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Research paper

Transcriptional down-regulation of c-*myc* expression in an erythroleukemic cell line, K562, and its doxorubicin-resistant variant by two topoisomerase II inhibitors, doxorubicin and amsacrine

Audrey Clary, Annick Larrue, Philippe Pourquier and Jacques Robert
Department of Medical Biochemistry and Molecular Biology, Université Victor Segalen Bordeaux 2, 146 rue Léo-Saignat, 33076 Bordeaux, France. Institut Bergonié, 180 rue de Saint-Genès, 33076 Bordeaux, France.

We have evaluated the effect of two topoisomerase II (Topo II) poisons, amsacrine and doxorubicin, on the expression of the c-*myc* oncogene, both at the mRNA and protein levels, in the leukemia cell line, K562, and its doxorubicin-resistant counterpart, K562 DoxR. We report in this study a concentration-dependent decrease in c-*myc* mRNA levels upon exposure of both cell lines to amsacrine and doxorubicin, with a more pronounced effect for amsacrine in the resistant line. In either case, c-*myc* down-regulation closely paralleled the drug-induced growth inhibition. We have also used the technique of PCR stop-assay to detect the occurrence of DNA breaks within the P2 promoter of the c-*myc* gene. We have shown that Topo II-mediated breaks induced by amsacrine are probably responsible for the down-regulation of c-*myc* in the resistant line. In addition, amsacrine induced apoptosis only in the resistant line while doxorubicin did not induce apoptosis in any cell line. These results suggest that c-*myc* is not involved in the resistance of K562 DoxR cells, but can induce the apoptosis pathway in these cells, while no drug-induced apoptosis could be detected in the sensitive line. [© 1998 Rapid Science Ltd.]

Key words: Amsacrine, c-*myc*, doxorubicin, K562, topoisomerase II.

Introduction

DNA topoisomerase II (Topo II) is a nuclear enzyme involved in DNA replication, transcription and recombination, and in chromatin organization.[1] Mammalian Topo II has been identified as the cellular target of various drugs such as anthracycline antibiotics, aminoacridines and epipodophyllotoxins. These drugs have been shown to interfere with the religation activity of Topo II via stabilization of the DNA-Topo II 'cleavable complex'.[2] Stabilization of cleavable complex and concomitant formation of DNA strand breaks have been shown to correspond with drug cytotoxicity and/or antiproliferative activity. However, it has been suggested that Topo II-induced DNA breaks were not distributed evenly along the DNA molecule and preferentially occurred in hypersensitive genomic sites.[3] Therefore, intensity of drug-induced DNA cleavage by Topo II at a given site depends on both drug concentration and DNA sequence.[4] For instance, it has been shown that amsacrine induced preferential breaks within the c-*myc* oncogene and alters c-*myc* expression in different cell lines, such as MCF7 and N417.[5–8]

A general mechanism of resistance to several Topo II-interfering drugs has been attributed to the overexpression of P-glycoprotein.[9] Other mechanisms of resistance to anthracyclines, epipodophyllotoxins and amsacrine have been identified; especially, qualitative and quantitative alterations of Topo II have been shown to occur in drug-resistant cells,[10] but a number of observations cannot be explained by the currently known mechanisms of resistance. One hypothesis is that the third partner of the cleavable complex, i.e. the DNA sequence itself, could also bear specific alterations in resistant cells, which could participate to the complete resistance phenotype.[11] Alterations in the structure and/or in the accessibility of sites involved in drug-induced double-strand nicking could explain some features exhibited by drug-resistant cell lines. A decreased ability to induce breaks within genes

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Correspondence to J Robert, Departement de biochimie médicale et biologie moléculaire, Université Victor Segalen Bordeaux 2, 146 rue Léo-Saignat 33076 Bordeaux Cedex, France. Tel: (+33) 557 57 17 51; Fax: (+33) 556 96 14 74

involved in cell proliferation or an increased ability to induce breaks within genes involved in cell death would both contribute to resistance to the drugs interfering with Topo II.

In order to explore whether such phenomena could be encountered in doxorubicin-resistant cell lines, we have examined in the present study the effect of doxorubicin and amsacrine on c-*myc* expression in K562 cells and in their doxorubicin-resistant variant (K562 DoxR), and the degree of gene-specific damage in the two cell lines as detected by PCR stop-assay.[12] We show in this paper that a down-regulation of the c-*myc* gene occurs upon drug exposure and is more important with amsacrine in the resistant cell line than in the wild strain. The MYC protein was consequently reduced upon drug exposure. We also show the existence of specific cleavage sites in the promoter P2 of the c-*myc* gene[13] in both cell lines, which is more pronounced in the resistant line. This study shows that c-*myc* may not play a role in resistance but rather plays a critical role in the apoptosis pathway of the doxorubicin-resistant cell line.

Materials and methods

Drugs and chemicals

Doxorubicin hydrochloride (Adriblastine®) was obtained from Pharmacia & Upjohn (Saint-Quentin-en-Yvelines, France), amsacrine (Amsidine®) from Parke-Davis (Courbevoie, France). Vincristine sulfate, 1 mg/ml aqueous solution (Oncovin®), was obtained from Lilly France (Saint-Cloud, France) and was stored at −20°C. All other chemicals were of reagent grade.

Cell lines and culture conditions

A human myelogenous leukemia cell line, K562,[14] and its doxorubicin-resistant variant, K562 DoxR,[15] were maintained in RPMI 1640 medium containing 10% fetal bovine serum and antibiotic mixture. The K562 DoxR resistant subline was continuously grown in the presence of 0.2 μg/ml of doxorubicin.

Growth inhibition

For the sensitive line, 1000 cells were seeded in each well of 96-well plates. Two days later, incubations with doxorubicin or amsacrine were performed for 2 h at appropriate concentrations ranging between 0.045 and 4.5 μM for amsacrine, and 0.027 and 2.7 μM for doxorubicin. For the resistant line, which has different growth characteristics, the seeding was of 2000 cells, the delay before drug incubation was 3 days, and the concentrations ranged between 8.9 and 890 μM for amsacrine, and between 6.44 and 644 μM for doxorubicin. In all cases, these concentrations represent the tenth of the IC_{50} and 10-fold the IC_{50}, respectively. At the end of incubations, the medium was removed, and after centrifugation the cells were washed with buffered saline and allowed to grow for 2 days (sensitive cells) or 3 days (resistant cells). At this time the surviving cells were estimated by MTT assay.[16]

cDNA probes

The 1.4 kb *Eco*RI-*Cla*I fragment of the human c-*myc* gene, containing the third exon,[17] was a generous gift of Dr JF Riou and was used as a probe recognizing a 2.4 kb human mRNA. As a control, a 3.3 kb *Hin*dIII fragment of the human β-actin gene[18] was used. cDNA probes were labeled with [α-^{32}P]dCTP (Amersham, les Ulis, France) using a random priming labeling kit (Amersham) according to the manufacturer's instructions.

RNA extraction and Northern blot analysis

Total cellular RNA was isolated by using the guanidine isothiocyanate method as described by Glisin *et al.*[19] Total RNAs were fractionated by electrophoresis in a 1% agarose gel containing 7% formaldehyde and were transferred to Hybond N membranes (Amersham) in 20 × SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7). Membranes prehybridization was performed for 20 h at 42°C in 50% formamide, 5 × SSC, 10 × Denhardt's solution, 50 mM phosphate buffer, pH 6.5, 1 mM EDTA, 0.1% SDS. Hybridization was then performed overnight in the same buffer containing the ^{32}P-labeled cDNA probe denatured at 100°C for 10 min. Membranes were washed twice at 65°C for 15 min in 1 × SSC, 0.1% SDS for the first wash and in 0.1 × SSC, 0.1% SDS for the second one. Autoradiography was performed at −80°C on Hyperfilm MP (Amersham) with intensifying screens. Autoradiograms were analyzed by densitometric scanning using the Densylab software (Bioprobe, Montreuil, France).

Stability of mRNA

K562 and K562 DoxR were incubated in the presence of doxorubicin or amsacrine at the IC_{50} value for 2 h;

then, 4 μM actinomycin D were added to the cells at time 0, and total cellular RNA was isolated at the following times: 15, 30, 45 and 60 min. Total RNA (20 μg) was electrophoresed on a 1% agarose gel and transferred to Hybond N membranes. The Northern blots were then probed with the third exon of the human c-*myc* gene as described above. The blots were then stripped and probed with β-actin.

PCR stop-assay

Cells (10^6) were incubated with doxorubicin or amsacrine for 2 h, then cells were washed and resuspended in 100 μl water, and the cell suspension was incubated at 100°C for 5 min. These cell lysates were stored at −20°C until use. PCR stop-assay was performed according to Oshita and Saijo.[12] This technique allows us to detect the lesions induced in sensitive or resistant cells incubated with various concentrations of doxorubicin or amsacrine. The PCR was carried out using the following primers, derived from the c-*myc* human gene. A 212 bp fragment of this gene, corresponding to the P2 promoter, was amplified with primers spanning the regions from nucleotide 2401 (5′-TGCCTCGAGAAGGGCAGG-3′) to 2613 (5′-CCCTATTCGCTCCGGATC-3′) and a 193 bp fragment of the 3′ region of the same gene was amplified with following primers: 5′-TTCGTTTCTTCCCCC-TCCCA-3′ and 5′-CACCTGCCTTCTGCCATTCC-3′. All PCR reagents were obtained from Perkin-Elmer (Roissy-Charles-de-Gaulle, France) and PCR was conducted in a thermocycler model 480 (Perkin-Elmer). A typical 50 μl reaction mixture contained 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1 mM $MgCl_2$, 0.2 mM of each of the dNTPs, 2 μM of each primer, 5 μl of cell lysate, 0.5 μl (2.5 units) of Taq polymerase and 2 μCi [α-^{32}P]dCTP. The initial heating step was performed at 94°C for 5 min followed by 30 cycles: 96°C for 1 min, 60°C for 30 s and 72°C for 30 s. The final cycle was followed by an extra 7 min of polymerization at 72°C and cooling to 4°C. The reaction products (20 μl) were separated on a 10% polyacrylamide gel. The gel was autoradiographed for 12 h. Autoradiograms were analysed by densitometric scanning using the Densylab software (Bioprobe).

Preparation of nuclear extracts

Exponentially growing cells (10^8) were suspended in a cold saline solution containing 0.02% EDTA and centrifuged at 1500 *g* for 5 min at 4°C. The cells were then resuspended in ice-cold hypotonic 10 mM Tris-HCl buffer, pH 7.4, containing 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40,[20] and, in addition, 10 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 5 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor, as recommended by Drake *et al.*[21] in order to minimize proteolysis. They were then incubated on ice for 30 min and the extent of cell lysis was monitored by microscopy. Nuclei were prepared after lysis by Dounce homogenization and by centrifugation at 1500 *g* for 5 min at 4°C. The nuclei were resuspended in 1 ml of hypertonic extraction buffer, pH 6.5, consisting of 2 mM K_2HPO_4, 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM dithiothreitol, 1 M NaCl and containing the proteolysis inhibitors mentioned before. Nuclear proteins were extracted for 60 min at 4°C. DNA and nuclear fragments were precipitated by centrifugation at 19000 *g* for 30 min at 4°C. Supernatant protein concentration was determined with the Bradford assay using bovine serum albumin as a standard. The 1 M NaCl extracts were fractionated after DNase I treatment for 30 min at room temperature and stored at −80°C in 30% glycerol until immunoblotting.

Immunoblot analysis of MYC

The 1 M NaCl nuclear extracts were equalized for protein content and mixed with Laemmli loading buffer,[22] and loaded onto a 10% SDS-polyacrylamide gel. Proteins were subjected to electrophoresis according to Laemmli[22] at 80 V overnight. The Rainbow colored protein molecular weight markers (Amersham) were run simultaneously to locate the position of the 64/67 kDa MYC isoform and the reactivity of the antibodies. Proteins were then transferred to Immobilon-P membranes (Millipore, Molsheim, France) by electroblotting at 2.5 mA/cm^2 for 1.5 h, using a transfer apparatus (Milliblot Graphite Electroblotter; Millipore) according to the recommendations of the manufacturer. Blots were preincubated overnight at 4°C in 5% non-fat dry milk in phosphate-buffer saline (PBS) containing 0.2% Tween 20. Membranes were then incubated with a mouse monoclonal antibody against human MYC (Boehringer Mannheim, Meylan, France), at a 1:100 dilution in PBS, for 2 h at room temperature. After three 10 min washes in PBS, blots were treated with an alkaline phosphatase-labeled rabbit antibody against mouse immunoglobulins (Dako, Trappes, France), at a 1:200 dilution in the same incubation buffer, for 1.5 h at room temperature. Membranes were then washed three times for 10 min and one time for 20 min in PBS. Immunoreactive bands were visualized by incubation for 5-10 min in 5-

bromo-4-chloro-3-indolylphosphate (BCIP) and nitro-blue tetrazolium, using BCIP/NBT tablets from Sigma Chimie, and rinsed with water to stop staining. Spots were quantified by densitometric scanning using an electronic dual transilluminator (Bioprobe) with the Densylab software (Bioprobe).

DNA fragmentation assay

Apoptosis is characterized by DNA fragmentation which was quantified by the filter elution method described by Fan *et al.*[23] with some modifications. Cells were labeled with [methyl-^{3}H]thymidine (Amersham; 0.5 μCi/ml medium) for 1.5-2 doubling times. After a chase period of 20 h in fresh label-free medium, $1\text{-}2 \times 10^6$ cells in each flask were treated with different concentrations of doxorubicin for 2 h or of amsacrine for 8 h at 37 C. The cells were centrifuged and washed immediately with pre-warmed buffered saline solution (PBS), and reincubated in fresh medium for 48 and 72 h. Cells were then centrifuged at 1500 *g* for 10 min at 4 C. A 1 ml aliquot of the medium was transferred to a liquid scintillation vial for radioactivity evaluation. Cell pellets were resuspended in 1 ml PBS and layered, in Swinnex funnels (Millipore), onto polycarbonate membranes, 25 mm in diameter and 2 μm pore size (Nucleopore). The cells were lysed *in situ* for 1 h in 5 ml of a solution containing 2% SDS, 0.1 M glycine, 0.025 M EDTA and 0.5 mg/ml proteinase K (Boehringer). Filters were then washed with 5 ml of a 0.04 M EDTA solution, pH 10. Fractions and washes were combined and an aliquot was counted for radioactivity. Filters were removed from the funnels heated for 1 h in 0.1 M HCl at 78 C, cooled at room temperature, put in liquid scintillation vials and 0.1 M NaOH was added before counting. Funnels were subsequently washed with 0.5 M NaOH and the counts were added to those provided by the filters themselves for a complete estimation of the tritium amount retained on the filters.

Results

Expression of c-*myc* gene

We have evaluated the effect of doxorubicin and amsacrine on c-*myc* expression, as assessed by Northern blotting, by comparison to a housekeeping gene, β-actin. We have first measured the basal expression of c-*myc* in order to detect any difference between the two cell lines. In untreated cells, there was no

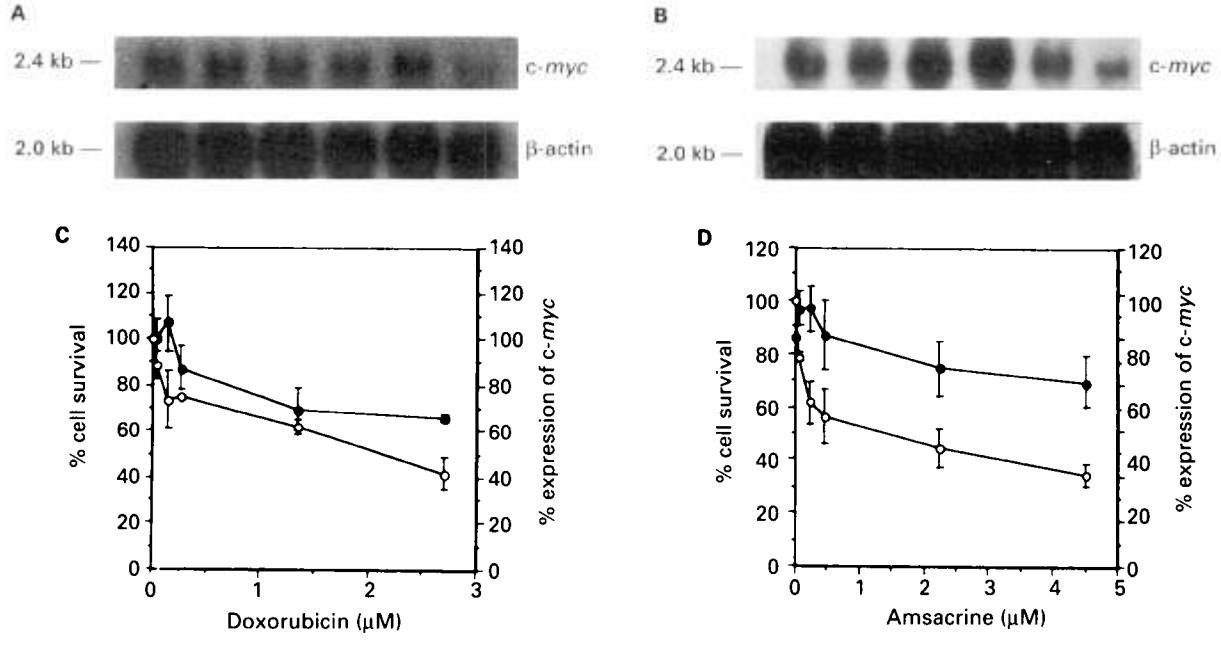

Figure 1. (A and B) Northern blots of c-*myc* transcripts after exposure of K562 cells for 2 h to various concentrations of doxorubicin (left) and amsacrine (right). Total RNA (20 μg) was electrophoresed on 1% agarose gel and transferred on Hybond membrane; the blot was then hybridized with the *Eco*RI–*Cla*I fragment of the third exon of the c-*myc* gene. The blots were stripped and probed with β-actin. (C and D) Quantification of the Northern blots from three independent experiments, as obtained by densitometric scanning using the bioprobe Densylab software. Results are presented as c-*myc* mRNA levels (per cent of untreated control) (●) and were compared to growth inhibition induced by the same drug exposure (○).

difference in c-*myc* mRNA level between the two cell lines (data not shown). Amsacrine and doxorubicin produced a specific decrease in c-*myc* expression in comparison to that of the β-actin gene: 2 h exposure to doxorubicin and 2 h exposure to amsacrine were followed in K562 cells by a reduction in c-*myc* expression, down to 60 and 80% of initial value, respectively (Figure 1). The c-*myc*/β-actin ratio decreased in a concentration-dependent manner, with a good parallelism to growth inhibition: with amsacrine, the coefficient of correlation between c-*myc* expression and cell survival was approximately 0.9.

To determine whether a similar inhibition of c-*myc* expression occurred in the resistant cell line, c-*myc* expression was also quantitated in K562 DoxR cells after exposure to concentrations of doxorubicin and amsacrine able to provide the same cytotoxicity as in sensitive cells. Figure 2 shows that doxorubicin induced a slight decrease in c-*myc* expression, down to 60% of the initial value, as in the sensitive line, whereas amsacrine induced an important decrease, down to 30% of the initial value. The coefficient of correlation between c-*myc* expression and cell survival was approximately 0.9.

In contrast, in both lines, there was no change in c-*myc* expression with similarly cytotoxic concentrations of vincristine, a drug wich does not interfere with Topo II (data not shown).

Stability of c-myc mRNA

In order to determine whether the decrease in c-*myc* transcript was related to an altered turn-over of the mRNA, we evaluated the influence of doxorubicin and amsacrine on the stability of the c-*myc* transcript by measuring the rate of disappearance of the c-*myc* mRNA in control and drug-treated cells. Cells were exposed for 2 h to 2.7 μM doxorubicin or 4.5 μM amsacrine (sensitive line) and to 322 μM doxorubicin or 44.5 μM amsacrine (resistant line), before treatment with 4 μM actinomycin D, dissolved in dimethylsulfoxide (DMSO). Figure 3(A and B) presents the results of representative Northern blots, showing the decrease of the c-*myc* message as a function of time in control and drug-treated cells, and Figure 3(C and D) shows the quantification of the Northern blots. Doxorubicin and amsacrine slightly altered the rate of disappearence of the c-*myc* transcript in the same manner in the sensitive line. When the data from three independent experiments were pooled and analyzed, the half-life of the c-*myc* mRNA was found to be 56.5 ± 5.1 min in the absence of drug, 36.3 1.0 min with amsacrine and 38.3 ± 10.1 min with doxorubicin. There was a statistically significant decrease in the half-lives of the c-*myc* mRNA upon exposure to both anticancer drugs, as calculated by Student's unpaired *t*-test ($p < 0.01$ and $p < 0.05$, respectively, for amsacrine and doxorubicin).

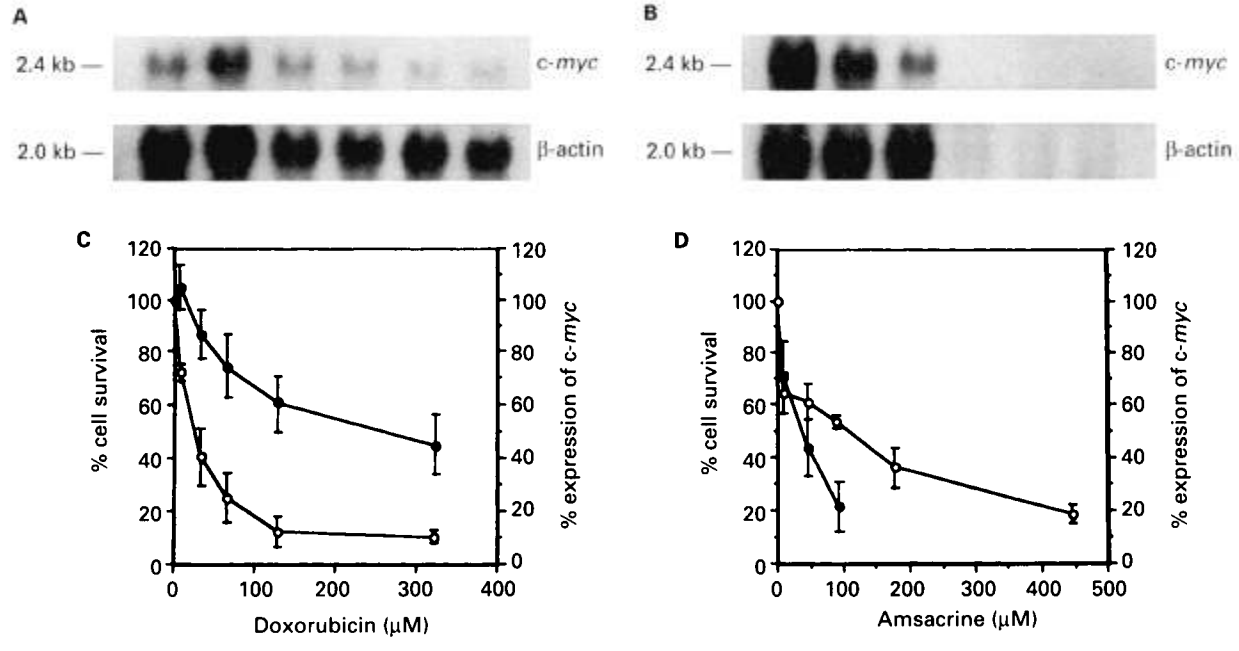

Figure 2. (A and B) Northern blots of c-*myc* transcripts after exposure of K562 DoxR cells for 2 h to various concentrations of doxorubicin (left) and amsacrine (right). Same legend as Figure 1. (C and D) Quantification of the Northern blots from three independent experiments. Same legend as Figure 1.

In resistant cells, the half-life of the c-*myc* transcript was 46.9 ± 2.0 min in the controls, 48.7 ± 5.3 min with doxorubicin and 78.3 ± 10.8 min with amsacrine (see Figure 3D). There was no significant difference between DMSO- and doxorubicin-treated cells (p=0.9), and on the contrary there was a statistically significant difference between DMSO- and amsacrine-treated cells ($p < 0.01$), indicating that this drug increased slightly the stability of the c-*myc* transcript.

DNA damage

Gene-specific damage in each cell line was analyzed by PCR stop-assay after 2 h exposures to doxorubicin and amsacrine. Two fragments of the c-*myc* gene were studied, the first one in the promoter P2 and the second one in the 3'-end of the gene as a control. In sensitive cells, 2 h exposure to amsacrine reduced the amplification signal of the P2 promoter down to 40% of the level measured in untreated cells, whereas a similarly cytotoxic exposure to doxorubicin did not alter significantly the level of the amplification signal (Figure 4). With both anticancer drugs, we noticed an important decrease in the promoter amplification signal of the resistant cells, down to 25% of that measured in untreated cells (Figure 5). There was no modification in the amplification of the control 3' fragment with either drug. PCR stop-assay shows that specific drug cleavage site(s) exist(s) in the promoter of the c-*myc* gene, DNA damage being more important in resistant cells than in sensitive cells and amsacrine being generally more active than doxorubicin.

Western blot analysis

In order to show whether the diminution of c-*myc* gene expression had a dowstream effect, we have measured the effect of doxorubicin and amsacrine on the MYC protein expression by Western blotting. Sensitive and resistant cells were exposed for 2 h to amsacrine and doxorubicin at the respective concentrations of 2.25 and 1.35 μM in the sensitive line, 64.4 and 44.5 μM in the resistant line, these values being roughly cytotoxic to the cells. Figure 6 shows the variation of the MYC protein: 2 h exposures to doxorubicin and amsacrine reduced MYC expression

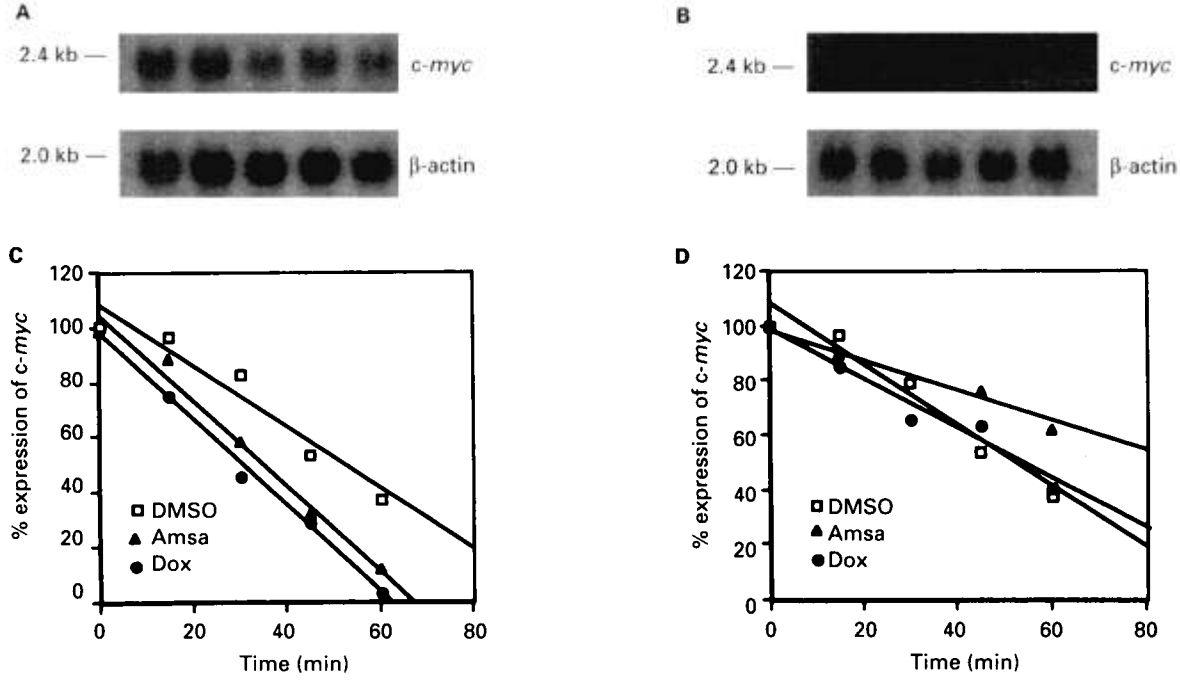

Figure 3. (A and B) Typical blots for the determination of c-*myc* transcript stability in K562 cells in control conditions (A) and after exposure to doxorubicin (2 h at 2.7 μM) (B). Actinomycin D (4 μM) was added to the cells at the end of drug exposure (time 0). Total cellular RNAs were then extracted after 15, 30, 45 and 60 min, electrophoresed, and blotted as in Figure 1. (C and D) Quantification of the blots. Drug exposure doses were 2.7 and 322 μM for doxorubicin and 4.5 and 44.5 μM for amsacrine, for the sensitive and resistant cells, respectively. DMSO control, □; doxorubicin, ●; amsacrine, ▲. The slope of the linear regression curve allowed us to determine the half-life of the c-*myc* transcript. Statistical analysis was performed by Student's unpaired *t*-test.

down to 50 and 30% of the control value, respectively, in both cell lines.

Evaluation of apoptosis

Since the decrease in c-*myc* expression and the decrease of cell suvival were correleted, we wanted to verify whether cells could undergo the apoptotic pathway, in order to approach the physiological significance of the decrease of c-*myc*. We have evaluated DNA fragmentation after 2 h exposures of both cell lines to several concentrations of doxorubicin and 8 h exposures of both lines to several concentrations of amsacrine. We have chosen 2 and 8 h exposures, respectively, because doxorubicin induced the same effects on c-*myc* expression whatever the exposure time, while amsacrine effects

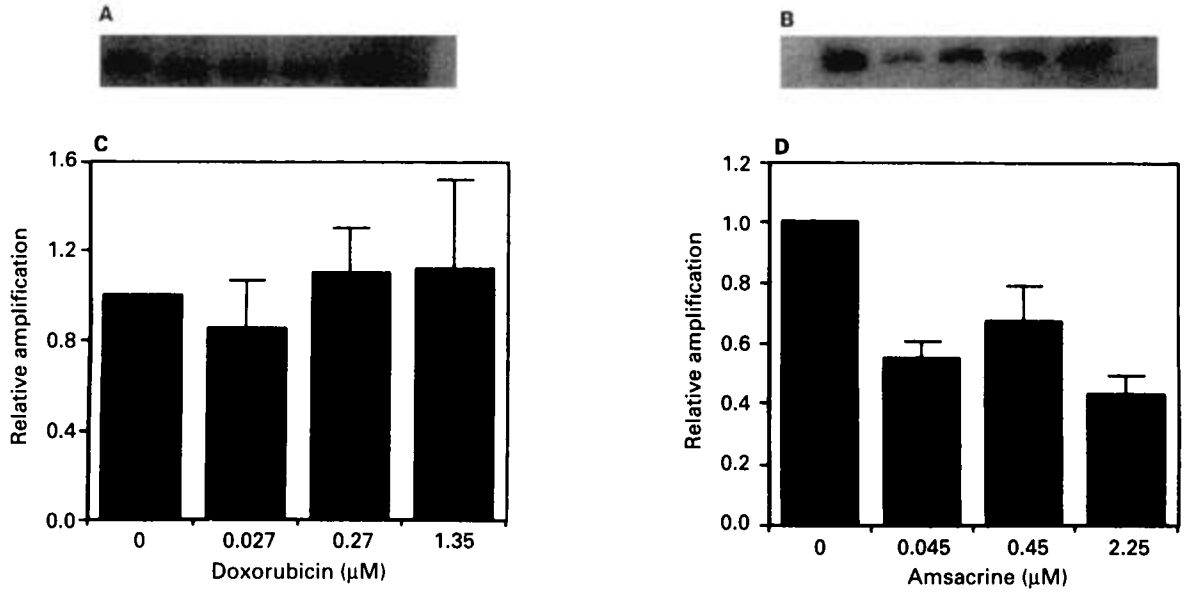

Figure 4. (A and B) Effect of doxorubicin (A) and amsacrine (B) on the amplification signal generated after PCR performed within the P2 promoter of the c-*myc* gene of K562 cells. The PCR was realized in the presence of [α-^{32}P]dCTP. The PCR products were separated by electrophoresis on 10% polyacrylamide and the gel was autoradiographed. (C and D) Quantification of PCR amplification products obtained above. The radioactivity from the gels was counted and expressed as percentage of the amplification obtained from undamaged DNA.

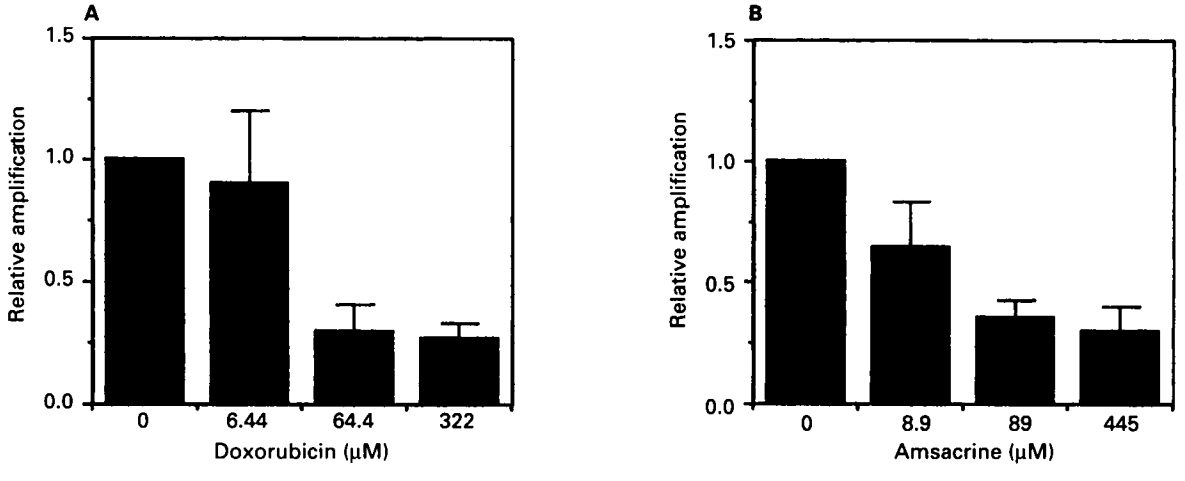

Figure 5. Quantification of the PCR amplification products obtained after PCR performed within the P2 promoter of the c-*myc* gene of K562 DoxR cells. Cells had been exposed to doxorubicin (A) or to amsacrine (B).

were more pronounced after 8 h exposure (data not shown). There was only a slight induction of DNA fragmentation after doxorubicin exposure of both cell lines and 48 or 72 h (Figure 7) reincubation in fresh medium, no difference was exhibited between the two lines. In contrast, amsacrine induced a slight DNA fragmentation in the sensitive line and a very important apoptosis in the resistant cell line (up to 82% of the cells at the highest concentration) as shown in Figure 7.

Discussion

Topo II inhibitors, such as doxorubicin and amsacrine, stabilize DNA-Topo II complexes, which results in the

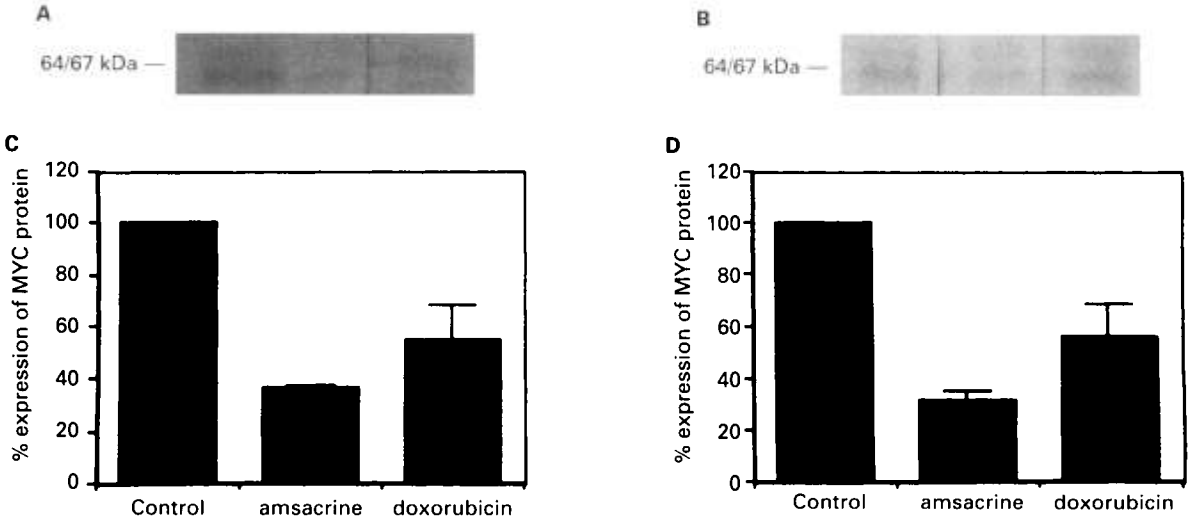

Figure 6. (A and B) Western blots of MYC protein in K562 cells (A) and K562 DoxR cells (B) in control conditions (left lanes) or after exposure to doxorubicin (middle lanes) and amsacrine (right lanes) at the IC_{50} concentration. (C and D) Quantification of the blots by densitometric scanning. Results are expressed as percent of untreated controls.

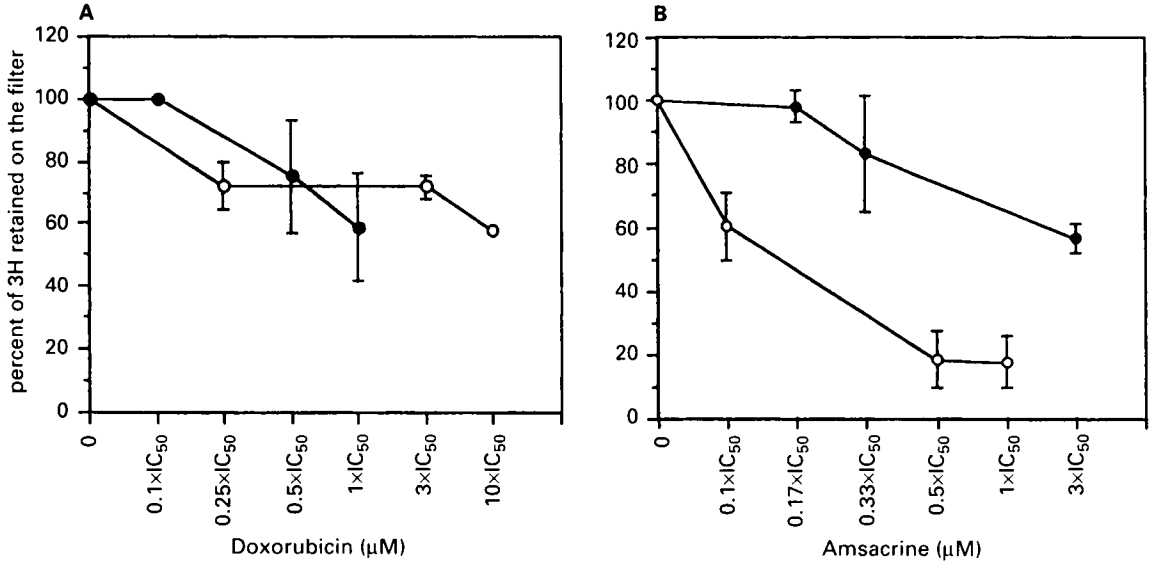

Figure 7. (A and B) Quantification of apoptosis induced by doxorubicin (A) and amsacrine (B) at different concentrations ($0.1 \times IC_{50}$, $0.25 \times IC_{50}$, $0.5 \times IC_{50}$, IC_{50}, $3 \times IC_{50}$, $10 \times IC_{50}$) in K562 sensitive (●) and K562 DoxR resistant (○) cells, as evaluated 48 h after a 2 h exposure to doxorubicin and 8 h exposure to amsacrine. Values are means ± SD of three independent experiments.

production of DNA strand breaks.[24,25] In this study, we have shown in two cell lines, K562 and K562 DoxR, that there was a drug-induced preferential damage in the c-*myc* gene, in comparison to a housekeeping gene. This damage leads to a decrease in c-*myc* expression, which appears more important in K562 DoxR cells than in sensitive cells. This decrease in c-*myc* expression is concentration dependent and is correlated to growth inhibition. Similar results have been obtained with other Topo II inhibitors in the MCF-7 cell line.[26,27] Our work indicates that the effects of both drugs on c-*myc* expression are not a consequence of alterations in transcript stability in the resistant cell line, while for the sensitive cells both drugs lead to a decrease in c-*myc* transcript stability. It has been already described[28-30] that the degradation of c-*myc* mRNA was dependent on *de novo* synthesis of a labile protein.

Since the stability of c-*myc* mRNA is not responsible for the decrease in c-*myc* expression observed in resistant cells, we have studied the effects of doxorubicin and amsacrine on the P2 promoter of the c-*myc* gene. The technique we have chosen is the PCR stop-assay used by Oshita and Saijo.[12] to detect specific damage after cisplatin exposure. We have shown the existence of a cleavage site in this promoter as already shown by Pommier *et al.*,[31] *in vitro* as well as in N417 cells. In particular, a cleavage site at nucleotide 2499 was shown to be more prominently cleaved by amsacrine than by doxorubicin. This should correspond to the presence, between nucleotides 2489 and 2506, of the 18mer consensus sequence described by Spitzner and Muller,[32] with a 16/18 matching.

The combined results from the c-*myc* stability studies and DNA damage in the P2 promoter lead to the conclusion that doxorubicin reduces c-*myc* steady-state mRNA levels due to the presence of a specific cleavage site in the P2 promoter. In addition, the participation of a post-transcriptional mechanism cannot be excluded in sensitive cells. Similar conclusions were obtained with amsacrine.

The reduction in c-*myc* mRNA levels leads to a decrease in the amount of the MYC protein in the same proportion for both cell lines, after 2 h exposures to doxorubicin and amsacrine. However, since c-*myc* expression was especially reduced upon amsacrine treatment of K562 DoxR cells as compared to sensitive ones, this suggests that the MYC protein amount is maintained in this line through the increase of mRNA stability. Such a regulation of oncogene protein levels has been shown to occur in other models.[20,33,34]

There is increasing evidence that alterations in the expression of different proto-oncogenes are part of the apoptotic response to cells to anticancer agents.[20,35] We have evaluated the apoptotic response of our cell lines after doxorubicin and amsacrine treatments in order to test the physiological significance of the decrease observed in MYC in K562 and K562 DoxR cells. At 48 h after drug exposure, only few sensitive cells underwent apoptosis, as already described in other studies upon etoposide treatment.[36,37] One explanation is that K562 cells do not produce *bcl*-2 but *bcl*-x, which functions as a repressor of apoptotic cell death.[38] On the contrary, amsacrine alone induced apoptosis in the resistant cell line, leading to an alteration of the cell death mechanism. It could be of interest to determine whether amsacrine treatment leads to a modification of *bcl*-x and p53 expression in K562 DoxR cells. This is presently under investigation in our laboratory.

Conclusion

Doxorubicin and amsacrine induced both a decrease in c-*myc* expression in the two cell lines K562 and K562 DoxR. In resistant cells, it is likely the consequence of the presence of a specific cleavage site in the P2 promoter, while in sensitive cells we cannot exclude the participation of a post-transcriptional mechanism. In addition, this study shows that, in the resistant cell line, amsacrine could induce apoptosis, while almost no apoptosis could be obtained in the K562 cell line with either doxorubicin or amsacrine.

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