 to maintain the G2 arrest after DNA damage [29]. Moreover, the p53 protein levels and DNA damage might determine not only the extent of apoptosis [18] but also cell cycle arrest in G2 [29,30]. It is worth noting that mitotic catastrophe might culminate in features of apoptosis, yet apoptosis and not mitotic death is promoted by wild-type p53 [30,31]. It has been proposed that genes involved in G1 arrest and apoptosis, for example p53, do not contribute to the sensitivity of solid tumors, which often are p53+ [19], to antitumor drugs. However, this does not appear to apply to cells of hematological origin, including Jurkat T lymphocytes. Our results support

Fig. 5. Phase contrast and laser confocal microscopy of the same fields showing the uptake of WP631 by Jurkat T lymphocytes. Results after 24-h continuous treatment (A,B) or 72 h (C,D). WP631 uptake was time-dependent (compare the autofluorescence of WP631 in C,D), and the drug was mostly located inside the nuclei. The unstained nucleoli can be observed.
the idea that when the expression of genes linked to apoptosis is inhibited [16], and the p53 protein levels consequently decrease (Fig. 6B), cells of hematological origin behave as p53−/− tumors, thus they die using a p53-independent pathway.

Strong inhibitors of transcription, such as WP631 [10,11,16], could produce cell death through mechanisms that are not only p53 independent, but also ‘dependent on the absence of p53’. Notwithstanding, the effects of WP631 on cell proliferation might be attained not simply by interfering with the transcription of some genes, but also by preventing some downstream events that brought the cells to the observed blockage in G2/M, which ultimately commit cells to die through mitotic catastrophe.

As WP631 appears to be quite inefficient as an inhibitor of topoisomerase II (unpublished observations), the decreased growth rates in Jurkat cells and the polynucleated cells (Fig. 7) may also be related to low formation of topoII-DNA complexes, which is generally associated with G2 arrest and the absence of programmed apoptotic death [5]. Be that as it may, daunorubicin and WP631 kill treated Jurkat T lymphocytes by distinct mechanisms. Namely, daunorubicin does it through a p53-dependent and relatively rapid apoptosis. In contrast, WP631 kills the cells through mitotic catastrophe: induction of transient arrest in G2/M followed by endoreduplication and polyploidy that results in multinucleation and impaired cytokinesis.
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References
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 IC$_{50}$ in Jurkat T cells; where IC$_{50}$ is the drug concentration that inhibits growth by 75%) resulted in cell-cycle arrest in G$_{1}$ and almost immediate apoptosis. In contrast, decreasing the drug concentration to 91 nM (close to the IC$_{50}$) caused G$_{1}$ 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 G$_{1}$/S cell-cycle checkpoint and the activation of apoptosis [2,16]. It is also involved in the halt in the G$_{2}$/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)
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/bj/372/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 IC50 in Jurkat T cells determined after 72 h continuous treatment [27]; where IC50 is the drug concentration that inhibits cell growth by 75%) or 91 nM daunorubicin (close to the IC50). The UltraspecRNA isolation reagent (Biotecx, 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. Subsaturating 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 μM 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 570 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 IC50) or 91 nM (close to the IC50 [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 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 1](image_url)  
**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 (IC50) or (B) cells treated with 182 nM daunorubicin (IC50) for 4 h.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Daunorubicin (nM)</th>
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<tr>
<td>ATM</td>
<td>+</td>
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<tr>
<td>p53</td>
<td>+</td>
</tr>
<tr>
<td>MDMX</td>
<td>=</td>
</tr>
<tr>
<td>BRCA1-associated ring domain 1</td>
<td>–</td>
</tr>
<tr>
<td>p21ras</td>
<td>+</td>
</tr>
<tr>
<td>N-Ras</td>
<td>+</td>
</tr>
<tr>
<td>p107a</td>
<td>–</td>
</tr>
<tr>
<td>p14ras</td>
<td>+</td>
</tr>
<tr>
<td>Cyclin D3</td>
<td>+</td>
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<tr>
<td>Cyclin E</td>
<td>+</td>
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<td>Cdc25A</td>
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<td>Rb</td>
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<tr>
<td>RB1</td>
<td>–</td>
</tr>
<tr>
<td>Rb2-3</td>
<td>–</td>
</tr>
<tr>
<td>E2F5</td>
<td>+</td>
</tr>
<tr>
<td>CDC-like kinase 2</td>
<td>+</td>
</tr>
</tbody>
</table>

* +, up-regulated; −, down-regulated; =, no change.
† As inferred from the semi-quantitative RT–PCR analysis (Figure 3).
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_{50} of daunorubicin, and approx. 20-fold in the presence of IC_{50}, whereas cyclin E was down-regulated 50 times (IC_{50}) 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_{50}), the drug increased abruptly the sub-G_{0}, which reached a peak within the

---

**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_{50} 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

---

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, hercgulin-β 3; 5, guanine nucleotide-binding protein α 13 subunit; 6, Pim-1; 7, Wilms' tumour protein; 8, ski-related oncogene snod; 9, N-myc; 10, DCC; 11, CD250 homologue; 12, FOS-related antigen 2; 13, ATM; 14, P38 kinase; 15, CDC-like kinase 2; 16, c-yes; 17, APC; 18, ETS oncogenes; 19, E2F; 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 1; 28, wingless-related MMTV integration site 2 protein; 29, jun-D; 30, jun-B; 31, APC; 32, v-Ki-RAS2B; 33, STAT2; 34, erbB4; 35, mitochondrial transcription factor 1; 36, p16-INK4A; 37, CBL-B; 38, ST4 oncofetal antigen; 39, delta lactoferrin; 40, FKBPs–rapamycin associated protein; 41, Frizzled homologue 5; 42, BRCA1-associated ring domain protein 1; 43, RB3; 44, TATA sequence-binding protein; 45, J1 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, RB1; 54, semaphorin III/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, rasf; 61, prohibitin; 62, cyclin D3; 63, Dsc2a homologue; 64, ski; 65, TBP-associated factor 250 kDa subunit; 66, p21/WAF1; 67, guanine nucleotide regulatory protein tim 1; 68, nucleoside-sensitive element DNA-binding protein; 69, c-fgr; 70, L-myc; 71, prefoldin A; 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-rat; 77, prefoldin 5; 78, smoothened homologue; 79, β-catenin; 80, ZAP70; 81, CDC-like kinase 3; 82, neurogenic locus notch homologue 1; 83, CD2-related protein CHED; 84, N-Ras; 85, SAP102; 86, rel A; 87, 130 kDa RB-associated protein; 88, PCTAIRE 3; 89, mihtr; 90, frizzled-related Frb2; 91, ABL2; 92, tumour necrosis factor receptor superfamily-member 108. 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: GAPDH5f, 5′-TCAGCCGCACTTTCTTGG-3′; GAPDHrev, 5′-CGTGGTGTTCTCTGTGTTTCAA-3′; p53dir, 5′-TCAGTCATCTCTCGAGTG-3′; p53rev, 5′-CTGTCGTCTCTTCATTC-3′; Rbdir, 5′-AGAAGGAGGTGCACTCGTAGG-3′; Rbrev, 5′-GCTGGCATCTTGGAGTTC-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-G1. The cell-distribution profiles would suggest that cells found in the S-phase underwent apoptosis after drug treatment (Figure 4A). Continuous exposure to the IC50 daunorubicin revealed that after 4 h treatment, most cells showed the same distribution as controls, with a prominent G0 peak. However, in the period up to 24 h, there was a progressive accumulation in G2/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 IC50 or IC75 for daunorubicin, cellular DNA fragmentation was also analysed 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.

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

<table>
<thead>
<tr>
<th>Daunorubicin (nM)</th>
<th>91</th>
<th>182</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>4 h</td>
<td>10 h</td>
</tr>
<tr>
<td>% DNA damage*</td>
<td>7.4 ± 5.2</td>
<td>7.2 ± 6.0</td>
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* Values are expressed as the percentage of untreated controls and represent the means ± S.D. for three experiments.
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 G0 or G1 (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](image-url) **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 senescent marker) using a phase-contrast microscope and photographed, after 16 h, at ×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 ×400 magnification, and they are shown at a final ×1300 magnification to display, in detail, the morphological changes characteristic of apoptosis, which include the condensation of chromatin and the formation of apoptotic bodies.
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, p21WAF1, ATM, cdc25A and Rb (Table 1) should induce cell
arrest at the G1/M checkpoint, as observed in the experiments
(Figure 4). From this point onwards, the transcription profiles
of p53, Rb, p16, p21WAF1 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,
senescent-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 IC50 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
casing 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 TNFα 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
NFκB 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 (IC50; 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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Variations in gene transcription induced by daunorubicin are of interest because they may involve changes in DNA damage and apoptosis. A senescence-like phenotype is associated with the expression of p16 and p53 in tumor cells undergoing terminal proliferation arrest after exposure to anticancer agents. Cancer Res. 59, 3761–3767


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ABSTRACT: The binding of Sp1 transcription factor to DNA is considered a potential target for small ligands designed to interfere with gene transcription. We attempted to distinguish the direct inhibition of the Sp1-binding to DNA in vivo (cell culture) from more indirect effects due to the network of pathways that modulate cell cycle progression, which may decrease transcription without direct interference with Sp1/DNA interactions. We tested whether the Sp3 protein, whose putative binding sequence overlaps the Sp1 site, can inhibit Sp1-activated transcription and interfere with drug–DNA interactions. A well-characterized model system consisting of a wtGLUT1 (wild-type glucose transporter 1) gene promoter, or a mutated mut2GLUT1 promoter, linked to a CAT (chloramphenicol acetyltransferase) reporter gene, was used to analyze the effects of overexpressed Sp1 and Sp3 transcription factors in transiently transfected Jurkat T lymphocytes. Bisanthracycline WP631, a potent inhibitor of Sp1-activated transcription in vitro, was assayed for its ability to specifically inhibit transcription in transfected Jurkat T lymphocytes. The mut2GLUT1 promoter was used to further discriminate between the WP631 interference with Sp1–DNA complexes and Sp3-induced inhibition, since the Sp3-binding site is canceled in this promoter and replaced by a high-affinity binding site for WP631.

Sp1 is a ubiquitous transcription factor that recognizes GC-rich DNA sequences, which are present in many promoters and have been shown to play a direct role in the regulation of transcription (1–6). Regulation of Sp1-dependent transcription may be affected by changes in Sp1 abundance, while protein phosphorylation has been implicated in changes in both DNA-binding and transcriptional activation (6). Sp1 can interact with various proteins involved in cell cycle regulation (7–9), and it is a key factor in the TGF-β-induced transcriptional stimulation of the proximal promoter of endoglin mediated by Smad proteins (10). Sp1 expression also increases during events associated with cell transformation (11).

DNA intercalators and some minor-groove binders can inhibit transcription by impeding the binding of transcription factors to certain promoters (12–15). In general, it is reasoned that drugs that merely prevent protein binding to promoter regions by steric blockage or distortion of DNA might not suffice to render a selective toxic targeting of tumor cells (16, 17). However, the presence of CG-rich tracts specifically recognized by Sp1 in several promoters suggests that DNA–Sp1 complexes are potential specific targets for some small drugs, which may impair Sp1-activated gene transcription, and thus control gene expression in vivo. Recent results obtained with bisanthracycline WP631 have revealed its high preference for targeting of Sp1-binding at nanomolar concentrations (15, 18, 19).

WP631 bisintercalates into DNA with a binding constant close to that of various transcription factors (20). These characteristics make WP631 more effective against some tumor cell lines, including a multidrug-resistant one (21, 22). There are grounds for considering that WP631 is a potent inhibitor of transcription through direct competition with the Sp1 transcription factor (10, 15, 19, 22). Results obtained with WP631 using cells in culture suggest that transcription events can be inhibited by WP631 with high specificity. WP631 and mitoxantrone inhibited collagen COL1A1 gene expression in fibroblasts, but WP631 showed a higher potency (19). The crucial role of Sp1 in the regulation of COL1A1 has prompted researchers to use WP631, at low nanomolar concentrations, to control the excess fibrotic process in fibroblast diseases (19).

Sp1 is the prototype member of a family of related transcription factors consisting of Sp1, Sp2, Sp3, and Sp4 (6). All four proteins exhibit very similar structural features. Molecular, genetic, and biochemical analyses have demonstrated that Sp2, Sp3, and Sp4 are not simply functional equivalents of Sp1. Both Sp1 and Sp3 are ubiquitously expressed in human cells, competing for common target sequences (23). Sp1 is considered a transcriptional activator, while Sp3 contains a transcription repressive domain and can behave as either a transcriptional activator or repressor (4, 6). Moreover, the behavior of the endogenous Sp1 and
Sp3 proteins depends on the specific ratio between these proteins (24). An intriguing aspect of the inhibition of Sp1-activated transcription in vivo is whether molecules such as WP631 can act synergistically with Sp3 as gene inhibitors or interfere with the physiological equilibrium between the Sp1-family proteins, since all of them recognize very similar DNA sequences.

The effects of WP631 in vivo are not easy to dissect, since the direct effects due to inhibition of the Sp1-binding to DNA should be distinguished from more indirect effects due to the network of pathways related to the control of progression through the cell cycle (8, 9), which may decrease transcription levels without a direct drug interference with Sp1–DNA interactions. For example, in Northern blot assays, it was observed that WP631 inhibits the transcription of c-myc, which contains a consensus Sp1 binding site, but also that of p53, which lacks putative Sp1 binding sites (18, 25).

To analyze the effects of WP631 on the Sp1-activated transcription in both the presence and the absence of the Sp3 protein, we have transfected Jurkat T lymphocytes with DNA plasmids bearing a chloramphenicol acetyltransferase (CAT) reporter gene, whose expression can be easily followed experimentally under the control of a promoter of the GLUT1 (glucose transporter1) gene promoter (26). GLUT1 is highly expressed in proliferating cells, and its 99 bp region upstream of the initiation site suffices to drive transcription (26–28). Sp1 strongly transactivates the GLUT1 promoter in cells. The Sp3 gene encodes three distinct transcription factors: a full-length Sp3 protein that can activate gene transcription and two internally initiated Sp3 isoforms described as inhibitors of gene transcription (29). However, this observation is not universal and might depend on the cellular context (6, 29). Sp3 full-length protein and Sp3 internally translated isoforms reduced the transcriptional activity of the GLUT1 promoter in several nonmuscle and muscle cells, which contributes to the decreased expression of GLUT1 during myogenesis (26). Both the wild-type wtGLUT1 promoter and the mut2GLUT1 promoter, mutated at the Sp3 binding site, were cotransfected with Sp1- or Sp3-expression plasmids to analyze all combinations between both protein factors, the presence of adequate targets, and the effects of bisanthraciline WP631 on transcription in Jurkat T lymphocytes.

**EXPERIMENTAL PROCEDURES**

WP631. Bisanthraciline WP631 was synthesized as previously described (21). A 500 µM WP631 stock solution was prepared with sterile 150 mM NaCl and diluted to the desired concentration using RPMI-1640 medium (Gibco) just before use.

Cell Culture Conditions. Jurkat T lymphocytes were maintained in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (Gibco), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM l-glutamine (Gibco), and 2 mM Hepes (pH 7.4), at 37 °C in a humidified atmosphere with 5% CO₂.

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1 Abbreviations: CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; MOPS 3-(N-Morpholino)propanesulfonic acid; MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo- lium bromide); PMSF, phenylmethyl-sulphonyl fluoride; PBS, phosphate-buffered saline.

**Figure 1:** Map of the proximal region of the GLUT1 promoter linked to a CAT reporter gene. The sequences of the −99 to −75 fragments of wtGLUT1 and mut2GLUT promoters (upper strands) are shown. The −93 to −85 region contains the CG box that binds Sp1 and Sp3. The mut2GLUT1 promoter contains a high-affinity WP631 binding site, CGTACG (33). Plasmids containing these promoters were used for transient transfection of Jurkat T lymphocytes. The figure also shows the expression of GLUT1 promoters transfected in Jurkat T lymphocytes. Data are expressed as relative CAT activity/β-galactosidase activity after 24 h incubation. Results are the means ± SD from four transfection experiments.

**Plasmids.** The expression plasmids pCMV-Sp1 and pCMV-Sp3 were kindly provided by Dr. R. Tjian (University of California at Berkeley) and Dr. J. M. Horowitz (Duke University Medical Center), respectively. Wild-type (wt) and mutant (mut2) pGLUT1-CAT reporter plasmids were a gift from Dr. A. Zorzano (University of Barcelona). These CAT reporter vectors contain the −99/+134 region of the rat GLUT1 genomic sequence cloned into the pCAT-basic vector (Promega) (26, 30). Partial sequences of the promoters relevant to the present study are presented in Figure 1. A pCMV-β-galactosidase expression plasmid was used as a control of efficiency in the transfection protocol and pCMV-Script vector (Stratagene) as a carrier.

**Cell Growth and Cell-Cycle Analyses.** The capacity of WP631 to interfere with the growth of Jurkat cells was determined using the MTT assay in the presence of various concentrations of WP631 at 37 °C for 24 h, as described elsewhere (31). Absorbance was determined at 570 nm using a SPECTRAMax-250 microplate reader (Molecular Devices). Viable cell number was determined on the basis of the exclusion of Trypan Blue dye (Fluka) using a hemocytometer.

After treatment with WP631 for various periods, the cells were harvested and stained with propidium iodide (Sigma) as described elsewhere (32). Nuclei were analyzed with a Coulter Epics-XL flow cytometer at the Serveis Científico-Tecnics of the University of Barcelona. Cell percentages at each phase of the cell cycle were estimated from their DNA content histograms after drug treatment.

**Transfections, Cotransfections, and Reporter (CAT ELISA) Assays.** Jurkat T lymphocytes were transfected with 2.5 µg of either the wild-type or the mut2 pGLUT1-CAT reporter plasmids and cotransfected with 5 µg of pCMV-Sp1 or pCMV-Sp3 expression vectors and 5 µg of pCMV-β-galactosidase expression plasmid to normalize for the efficiency of transfection. DNA total concentration was adjusted to 20 µg by the addition of parental “empty vector” pCMV-script (Stratagene) as a carrier. Cells subcultured at a density of 1 × 10⁶ cells/mL were transfected by electroporation using a Gene Pulser II (Bio-Rad) and maintained in 24-well plates at 37 °C for 48 h, in a humidified atmosphere with 5% CO₂.
Transfected cells were incubated, when required, with 60 nM WP631 (its IC\textsubscript{75} concentration in Jurkat T cells, determined after 72 h of continuous treatment (31)) for 24 h. Experiments were repeated 3–5 times.

Transfected cells (WP631-treated or untreated cells) were washed with ice-cold PBS and collected by centrifugation. The pellets were resuspended in 1 mL of a lysis buffer consisting of 10 mM MOPS and collected by centrifugation. Cell extracts were centrifuged at 15 000 g (4 °C) for 10 min, and the supernatants were stored at −80 °C.

Total protein in cell lysates was quantified by the Bradford assay (Bio-Rad). CAT activities of about 50 µg of cell extracts were determined with the CAT ELISA kit (Roche Diagnostics). Absorbance was measured at 405 nm, using 492 nm as a reference wavelength, in a SpectraMax-250 microplate reader (Molecular Devices). β-Galactosidase activities were measured using the β-Gal Reporter Gene Assay (Roche Diagnostics) and a Sirius luminometer (Bertold Detection Systems).

**Western Blot Analysis.** Jurkat cells transfected with either the wild-type or mut2 GLUT1 promoters and the Sp1 and Sp3 expression vectors were used to obtain cell extracts, as described above. Equal amounts of protein (50 µg) were separated by SDS-polyacrylamide on 10% gels and transferred to Optitran BA-S85 membranes (Schleicher and Schuell). The blots were incubated with 5 µg mL−1 rabbit polyclonal anti-Sp1 or anti-Sp3 antibodies (both from Santa Cruz Biotechnology). The membrane was washed and incubated with a secondary peroxidase-conjugated anti-rabbit IgG antibody, and the bound antibody was detected by chemiluminescence using luminol (Sigma). Signal intensities were quantified in a Molecular Dynamics computing densitometer and used to determine the relative Sp1 and Sp3 contents in the different transfection experiments.

**Electrophoretic Mobility Shift Assays.** EMSA assays were performed in a buffer of 10 mM Tris-HCl (pH 7.4) containing 50 mM KCl, 1 mM MgCl\textsubscript{2}, 5 µM ZnCl\textsubscript{2}, 2 mM EDTA, 0.5 mM dithiothreitol, 30 µg/mL bovine serum albumin, 0.1% Nonidet NP-40, and 5% glycerol. A typical reaction contained about 20 ng of pure Sp1 protein (Promega) and 1500–3000 cpm (about 2 nmol in bp) of a radioactively end-labeled HindIII-AvaII 62-mer fragment from either promoter (Figure 1), in the presence of 0.5 µg of poly[d(I–C)] (Roche Diagnostics). In reactions containing WP631, the Sp1 protein and the drug were added at the same time to mimic the conditions of the transcription experiments. Following 20 min of incubation, the samples were analyzed on 4.5% nondenaturing polyacrylamide gels containing 45 mM Tris-borate, 1 mM EDTA, and 0.1% Nonidet NP-40 (pH 8.3). After running at 12 V/cm, the gels were dried under vacuum and subjected to autoradiography. Quantitative analysis was performed with a Molecular Dynamics computing densitometer.

**RESULTS**

We were interested in analyzing changes in gene transcription under strict conditions of cell growth in the presence of WP631. Jurkat T lymphocytes were chosen because with this cell line we had performed previous experiments on the pharmacological effects of WP631, including the effects of the drug on the transcription of genes involved in the control of cell cycle (18). Three types of experiments were performed to analyze whether bisanthraniloylnaphthalene WP631 inhibits the Sp1-activated transcription of GLUT1 promoters in Jurkat T cells. First, WP631 was added to test the inhibition of a CAT gene reporter under the control of the wild-type wtGLUT1 promoter or the mutated mut2GLUT1 promoter, which lacks canonical Sp3 binding sites (Figure 1) (26), in transiently transfected cells, in experiments driven by the intracellular (endogenous) Sp1 and Sp3 and the basal transcription machinery. Second, WP631 was used to inhibit transiently transfected cells with either the wtGLUT1 or mut2GLUT1 promoters, in cells also cotransfected with an Sp1- or an Sp3-expression plasmids, or both, to obtain various Sp1/Sp3 ratios. Third, EMSA analysis was used to assess the differences in Sp1-binding affinity for the two GLUT1 promoters in vitro and to elucidate how the Sp1 binding to the promoter regions was affected at the molecular level by the presence of increasing concentrations of WP631.

**Cell Growth in the Presence of WP631.** Proliferation of Jurkat T lymphocytes treated with three concentrations of WP631 (60 nM, 600 nM, and 1 µM) for 24 h is presented in Figure 2A. WP631 at a concentration of 60 nM (which corresponds to its IC\textsubscript{75}, as determined after 72 h of treatment (31)) did not alter the percentage of proliferating cells compared to the untreated control. Only 55% cells treated with 600 nM were proliferating, and 40% were proliferating in the presence of 1 µM WP631. However, the MTT assay fails to distinguish between growth arrest and a reduction in cell number due to cell death. Therefore, the number of viable cells was also determined as the capacity of these cells to exclude the Trypan Blue dye in the experimental conditions shown in Figure 2. Trypan Blue assays indicated that about 5% (600 nM) and 13% (1 µM) cells died during the 24 h treatment. Since the number of proliferating cells decreased after 24 h of treatment with 600 nM or 1 µM while most of these cells remained viable, we also analyzed the cell-cycle distribution to identify the phase of the cell cycle at which proliferation stopped. Figure 2B displays the cell cycle distribution of Jurkat cells treated with various concentrations of WP631 for 24 h. Cells accumulated, in a dose-dependent manner, in G2 after 24 h of treatment (Figure 2B). Cells in G2 were committed to die after longer incubation time (data not shown) in agreement with our previous observations (31). Therefore, experiments on gene transcription under strict
cotransfection with an Sp3-expression plasmid should produce a large reduction of the Sp1/Sp3 ratio, which might alter the activity of either wtGLUT1 and mut2GLUT1 promoters, as described below.

**WP631 Inhibits the Expression of GLUT1 Promoters Driven by the Endogenous Transcription Machinery.** Results plotted in Figure 4 (bars wt and mt, and their equivalent bars in Figure 1) would indicate that the transient expression of the construct containing wtGLUT1 was more activated by the endogenous cell transcription machinery. The presence of 60 nM WP631 in the cell culture media sufficed to inhibit, by about 30%, the CAT reporter activity linked to either promoter under the control of the endogenous Sp1 and Sp3 and the basal transcription machinery of lymphocytes (cf. bars wt and mt versus wt + W and mt + W in Figure 4). WP631 and endogenous Sp1 and Sp3 protein factors might compete for DNA binding, with almost identical WP631 efficiency, to attenuate the transcription of both promoters. A lower WP631 concentration, 6 mM, was also assayed, but it did not alter the GLUT1-driven transcription significantly (not shown).

**In the Presence of Cotransfected Sp1 and Sp3, the Transcription of wtGLUT1 and mut2GLUT1 Promoters Was Differently Inhibited by WP631.** We compared the relative CAT activities in cells transfected with GLUT1 promoters in the presence/absence of cotransfected Sp1 and/or Sp3 and WP631 (Figure 4, bars a−l).

Overexpressed Sp1 activated gene transcription similarly in experiments using both promoters (Figure 4, bars a and c), which was inhibited by bisanthracycline WP631, yet with distinct effectiveness: 30% inhibition versus 100% inhibition (Figure 4, bars b and d). The effect of WP631 was stronger on the mut2GLUT1 promoter, which contains a high-affinity CGTACG binding-site for WP631 (Figure 1) (33). For the sake of comparison, Figure 4 displays, side by side, the experimental conditions with and without WP631. WP631 and overexpressed Sp3 did not suffice to completely inhibit the transcription of the wtGLUT1 promoter in vivo in the presence of overexpressed Sp1 (Figure 4, bar j), but they did when the mutant promoter was used (Figure 4, bar l). In all cases analyzed, the presence of WP631 significantly inhibited gene transcription (Student’s t-test, *p* < 0.05; see legend to Figure 4), except when the wtGLUT1 promoter was cotransfected with both expression plasmids. In this case, the drug did not significantly alter the transcription levels (Figure 4, bars i and j), which is further analyzed in the Discussion section. Taken together, these results are in agreement with the view that the Sp1/Sp3 ratio determines the transcription levels of GLUT1 promoters in proliferating cells (26–28) and the three distinct gene isoforms encoded by the Sp3 expression plasmid ((29) and Figure 3) act as repressors on GLUT1 promoters (26).

**Sp1/Sp3 Protein Ratio Determines the wtGLUT1 Promoter Activity in Transfected Lymphocytes and Modulates the Effects of WP631.** The control of transcription by the Sp1/Sp3 ratio was corroborated using the wtGLUT1 promoter, whose activity was inhibited by Sp3 in the absence of cotransfected Sp1 (Figure 4, bar e), which correlates with the changes in protein levels detected by Western blot (Figure 3). In addition, the presence of Sp3 protein and WP631 abrogated the transcription activity of the wtGLUT1 promoter (Figure 4, bar f). Cotransfection with both protein-expression
plasmids almost halved the CAT reporter activity driven by the wtGLUT1 promoter (cf. bars a and i in Figure 4), suggesting that overexpressed Sp3 partially inhibited Sp1 binding to the wtGLUT1 promoter. WP631 did not act efficiently when the two proteins were overexpressed (Figure 4, bar j), in contrast to what was observed in the experiments performed without cotransfection with protein expression plasmids (cf. bars wt + W and j in Figure 4). In any case, the results obtained with the wtGLUT1 promoter do not allow us to differentiate clearly the inhibition by Sp3 from that caused by the drug.

Using mut2GLUT1 Promoter Provides Direct Dissection of WP631 Effects on Sp1-Activated Gene Expression in Vivo. The capacity of WP631 to inhibit DNA–Sp1 interactions in vivo was further supported by experiments using the mut2GLUT1 promoter, whose transcription was completely inhibited by WP631 (Figure 4, bars h and l). We would like to highlight that the Sp3-binding site is canceled in this promoter (26, 30), while EMSA experiments showed, see below, that Sp1 can bind to this promoter yet with a lower affinity than to the wild-type promoter (Figure 5A), in agreement with the observed Sp1-activated transcription of the mut2GLUT1 promoter (Figure 4) (26).

In the presence of overexpressed Sp1, WP631 reduced the Sp1-activated transcription levels in the mut2GLUT1 promoter by more than about 100% (Figure 4, bar d). This suggests that the presence of a specific drug-binding site accounted for the differences observed. We found the same degree of inhibition by WP631 in lymphocytes cotransfected with Sp1 plus Sp3, in agreement with the fact that Sp3-binding site is canceled in this promoter (26) (cf. bars d and l versus bars c and k in Figure 4). Unexpectedly, cotransfection of Jurkat T cells that contained the transfected mt2GLUT1 promoter with Sp3-expression plasmid resulted in a lower activity compared to the same promoter driven by the endogenous proteins (cf. bar g versus bar mt in Figure 4). This behavior may be accounted for by postulating that the cotransfected “extra” Sp3 (Figure 3) inhibited transcription by squelching intracellular Sp1; thus, the effective concentration of Sp1 that can bind to the mut2GLUT1 promoter was reduced. When the two transcription factors were cotransfected, the mut2GLUT1 promoter activity was higher than in the presence of overexpressed Sp3 (cf. bars k and g in Figure 4). In fact, the CAT activity measured after cotransfection was higher (Figure 4, bar k) than in the mutant promoter driven by the endogenous proteins (Figure 4, bar mt), a result that emphasizes the importance of the relative Sp1 and Sp3 levels (26) in the behavior of the GLUT promoters.

In contrast to the transcription driven only by the endogenous cell factors, WP631 exerted a stronger effect on the mut2GLUT1 promoter, regardless of the presence of cotransfected Sp1, Sp3, or Sp1 plus Sp3 (Figure 4, bars d, h, and l). Our data support that WP631 was the unique active inhibitor of the mutated promoter and that it directly inhibited the Sp1–DNA interaction in vivo.

Sp1–Promoter Complexes Are Distinctly Susceptible to WP631 in Vitro. Figure 5 shows band shift (EMSA) analyses of the Sp1 binding to 62-mer fragments of the wtGLUT1 and mut2GLUT1 promoters (Figure 1), which contain consensus protein-binding sites. Cancellation of the Sp3 site in mut2GLUT1 did not destroy the overlapping Sp1-binding
site but altered the putative Sp1-binding sequence (26), yet Sp1 transactivated the mutated promoter (Figure 4). Experiments performed in parallel using both promoter fragments demonstrated that the Sp1 binding to the wild-type promoter was about 6-fold higher than to the mutant promoter in the presence of a large excess of competing DNA (poly[d(I-C)]) (Figure 5A).

Figures 5B and 5C show representative examples of experiments carried out in duplicate, in which WP631 inhibits the formation of Sp1–DNA complexes. When a fragment of the wild-type promoter was used, 600 nM WP631 inhibited the formation of Sp1–DNA complex by about 40%. The same WP631 concentration inhibited completely the Sp1–DNA interaction when the mutant promoter was used (Figures 5A and 5B). These results suggest that the protein binds tighter to the wtGLUT1 promoter. In the EMSA experiments, 300 nM WP631 achieved 35% inhibition of the Sp1–DNA band for the wild-type promoter, while approximately the same inhibition required only 150 nM WP631 in the mut2GLUT promoter, in which 300 nM drug produced a 47% inhibition (Figures 5B and 5C). These results were consistent with previous studies on Sp1 binding to each promoter, which revealed stronger Sp1-binding to wtGLUT1 (26), and they are also in agreement with the distinct susceptibility of the two promoters to transcription inhibition by WP631. Nevertheless, the drug concentrations required to disrupt the complex in vitro (Figure 5) and to inhibit the CAT reporter activity (Figure 4) were not quantitatively comparable because of the differences in experimental conditions.

**DISCUSSION**

Previous studies have shown that WP631 is a potent inhibitor of transcription, as this bisanthracycline inhibits Sp1-activated transcription at low nanomolar range concentrations both in vitro and in vivo (cell culture) (15, 18, 19, 22). However, certain drugs can lose “potency” as assay conditions become more complex (13, 34). Therefore, we aimed to dissect the inhibiting effects produced by the drug from those arising from the presence of some transcription factors binding to overlapping sequences. We have shown that the conditions used in our experiments (60 nM drug, CAT determination performed 24 h after transfections) were essential to ensure that cells proliferated in the presence of the drug (Figure 2) and that the cotransfected expression plasmids were functional. These observations are consistent with the view that transcriptional activity depends on a given Sp1/Sp3 ratio, which can be modified by transient expression of plasmids (Figures 3 and 4), and it is subjected to changes in cell cycle and cellular conditions (6). They are also consistent with the view that cells with enhanced growth are more susceptible to changes in gene expression produced by WP631 than barely proliferating cells (35).

We sought to gain new insights into the WP631 effects on transcription in vivo by testing whether the Sp3 protein, whose putative binding sequence overlaps the Sp1 site, inhibits Sp1-activated transcription and interferes in drug–DNA interactions. We took advantage of the mut2GLUT1 promoter that lacks the Sp3-binding site (26), while encompassing a high-affinity binding site for WP631 (CGATCG) elucidated by NMR and X-ray studies (33, 36), to dissect the transcription attenuation produced by the drug (Figure 4).

Sp3 has been described as a bifunctional transcription regulator because it can repress Sp1-mediated transcription by competing with Sp1 for their overlapping binding sites or behave as an activator of gene transcription (4, 24, 37). The Sp3/Sp1 ratio is known, for example, to modulate the
transcription of the GLUT1 promoter during myogenesis (26, 27). In this paper, we have differentiated some specific effects of Sp1, Sp3, and WP631 on the transcription of the GLUT1 promoter (Figure 4). High levels of endogenous Sp1 have been described in Jurkat T cells (38), which may limit the response to additional (transiently expressed) Sp1, yet about a 12% increment in the Sp1 protein levels can be detected after cotransfection experiments (Figure 3). Thereby, the similar levels of CAT reporter activity observed under the control of the wtGLUT1 promoter (Figure 1, and Figure 4, bars wt and a). However, coexpression of Sp1 and Sp3 altered the situation in transiently transfected Jurkat T cells. The presence of overexpressed Sp3 (Figure 3) reduced significantly the Sp1/Sp3 ratio and inhibited wtGLUT1 promoter activity even in the presence of additional Sp1 (Figure 4, bars a and i), which resembles the regulation of the GLUT1 promoter in some cells (26). These results are in agreement with the view that the Sp1/Sp3 ratio, which changed depending on the cotransfected plasmids (Figure 3), determines the transcription levels of GLUT1 promoters in proliferating cells (26–28) and in accordance with the view that the three distinct gene products encoded by the Sp3 expression plasmid (29) act as repressors in GLUT1 promoters (26). Inside cells, the binding of one or another of these protein factors to the overlapping sites may depend not only on their relative concentrations but also on the intrinsic affinities of Sp1 and Sp3 for their putative sites. The finding of gene repression after the cotransfection with wtGLUT1 promoter is consistent with previous studies showing that Sp3 efficiently competes for the binding to the wtGLUT1 promoter (26, 28). Sp3 partially inhibited the activation by Sp1 in this promoter (Figure 4).

WP631 shows a clear preference for interfering with Sp1–DNA complexes (10, 15, 18, 19, 39). Our results support the hypothesis that it is an extremely potent inhibitor of Sp1 binding in vivo (19, 22) and also indicate that drug effects depend on two key elements: the relative levels of Sp3 protein and the exact composition of the Sp1 binding site, since the drug effects on activated transcription differed in wtGLUT1 and mut2GLUT1 promoters (Figure 4). WP631 binds better to the mutant promoter (Figure 5), which encloses a high-affinity binding site (Figure 1). This is consistent with the fact that higher concentrations of the bisanthracycline were required to disrupt the tighter binding of Sp1 to the wtGLUT1 promoter in vitro (Figure 5). Previous experiments, using an unrelated promoter, showed the strong capacity of WP631 to inhibit Sp1–DNA complexes, even in the absence of a canonical 6 bp binding site for WP631 (15), thus suggesting that WP631 binding is facilitated by Sp1-induced bending (40).

As mentioned in Results, the experiments using wtGLUT1 cotransfected with Sp1 and Sp3 plasmids brought about a condition in which the presence of added WP631 in transiently transfected Jurkat T lymphocytes did not abrogate gene activity, since the drug did not compete efficiently when the proteins were in excess (Figure 4, bars i and j). WP631 may thus be incapable of increasing the inhibiting capability of Sp3 because the protein and the drug can compete for overlapping binding sites. Further discussion on the origin of the competition between both proteins and WP631 can be only tentative. Figure 6 presents a model in which DNA bending by Sp3 can be required for correct (efficient) drug binding, as suggested for a Sp1–WP631–DNA complex (15). The WP631 molecule is rather flexible and may monointercalate at isolated CG sites, yet with a lower binding affinity than when bisintercalating at 6 bp CG-rich tracts (41). We envisage a scenario, schematized in Figure 6, in which, to some extent, the presence of drug molecules, monointercalated in some neighboring CG sites, should alter protein binding after that drug intercalation unwinds the double helix, thereby changing the Sp3-binding affinity, which in turn allows the binding of overexpressed Sp1. DNA bending can be induced by Sp1 or Sp3 upon binding (40), and the change in DNA structure would facilitate the formation of bisintercalated WP631–DNA complex. The DNA unwinding produced by bisintercalated WP631 is known to displace the Sp1 binding to DNA (15). It is worth noting that WP631 completely abrogated the mut2GLUT1 activity in vivo, even though Sp3 did not bind to the mutated promoter. Simply, the protein-induced bending of DNA was not required because the promoter encompassed a specific 6 bp drug-binding site (Figure 1).
Neither of the techniques used here can establish the nature of the structural variations occurring after binding by itself, but the magnitude of Sp1-induced DNA bending is known to have key consequences on drug binding (15). In any case, the protein-induced curvature of a particular promoter may depend on the exact composition of the G-box and the neighboring sequences (42). Sp1 binds to the wtGLUT1 promoter better than to the mut2GLUT1 promoter (Figure 5) (26), while WP631 appears to better inhibit the Sp1-binding to the mut2GLUT1 promoter by two complementary, Sp3-independent mechanisms. First, WP631 binds tightly to the mut2GLUT1 promoter that contains a high-affinity CGATCG binding site. Second, it competes more efficiently with Sp1 (Figure 5), which, in turn, displays a looser binding to the mut2GLUT1 promoter. 

The effects of WP631 on both transiently expressed GLUT1 promoters were considered to be independent of those on other intracellular genes, which either contain Sp1 binding sites (including an active endogenous GLUT1 promoter in lymphocytes) or can be indirectly repressed through the complex network of control pathways in the cell. Inside the cells, several genes involved in cell cycle control may be regulated by Sp1 and/or Sp3 and by some gene products that could directly interact with Sp1 (8, 9). Our approach has allowed us to dissect WP631 and Sp3 effects on Sp1-transactivated transcription in vivo. The concentration of WP631 used in the experiments with Jurkat T lymphocytes (60 nM WP631) is lower than that normally used to inhibit transcription by other DNA-binding ligands (12, 13, 43), and thus it is more likely to be attained in physiological conditions (19, 22, 31). In fact, an analysis of the WP631 uptake rate revealed that even smaller concentrations of the drug may be located inside the nuclei of cells treated with this drug concentration (31), and thus the transcription-inhibiting effect may well be due to a rather lower intracellular concentration of WP631, within the nanomolar range.

Collectively, our results show that WP631 specifically inhibits Sp1-activated transcription and may compete with Sp3 binding to finally inhibit transcription in vivo. Hitherto, only the unrelated drug cetelesicin 743, which does not intercalate into DNA (16, 44), also recognizes Sp1–DNA at low nanomolar concentrations and inhibits transcription (45).

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Circumvention of the multidrug-resistance protein (MRP-1) by an antitumor drug through specific inhibition of gene transcription in breast tumor cells

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Abstract

Multidrug resistance protein 1 (MRP-1) confers resistance to a number of clinically important chemotherapeutic agents. The promoter of the mrp-1 gene contains an Sp1-binding site, which we targeted using the antitumor drug WP631. When MCF-7/VP breast cancer cells, which overexpress MRP-1 protein, were incubated with WP631, the expression of the multidrug-resistance protein gene decreased, which did not occur with doxorubicin. The inhibition of gene expression was followed by a decrease in the activity of the MRP-1 protein. The IC75 for WP631 (drug concentration required to inhibit cell growth by 75%) circumvented the drug pump efflux, without addition of resistant modifiers. After treatment with WP631, MCF-7/VP cells were committed to die, although they lacked functional caspase-3, by mitotic catastrophe, while they were resistant to doxorubicin. This is the first report on an antitumor drug molecule inhibiting the mrp-1 gene directly, rather than being simply a poor substrate for the transporter-mediated efflux. However, both situations appeared to coexist, thereby a superior cytotoxic effect was attained. Ours results suggest that WP631 offers great potential for the clinical treatment of tumors displaying a multidrug resistance phenotype.

Keywords: Multidrug resistance; MRP-1; Breast cancer; Mitotic catastrophe; WP631
Introduction

Multidrug resistance is a major impediment to the successful treatment of cancer. Although the cellular bases underlying drug resistance are not fully understood, several factors that contribute to its development have been identified [1]. Tumor cells often gain drug resistance through the overexpression of membrane transport proteins that effectively efflux anticancer drugs [1, 2]. Increased transmembrane efflux of antitumor drugs is one of the best-characterized mechanisms of MDR and is mediated through the overexpression of ATP-binding cassette (ABC) transporter superfamily members, for example P-glycoprotein (MDR-1) and the multidrug resistant-associated protein (MRP-1) [1, 3-5]. The mrp-1 gene encodes for a transmembrane protein [6], originally isolated from a doxorubicin-selected lung cancer cell line [7], and confers the ability to reduce drug accumulation in cells [1]. MRP mediates resistance to several anthracyclines, Vinca alkaloids and etoposide, but not to taxanes [4] and camptothecin [5]. MRP-1 is overexpressed in some patients with relapsed acute leukemia [8] and it is expressed in patients suffering invasive breast cancer, offering useful prognostic information [9].

MCF-7/VP breast cancer cells were originally derived from MCF-7 cells after selection with etoposide (VP-16), and they have been shown to overexpress the mrp-1 gene [10]. MCF-7/VP cells have often been used to evaluate the effects of several drugs on multidrug resistance [3, 10-12]. Parental MCF-7 cells are more susceptible to doxorubicin than to bisanthracycline WP631, which appears to be more cytotoxic in MCF-7/VP cells [13].

There is convincing evidence that highly lipophilic molecules are poor substrates for the MRP-1 pump efflux [2, 11]. Some anthracyclines [2, 11], including the novel bisanthracycline WP631 [13], circumvent to some extent the MRP-1 pump efflux in MCF-7/VP cells, thus enhancing the cytotoxic effect at nanomolar concentrations. We sought to gain insight into the mechanisms used by WP631 to exert its cytotoxicity in MRP1-expressing breast carcinoma cells. We, along with others, have documented that WP631 is a potent inhibitor of basal and Sp1-activated transcription [14-18]. The mrp-1 gene promoter contains an Sp1 binding site, which is
involved in the regulation of its gene transcription [19]. Therefore, we aimed to determine whether WP631 directly inhibits the expression of \textit{mrp-1}. Bisanthracycline WP631 binding to DNA would thus reduce the MRP-1 mRNA produced by the cells and thereby decrease, in turn, the MRP-1 pump efflux.

**Materials and methods**

**Doxorubicin and WP631**

WP631 was synthesized as described elsewhere [13]. Solutions containing 500 \( \mu \)M doxorubicin (Sigma, St. Louis, MO) or WP631 were prepared with sterile 150 mM NaCl, maintained at -20 °C, and brought to the final concentration with RPMI 1640 medium just before use. Exponentially growing cells subcultured at a density of 5 x 10^4 cells/ml were incubated with various concentrations of doxorubicin or WP631 at 37 °C for times ranging from 24 to 96 hours.

**Cell line and culture conditions**

MCF-7/VP breast tumor cells were a gift of Dr. K. H. Cowan (University of Nebraska Medical Center). Cells were maintained in RPMI 1640 medium (Gibco, Life Technologies, Prat de Llobregat, Spain) supplemented with 10% fetal bovine serum (Gibco) 100 U/ml penicillin, 100 \( \mu \)g/ml streptomycin, 2 mM L-glutamine (Gibco) and 2 mM HEPES (pH 7.4), at 37 °C in a humidified atmosphere with 5% CO\(_2\).

**Cytotoxicity assays**

The effects of doxorubicin and WP631 on MCF-7/VP cell growth were determined by the MTT dye assay [20] in 96-well microtiter plates with flat-bottomed wells in a total volume of 100 \( \mu \)l. After incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to each culture (15 \( \mu \)l (50 \( \mu \)g)/well). The dark-colored crystals produced by proliferating cells were solubilized with 30 mM HCl in 2-propanol. Absorbance was determined at 570 nm using a SPECTRAmax 250 microplate reader (Molecular Devices, Sunnyvale,CA). Viable cell number was determined using the exclusion of trypan blue dye (Sigma) and a
Assessment of multidrug resistance gene expression

Total RNA was isolated, after 24 h, from control cells (those to which no drug was added) and cells treated with either the IC50 for doxorubicin, the IC50 or the IC75 for WP631 using the Ultraspec RNA isolation reagent (Biotecx, Houston, TX) following the vendor’s guidelines. RNA samples were treated with RNAse-free DNAse I (Roche Diagnostics, Mannheim, Germany) and phenol-extracted. The occurrence of transcripts of both \textit{mdr-1} and \textit{mrp-1} genes in MCF-7/VP cells was assayed by RT-PCR in the absence of any drug, using the primers indicated in the legend to Fig. 1. Changes in \textit{mrp-1} gene expression were analyzed in the presence of doxorubicin or WP631 by semi-quantitative RT-PCR using the housekeeping \textit{GAPDH} gene as an internal normalization standard. The primers used (0.6 µM) are indicated in the legend to Fig. 1. Sub-saturating RT-PCR conditions were adjusted to 10 ng of total RNA and 25 amplification cycles, with the annealing reactions carried out at 50° C for 1 min, using the OneStep PCR kit (Qiagen, Servicios Hospitalarios, Barcelona, Spain) following the manufacturer’s instructions. PCR samples were electrophoresed in a 2% agarose gel, stained with ethidium bromide and quantified using a Molecular Dynamics densitometer (Sunnyvale,CA).

Measurement of drug transport activity mediated by MDR and MRP1 proteins

Drug transport activities of the multidrug resistance protein MDR-1 (P-Glycoprotein) and MRP-1 were assayed using the MultiDrugQuant Assay kit (Chemicon International, Pacisa-Giralt, Barcelona, Spain), which is based on the measurement of the intercellular fluorescence of the fluorogenic dye calcein using a flow cytometer. Selective inhibitors, provided within the kit, allowed us to measure both MDR-1 and MRP-1 multidrug activities separately. Changes in the intracellular green fluorescence of calcein were measured in living, propidium iodide-negative, cells in a Coulter Epics-XL flow cytometer (Coulter Corporation, Hialeah, FL) using the equipment sets described in the kit instructions. Measurements were made on cells treated with the IC50 or IC75 for WP631 or the IC50 for doxorubicin and compared with untreated cells.
MRP-1-related activity was calculated following the manufacturer’s instructions.

**Quantification of intracellular drug accumulation**

Cellular accumulation of doxorubicin or WP631 was quantified spectrofluorimetrically as described elsewhere [21]. In brief, cells were incubated with various concentrations of either doxorubicin or WP631 for the times indicated in the legend to Fig. 2D. They were then rinsed three times with ice-cold RPMI 1640 medium, and the drugs were extracted from the cells using 2 ml of 80 mM HCl in 2-propanol for 16 h at 4°C. The concentrations of the two drugs were measured using a Shimadzu RF-1501 spectrofluorophotometer (Shimadzu, Columbia, MD) with an excitation wavelength of 480 nm and an emission wavelength of 555 nm. The fluorescence intensity emitted was translated into concentrations of drug using a doxorubicin or WP631 standard curve, and expressed as ng drug/10^7 viable cells, assessed before and after treatment by exclusion of trypan blue dye.

**Cell cycle distribution**

After treatment with doxorubicin or WP631 for various periods of time, the cells were harvested and stained with propidium iodide (Sigma) as described elsewhere [22]. Nuclei were analyzed with a Coulter Epics-XL flow cytometer (Coulter Corporation, Hialeah, FL) using the 488 nm line of an argon laser and standard optical emission filters. The percentages of cells at each phase of the cell cycle were estimated from their DNA content histograms after drug treatment.

**Cytological analysis of multinucleated cells**

A CompuCyte Laser Scanning Cytometer (Compucyte; Cambridge, MA) was used for morphological observation of multinucleated cells. The presence of multinucleated cells was assessed on microscope slides containing samples prepared as described above for flow cytometry. After establishing a scan area, the slides were analyzed using a 40x objective and 5 mW of Argon laser power. The entire cell preparation was examined. Using the WinCyte 3.4 software (Compucyte), a cell gallery was created by relocation of cells from each of the major
peaks in the histogram of integrated red fluorescence. Polyploidy was determined by setting an appropriate histogram gate, while the morphology was established under the microscope. We deemed that enlarged cells containing multiple evenly-stained nuclei (polyploid, multinucleated cells) underwent mitotic catastrophe [23, 24], provided that they did not recover in fresh drug-free medium.

For the analysis of mitotic figures in drug-treated cells, about 50-100 µl of cells in RPMI medium were dropped on clean slides with a Pasteur pipette and left to air-dry. Cells were fixed using Carnoy’s solution (3 vol. methanol, 1 vol. glacial acetic acid). Slides were stained with Leishman’s stain (BDH, Poole, UK) diluted in methanol for 30 min, and examined under a Carl-Zeiss Axiophot microscope.

**Clonogenic and proliferation assays**

After 96-h continuous treatment with either the IC50 for doxorubicin or the IC50 for WP631, MCF-7/VP cells were allowed to grow for 16 days in drug-free fresh medium. Cells that remained attached were harvested to assay their clonogenic capacity. They were subcultured at a seeding density of $10^4$ cells/60 mm diameter dish in experiments performed in triplicate. A fresh culture of $10^4$ cells was used as control, untreated, cells because the control used up to 96 h plus 12 days showed that most cells were dead owing to nutrient shortage (see Results for details), and so we could not use them during clonogenic experiments. This allowed us to obtain isolated cells for clonogenic assays, clonogenicity being measured as the number of colonies containing 50 or more cells compared with the total number of colonies. Cells were maintained at 37°C, with fresh medium added every 2-3 days. In this way, we may expect exponentially growing cells to raise clones containing more than 50 cells after 11 days. One of the dishes was used to assay the presence of cell division using a fluorescent dye, as described in the following paragraph, and viability was determined by the exclusion of trypan blue dye on a hemocytometer. After three days, the remaining two dishes were used to measure the number of viable cells, determined by the exclusion of trypan blue, and to assay the cell cycle distribution.
Cells grown as described in the previous paragraph were labeled with Carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) and plated at 10⁶ cells to analyze cell division and cell-cycle distribution. They were allowed to recover in drug-free medium for up to 3 days, and cytoplasmic fluorescence was quantified using a Coulter Epics-XL flow cytometer. Covalently bound CFSE was evenly distributed into daughter cells, allowing the discrimination of successive rounds of division [25].

Results

Early studies have demonstrated that WP631 is much more cytotoxic against the MCF-7/VP cell line than doxorubicin, suggesting that WP631 can circumvent multidrug resistance in this breast tumor cell line [13]. We have both confirmed these initial cytotoxicity experiments and determined the IC50 (drug concentration required to inhibit cell growth by 50%) for both drugs, as well as, for the very first time, the IC75 (drug concentration required to inhibit cell growth by 75%) for WP631 and doxorubicin. The IC50 were 306.4 ±1.3 nM for doxorubicin and 122.1 ±0.1 nM for WP631 respectively. The IC75 value obtained for doxorubicin was higher than 3 µM, but only 594.3 ±3.2 nM for WP631. As expected, WP631 achieved higher cytotoxicity than doxorubicin, although the WP631 concentration required to halve cell growth was barely lower than that obtained with another MCF-7/VP clone [13]. The rather high concentration of doxorubicin needed to inhibit proliferation by 75% agreed with the known resistance of the MCF-7/VP cells to doxorubicin [10]. This micromolar doxorubicin concentration was not used in the following experiments, since it represents a supraclinical concentration.

RT-PCR analysis of the expression of mdr-1 and mrp-1 genes in exponentially growing MCF-7/VP cells showed that the cells expressed mrp-1, but not the mdr-1 gene, at least significantly (Fig. 1A), in agreement with previous reports of scarce MDR-1 activity in these cells [10, 26]. Figure 1B shows a partial sequence of the mrp-1 promoter, which contains a multiple Sp1 binding site [19]. This CG-rich sequence may be targeted by WP631, and thus the
drug would compete with Sp1 protein for their common binding sites, as observed for other gene promoters [14-16]. MCF-7/VP cells were incubated with the IC50 doses for doxorubicin or WP631 and the IC75 dose for WP631, to analyze by semiquantitative RT-PCR the effects of these anthracyclines on the expression of the *mrp-1* after 24 and 96 h of continuous treatment with the drugs (Fig. 1C). After 24-h treatment with 594 nm WP631 (IC75), *mrp-1* expression was inhibited by about 20%, while *mrp-1* gene expression was inhibited by more than 50% after 96 h of continuous treatment (Fig. 1C). The 50% inhibition scored in the presence of WP631 may represent a lower limit, and higher inhibition may occur in some cells.

After observing the dose-dependent effects of WP631 on gene transcription, we tested whether the interference of the drug with gene expression was followed by a lower activity of the multidrug resistance-associated MRP-1 protein, which may explain the capacity of WP631 to overtake the pump efflux effect. We took advantage of the fluorescence of intracellular calcein to determine, by flow cytometry, the accumulation of this molecule in the presence of well-characterized MDR-1 (which was not present in MCF-7/VP cells—see above-) and MRP-1 inhibitors, and WP631 or doxorubicin. Figure 2 shows the effects of ‘inhibitor 1’ (provided within the MultiDrugQuant assay kit—see Materials and methods—) on the efflux by both MDR-1 and MRP-1 in the absence/presence of any of the anthracyclines used in this study, while ‘inhibitor 2’ (also provided within the kit) was known to affect solely the MRP-1 pump efflux. Since MCF-7/VP cells did not express *mdr-1* significantly (Fig. 1A), the flow cytometry profiles in the presence of both ‘inhibitors’ coincided after 24 h and 96 h, in the absence of either doxorubicin or WP631 (cf. columns *a* and *b* in Figs. 2A and 2B). After 24-h treatments, and with the IC50 for both drugs, changes in the effect on intracellular fluorescence (calcein content) were negligible (column *c* in Fig. 2A). All cytometric fluorescence profiles analyzed are merged in column *d* of Fig. 2A. In the presence of doxorubicin, protein activity was slightly enhanced (Fig. 2C), while with the IC75 for WP631, it decreased by about 20% (Fig. 2A and 2C).

Figure 2B shows the effects of doxorubicin and WP631 on intracellular calcein fluorescence...
after 96-h treatments. While doxorubicin slightly affected the transporter-mediated efflux, the two concentrations of WP631 reduced MRP-1 activity differently (column c in Fig. 2B). The IC50 for WP631 split the cell population into two parts, some cells showing little pump efflux and others MRP1-mediated efflux (column d in Fig. 2B). It is remarkable that the IC75 for WP631 reduced MRP-1 activity by more than 50% after 96 h of treatment, as depicted by the merging of fluorescence profiles around a single maximum in column d of Fig. 2B. All the effects produced by WP631 were unequivocally ascribed to MRP-1, since the efflux inhibition caused by WP631 and ‘inhibitor-1’ together, and that caused by WP631 alone merged in the same cytometric maximum of intracellular calcein fluorescence. WP631 affected MRP-1 activity in MCF-7/VP breast cancer cells, in agreement with similar changes found in mrp-1 transcription (Fig. 1C). Transcription was halved after 96 h, which agrees with the lower MRP1-efflux activity detected after 96 h. Moreover, the results analyzed in Figs. 2A and 2B confirmed that there was no MDR-1 activity.

It is noteworthy that the experiments shown in Figs. 2A and 2B measured the intracellular calcein content rather than the uptake and/or release of WP631. The lack of changes in calcein content after treatment with WP631 may be due to some sort of saturation by this drug of the MRP-1 transporter-mediated efflux that was somewhat trying to eject the bisanthracycline. Thus, the levels of calcein may remain unaltered because not enough molecules of transporter were available. However, this does not appear to be the case because the transporter-mediated efflux prevented the accumulation of doxorubicin, while there was enough MRP-1 protein to eject and important portion of both the intracellular calcein and doxorubicin (Fig. 2). We previously demonstrated using Confocal laser microscopy WP631 is accumulated in the nuclei of susceptible cells [21].

To confirm that the effect on multidrug resistance was mediated by the presence of WP631 inside the cell, we directly measured WP631 accumulation in MCF-7/VP cells (Fig. 2D). The time-dependent uptake of doxorubicin and WP631 by MCF-7/VP cells, as determined
spectrofluorimetrically (Fig. 2D), was correlated with time-dependent changes in MRP-1 activity (Fig. 2C). It was evident from the very beginning of the incubation with WP631 that this drug circumvented the MRP-1 pump efflux. WP631 accumulation inside cells was equivalent after 48 h, in spite of the drug concentrations in the culture medium (Fig. 2D). However, cells accumulated rather low quantities of doxorubicin, owing to the MRP1-mediated efflux, regardless of the incubation time (Fig. 2D). Moreover, the IC75 resulted in an enhancement of WP631 uptake, which reached a maximum after 96-h treatment, while after about 48-h treatment with the IC50, the drug was progressively ejected out of the cells. The experimental observation agreed with the requirement of ‘sufficient’ intracellular drug accumulation to inhibit Sp1-driven transcription of mdr-1 (cf. Figs. 1C and 2D).

Given that the IC75 WP631 inhibited mrp-1 gene transcription and circumvented the resistant phenotype (Figs. 1 and 2), we explored whether MCF-7/VP cells were prompted to rapid cell death after the drug accumulated in sufficient amounts. As MCF-7 breast cell lines do not express caspase-3 [27], we aimed to understand how WP631 achieved its high cytotoxicity, despite the lack of apoptotic response via caspases. The MTT assay was used to determine the cytotoxicity of WP631 and doxorubicin (see above). However, this assay fails to distinguish between growth arrest and a reduction in cell number due to cell death. Therefore, to determine the capacity of doxorubicin and WP631 to produce MCF-7/VP cell death, adherent cells were loaded on a hemocytometer, and the number of viable cells was scored, based on the exclusion of trypan blue by viable cells. Figure 3A shows a time-course analysis of the percentage of cell death, compared with untreated cells, for a treatment that lasted 264 hours (96 h of continuous treatment, followed by 7 days in fresh drug-free medium). At both WP631 concentrations, there was a clear correlation between the percentage of cell death after 48 h (Fig. 3A) and the time-dependent accumulation of WP631 shown in Fig. 2D. When the IC75 for W631 was used, the number of dead cells reached 100% of the population after 96 h (Fig. 3A), while surviving (resistant) cells were detected when the IC50 for WP631 was used (Fig. 2). Moreover,
doxorubicin neither accumulated (Fig. 2D) nor committed a large number of cells to die (Fig. 3A).

We used flow cytometry to evaluate the effects of the IC50 for doxorubicin, and the IC50 and IC75 for WP631, on MCF-7/VP cell cycle progression. Untreated, control, adherent cells maintained an almost uniform distribution for more than 9 days (Fig. 3B). The IC50 for doxorubicin unpaired the cell cycle inducing G2/M arrest up to 96 h after treatment (Fig. 3B), which was followed by polyploidy (around 6 %) and a small sub-G1 peak of dead cells. Polyploidy corresponded to multinucleated cells (Fig. 5), which re-entered the cell cycle after recovering their proliferating potential, which supports the resistance of MCF-7/VP cells to doxorubicin. These results agree with the finding that resistant cells, selected by the IC50 doses of either anthracycline, were able to return to the diploid state and released mitotic descendents (Table I). The presence of polyploidy in resistant cells (i.e. those surviving after treatment with the IC50 for doxorubicin) (Fig. 3B), and of multinucleated cells (Fig. 5A), suggests that these cells entered an endocycle that provided an alternative to mitotic catastrophe. Cells treated with the IC75 for WP631 died after undergoing around 69% polyploidy (Fig. 3B, 96 h plus 3 days population), including 8n-ploidy, which was followed by multinucleation (Fig. 5B), the presence of aberrant mitotic figures, and, eventually, cell death by mitotic catastrophe (Figs. 3A and 5).

Flow cytometry analyses revealed, in keeping with trypan blue exclusion measurements, that the IC75 for WP631 committed the whole cell population to die, thus yielding a 100% cytotoxic effect on MCF-7/VP breast cancer cells (Fig. 2C). The time-dependent cytotoxic effects correlated agreeably with the changes in drug accumulation described above, which suggests that a more direct circumvention of the MRP-1 pump efflux, other than the effect on mrp-1 transcription, was also taking place. Figure 3C shows the flow cytometry analysis of both adherent and floating cells after a longer period of incubation in fresh medium. The IC50 of both WP631 and doxorubicin committed floating cells to die from G2. In the intervening time, adherent cells behaved after treatment as control ones (Fig. 3C), suggesting that MCF-7/VP cells
were split into two populations by the IC50 for WP631: sensitive (floating), and resistant (adherent) cells (Fig. 3C).

MCF-7/VP cells were treated with the fluorescent compound CFSE (see Materials and methods) to investigate whether the cells that remained attached after 7 or 12 days still proliferated following the change to a fresh drug-free medium (Fig. 3C). CFSE incorporated stably into the cell cytoplasm, and it was evenly divided between daughter cells upon division. Therefore, cell division was easily followed cytometrically by the changes in fluorescence (Fig. 4). Unlike in MCF-7/VP cells treated with the IC75 for WP631, which were committed to die by mitotic catastrophe, the analysis of cell division using CFSE showed that cells treated with the IC50 for either drug, which remained adhered, proliferated. Adherent cells predominantly in G1 phase underwent several divisions as shown by the decreasing amount of CFSE fluorescence, which made control, untreated cells, and those treated merge at the same fluorescence maximum (Fig. 4B). These cells, resistant to doxorubicin, and those selected by the low dose treatment with WP631, showed almost 100% clonogenic capacity. They grew to a confluence higher than 80% (Table 1) when they were allowed to recover in fresh drug-free medium.

Discussion

In this paper, we show that the antitumor drug WP631 inhibits the expression of the \textit{mrp-1} gene. The ability of WP631 to circumvent MRP-1 resistance \textit{via} direct inhibition of its gene transcription represents the first case, to our knowledge, in which an antitumor drug reaches modulation of a multidrug resistance phenotype through direct gene inhibition, rather than because the drug is a poor substrate for the transporter-mediated efflux, and without requiring the presence of resistant modifiers. MRP-1 inhibition appears to be dose-dependent, reaching more than 50 \% when MCF-7/VP breast cancer cells are treated with 594 nM WP631 (IC75). The ability of WP631 to both inhibit gene transcription and circumvent multidrug resistance is substantiated by a concomitant decline in the pump efflux activity of MRP-1. Therefore, cells treated with WP631 were committed to die within a few days after continuous drug treatment,
while MCF-7/VP cells were resistant to doxorubicin because the IC75 for this anthracycline was higher than 3 µM. Cells treated with the IC75 for WP631 were committed to die, after 3 days of treatment, through the accumulation of mitotic abnormalities, which resulted in mitotic catastrophe (Fig. 5). A loss of caspase-3 function is frequently observed in clinical samples of breast cancer [28]. Remarkably, MCF-7/VP cells died through mitotic catastrophe (Fig. 5) rather than apoptosis, as expected in caspase-3 deficient cells. The results obtained with doxorubicin (Figs. 2 and 3) were in line with that cells surviving to an insult might sometimes undergo somatic reduction, to return to the diploid state and release mitotic descendants [29]. The re-incorporation of some surviving cells into the cell cycle, after treatment with moderate (IC50) doses, may contribute to carcinogenesis, as cells still proliferated (Table 1 and Fig. 4). Cells treated with the IC50 for WP631 or doxorubicin proliferated after treatment, when they were incubated in fresh drug-free medium, and showed clonogenic capacity for more than 30 days. Since cells that survived to the insult may harbor repair capacity, our results emphasize the need to reach sufficient intracellular cell accumulation in order to obtain the desired therapeutic effect, even for a potent cytotoxic drug like WP631.

WP631 appears to accumulate in MCF-7/VP cells and circumvent the multidrug-resistance pump efflux, acting as a potent cytotoxic, without the requirement of additional molecules to modify multidrug resistance. This finding may be of potential clinical interest in the search for alternative treatment of carcinomas expressing MRP-1, since clinical trials conducted to evaluate the efficacy of inhibitors of multidrug resistance in tumors have often shown that the serum levels required to reverse or block the antitumor drug efflux may be hard to achieve [2]. There is evidence that lipophilic molecules are poor substrates for both MRP-1 and MDR-1 [11, 30, 31] owing to the preferential partition into lipidic structures, thus avoiding drug interaction with the efflux pump [2, 11]. This chemical property has been exploited in the design of improved anthracyclines. WP631 is a bisanthracycline that bears two chromophores [13, 32], and it is known to bind to DNA with high affinity, which is mainly driven by hydrophobic interactions.
Therefore, part of the WP631 cytotoxicity against cells expressing MRP-1 may arise from partial circumvention of the MRP-1 pump efflux given its lipophilic structure, which would make it a much poorer substrate for the MRP-1 protein, thus explaining its lower resistance compared to doxorubicin. Indeed, both IC50 and IC75 doses produced comparable accumulation of WP631 after 48 h, and MCF-7/VP cells were similarly committed to die by both concentrations (Fig. 3A). The higher accumulation of WP631 after short-time incubations (Fig. 2D) may be in part attributed to a reduced pump efflux, as observed for other lipophilic anthracyclines [2]. The inhibition of mrp-1 transcription can affect protein activity after longer incubation times, since it may require the translation of changes in gene transcription to lower amounts of functional protein to decrease MRP-1 activity (Fig. 2D).

In summary, WP631 may circumvent MRP-1 through its ability to avoid the efflux pump, but the direct effect of WP631 on gene transcription produces its superior cytotoxicity. In agreement with our findings on the importance of inhibiting mrp-1 gene transcription, it has been recently reported that the expression of MRP-1 in glioma cells can be reduced by an antisense oligonucleotide, thus increasing the sensitivity of tumor cells to chemotherapy [33]. Since both MRP and MDR proteins are the product of genes regulated by Sp1 [19, 34], and Sp1-binding to DNA is strongly inhibited by WP631 (Fig. 1 and [14, 16]), we foresee WP631 as a drug with potential clinical interest in the treatment of certain tumors displaying multidrug resistance phenotypes.

Acknowledgements

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S. M. was recipient of a doctoral fellowship from the CIRIT, Generalitat de Catalunya.

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LEGENDS TO FIGURES

Fig. 1. Expression of multidrug resistance genes in the MCF-7/VP breast cancer cells. (A) RT-PCR analysis of the expression of \textit{mdr-1} and \textit{mrp1} genes in MCF-7/VP cells. The specific primers used for RT-PCR amplification were: MDRdir, 5’-CCCATCATTGCAATAGCAGG-3’; MDRrev: 5’-GTTCAAACTTCTGCTCCTGA-3’; MRPdir, ACGGTCGGGGAGATTGTCAAC; MRPrev, GCCCAGATTCAGCCACAGGAG-3’. There was no \textit{mdr-1} expression in MCF-7/VP cells. (B) Partial sequence of the proximal promoter of the human \textit{mrp-1} gene (adapted from [19]; Ensembl gene ENSG00000103222) in which the presence of a multiple Sp1-binding site is highlighted. This CG-rich sequence also represented potential binding sites for bisanthracycline WP631. (C) Semiquantitative RT-PCR analysis of the effect of the IC$_{50}$ and IC$_{75}$ of WP631 and the IC$_{50}$ for doxorubicin on the transcription of the \textit{mrp-1} gene after 24 h and 96 h of continuous treatment. The housekeeping \textit{GAPDH} gene was co-amplified as internal control using the primers: GAPDHdir, 5’-TCAGCCGCATTCTTCTTTTG-3’ and GAPDHrev, 5’-TGATGGCATGGACTGTGGT-3’.

Fig. 2. Multidrug resistance activities and drug accumulation. (A) Flow cytometry analysis of the fluorescence of intercellular calcein after 24-h treatment with doxorubicin or WP631, as indicated to the left margin. Column \textit{a} corresponds to experiments performed in the presence of ‘inhibitor 1’ (Chemicon International), which is known to inhibit both the MDR-1- and MRP-1-mediated resistance pump efflux (see main text for details). Column \textit{b} corresponds to experiments performed in the presence of ‘inhibitor 2’ (Chemicon International), which is known to inhibit the MRP-1 resistance pump efflux. Column \textit{c} corresponds to experiments performed in the presence of WP631 or doxorubicin alone. Column \textit{d} shows a merge of the cytometric profiles in panels \textit{a} to \textit{c} allowing us to determine whether doxorubicin and WP631 influence intracellular calcein accumulation. (B) Flow cytometry analysis of the fluorescence of intercellular calcein after 96-h treatment with doxorubicin or WP631, as indicated to the left margin; other details as
for panel (A). (C) Quantification of relative MRP-1 activity, obtained from the cytometric profiles shown in (A) and (B), after treatments for 24 h (black bars) or 96 h (gray bars). (D) Quantitative determination of the uptake of WP631 and doxorubicin in MCF-7/VP cells. Cells were continuously treated with either 307 nM doxorubicin or 122 nM WP631 for 24, 48 and 96 h. Data are relative uptakes, normalized to $10^7$ cells, represented as mean ± SD for three independent experiments.

Fig. 3. Cell death and cell cycle distribution. (A) Plot showing the percentage of cell death, as determined by trypan blue staining of MCF7/VP cells treated with the IC50 for doxorubicin, or the IC50 or IC75 for WP631. Cell were continuously treated with the drugs up to 96 h, and maintained in fresh drug-free medium for the times indicated in the x-axis. (B) Effects of doxorubicin and WP631 in the cell cycle distribution of MCF-7/VP cells. The profiles correspond to adherent cells. Accumulation in G2 phase and polyploidy can be observed according to the treatment. (C) Cytometric comparison of the cell cycle traverse of cells treated with doxorubicin or WP631 for 264 h (96 h of continuous treatment followed by 7 days in fresh drug-free medium) or for 336 h (96 h of continuous treatment followed by 12 days in fresh drug-free medium). Flow cytometry profiles for both adherent and floating cells are displayed. With the IC75 dose of WP631, all the cells floated in the cell culture, and they corresponded to dead cells in panel (A).

Fig. 4. Analysis of proliferating and growth-arrested MCF-7/VP cells. (A) Flow cytometry profiles of cells treated with the IC50 for either doxorubicin or WP631. The profiles were obtained 30 days after incubating previously drug-treated cells (for 96 h) in free-drug medium, which a change to new fresh medium every 2-3 days. Adherent cells, which survived to drug treatment, showed a synchronized profile with cells predominantly in G1 phase. (B) Adherent cells were incubated with CFSE (Molecular Probes) after drug treatments and allowed to grow.
Proliferating cells divided the cytoplasmic fluorescence evenly. (A). The profiles shown in this panel demonstrate that cells divided normally, since the CFSE fluorescence inside cells treated with either the IC50 for doxorubicin or WP631 merged with control, untreated, cells (see Table 1).

Fig. 5. Changes in the morphology of MCF-7/VP cells after drug treatment. Morphological detection of multinucleated MCF-7/VP cells in the presence of the IC50 for doxorubicin (A), or IC75 for WP631 (B). (C) Examples of the morphology of MCF-7/VP adherent cells (upper panel), together with various abnormal mitotic figures (lower panel) observed after treatment with the IC75 for WP631. Panels A and B were obtained by Laser Scanning Cytometry. Panel C was obtained by microscopy (Leishman’s staining; photographed at 40 x magnification).
Table 1. Results of a clonogenic assay of MCF-7/VP breast cancer cells treated with 307 nM (IC$_{50}$) doxorubicin or 122 nM (IC$_{50}$) WP631$^{(a)}$.

<table>
<thead>
<tr>
<th>Days after treatment$^{(b)}$</th>
<th>Viable Cells</th>
<th>Dead Cells</th>
<th>% Viability</th>
<th>% Confluence</th>
</tr>
</thead>
<tbody>
<tr>
<td>27  Control (untreated)</td>
<td>4.1(±0.4) x 10$^{6}$</td>
<td>1.1(±0.1) x 10$^{5}$</td>
<td>97.5(±9.5)</td>
<td>&gt; 80</td>
</tr>
<tr>
<td>27  IC$_{50}$ doxorubicin</td>
<td>4.0(±0.5) x 10$^{6}$</td>
<td>9.8(±0.1) x 10$^{4}$</td>
<td>97.6(±11.2)</td>
<td>&gt; 80</td>
</tr>
<tr>
<td>27  IC$_{50}$ WP631</td>
<td>3.4(±0.3) x 10$^{6}$</td>
<td>9.6(±3.8) x 10$^{4}$</td>
<td>97.2(±8.3)</td>
<td>&gt; 80</td>
</tr>
<tr>
<td>30  Control (untreated)</td>
<td>8.9(±0.6) x 10$^{6}$</td>
<td>2.7(±1.9) x 10$^{5}$</td>
<td>97.1(±6.3)</td>
<td>&gt; 90</td>
</tr>
<tr>
<td>30  IC$_{50}$ doxorubicin</td>
<td>7.9(±0.8) x 10$^{6}$</td>
<td>5.5(±1.3) x 10$^{5}$</td>
<td>93.9(±8.9)</td>
<td>&gt; 90</td>
</tr>
<tr>
<td>30  IC$_{50}$ WP631</td>
<td>9.0(±0.4) x 10$^{6}$</td>
<td>4.4(±1.6) x 10$^{5}$</td>
<td>95.4(±4.3)</td>
<td>&gt; 90</td>
</tr>
</tbody>
</table>

$^{(a)}$ The number of cells per colony after 27 and 30 days of incubation in fresh drug-free medium, following 96-h continuous treatment with the drugs, was determined using trypan blue exclusion on a hemocytometer (mean ± standard deviation, three independent experiments).

$^{(b)}$ After 96-h continuous treatment, cells were allowed to recover in fresh medium for 12 days. Surviving cells were seeded as single cells and allowed to growth for 27/30 days, with fresh medium changes every 2-3 days. Colonies containing 50 or more cells were considered to rise from the cells that maintained clonogenic capacity (see main text for details). After treatment with the IC$_{50}$ for either doxorubicin or WP631, adherent cells showed almost 100% clonogenicity.
FIG. 1
FIG. 2
FIG. 3
FIG. 4

A.

Control  IC_{50} Doxorubicin  IC_{50} WP631

B.

Control  IC_{50} Doxorubicin  IC_{50} WP631  Merge