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 Δ*erg6* 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 co-ordinate 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 Δ*erg6* 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 $CGGN_{11}CCG$, 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 *ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*) and its Δ*pdr1*, Δ*pdr2*, Δ*pdr5* and Δ*erg6* derivatives were obtained from EUROSCARF, Frankfurt, Germany. All reporter plasmids are derivatives of pSFLΔ-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 *Kpn*I site of pSFLΔ-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, Δ*erg6* cells transformed with plasmid pGAL were grown overnight in YEP medium (identical to YPD but without glucose) plus 2% raffinose. After adjusting the D_{600} to 0.1–0.2, 1% galactose was added together with daunorubicin. For oestrogen induction, Δ*erg6* 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.

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

Binding motifs are underlined; daunorubicin putative binding sites are shown in bold. I, inducible; C, constitutive.

Plasmid	Activating sequence	Binding factor	Type
pGAL	**TCG**GAGGAGAGTCTTC**CGA**GT	Gal4p	I
pRPG2d	**TCG**ACACCCATACATTTACACACACCCATACATTT	Rap1p	C
pVITB2x	AGTCACTGTGACC	Oestrogen receptor	I

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. Subsaturating RT-PCR conditions were adjusted to 10 ng of total RNA per reaction and to 25 amplification cycles. Tubuline (*TUB1*) was used as a reference gene. Primers used were as follows: TUB1 upper primer, 5′-AAGGGTTCTTGTTTACCC-3′; TUB1 lower, 5′-GCCATGTATTTACCATCT-3′; GAL1 upper, 5′-CCAAGACCATTAGCCGAAA-3′; GAL1 lower, 5′-GACGGCGCAAAGCATATCA-3′; GAL3 upper, 5′-GGCCATAGATCCGTCTGTGT-3′; GAL3 lower, 5′-CTAGTGCTGCCGCGCAAGT-3′; GAL7 upper, 5′-GGTCAACAGGAGGCTGCTT-3′; GAL7 lower, 5′-CGAGCCTAACGGCAGCATA-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

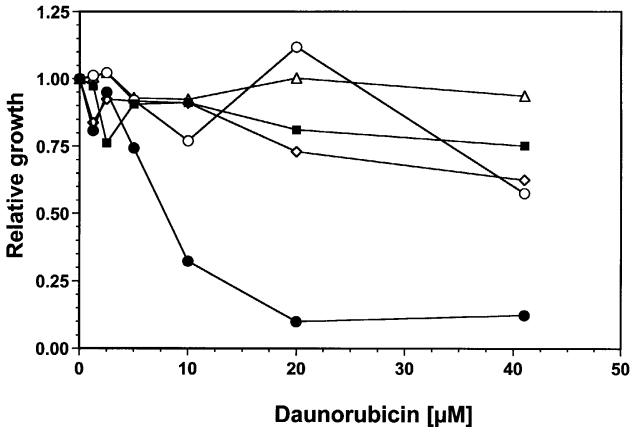

Figure 1 Effect of daunorubicin on the growth of yeast cells with different genetic backgrounds

Yeast BY4741 cells (■) and Δ*erg6* (●), Δ*pdr1* (○), Δ*pdr3* (△) and Δ*pdr5* (◇) derivatives were treated for 16 h with various concentrations of daunorubicin. Drug effects on growth are represented as the percentage of D_{600} 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).

	Viability (%)	
Daunorubicin (μM)	6 h	24 h
0	100	100
3	74.9 ± 8.1	98.1 ± 12.3
10	54.0 ± 13.4	57.8 ± 23.9
25	48.0 ± 4.1	17.8 ± 6.5
50	36.2 ± 7.1	11.9 ± 7.2

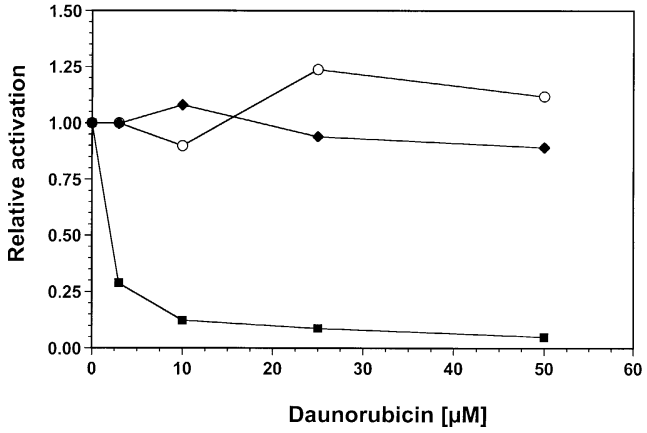

Figure 2 Effects of daunorubicin on the transcription of reporter plasmids

The yeast Δ*erg6* strain were transformed with the plasmids pGAL (■) or pRPG2d (◆), or co-transfected with the plasmids pVITB2x and pH5HE0 (○). Transformed cells were treated with various daunorubicin concentrations for 6 h; for inducible systems, appropriate inductors (galactose for the pGAL-transformed strain and oestradiol for the pVITB2x/pH5HE0 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 IC_{50} 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 enzymic 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.

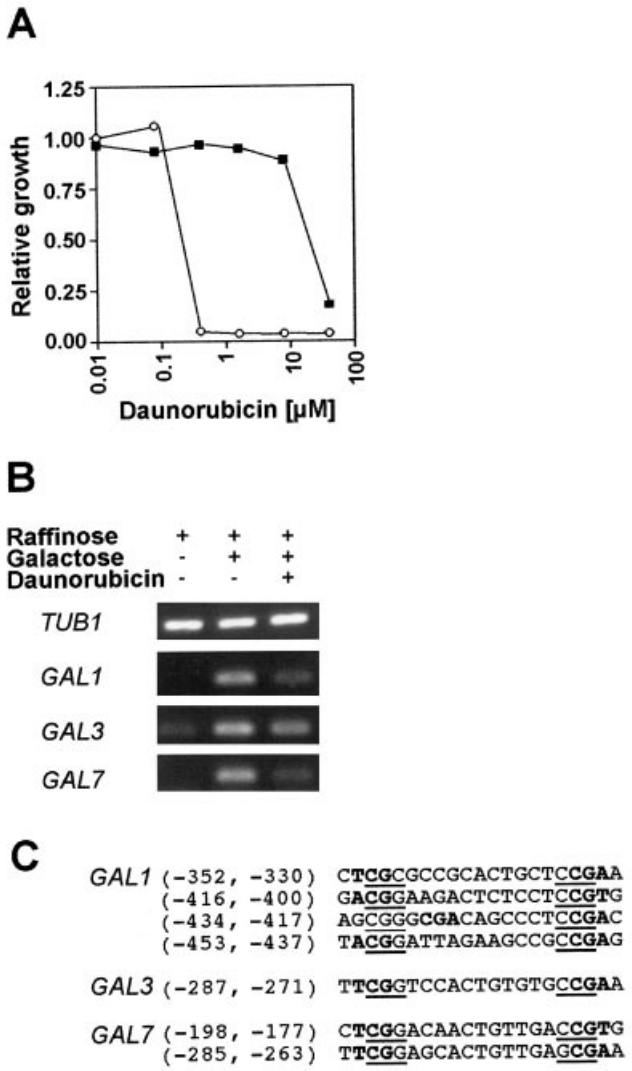

Figure 3 Effects of daunorubicin on galactose utilization

(**A**) Inhibition of growth in galactose. Δ*erg6* yeast cells grown in glucose as a carbon source (YPD) were transferred either to a fresh YPD medium (■) or to a YEP/galactose medium (○) containing various concentrations of daunorubicin. Values on the *y*-axis represent relative cell growth (taking the D_{600} of the untreated control as 1) after 16 h of incubation at 30 °C. (**B**) Inhibition of activation of *GAL* genes by daunorubicin. Culture conditions are described at the top of the panel. Daunorubicin concentration was adjusted to 3 μM. The picture shows semi-quantitative RT-PCR products corresponding to *GAL1*, *GAL3* and *GAL7* genes. The constitutive *TUB1* gene is included as a reference. (**C**) Sequences of the UASgal sites in *GAL1*, *GAL3* and *GAL7* promoters. Gal4p-recognition motifs (CGG) are underlined, whereas high-affinity daunorubicin-binding sites (WCG) are marked in bold. SCG sites, which may also constitute daunorubicin-binding sites, are found in several of these UASgal sequences.

The direct effects of daunorubicin on the transcription of several *GAL* genes are shown in Figure 3(B). A single culture of the Δ*erg6* 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*-specific primers 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 steps 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 inherent 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 cytoxicity of several drugs in yeast [27]. We found that only Δ*erg6* strains, but not strains lacking the PDR function, were sensitive to daunorubicin concentrations in the therapeutical range. Δ*erg6* 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 Δ*erg6* 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

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 bisanthracyclines *in vivo* 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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