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Down-regulation of *bcr-abl* and *bcl-*x_L Expression in a Leukemia Cell Line and Its Doxorubicin-Resistant Variant by Topoisomerase II Inhibitors

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ABSTRACT. K562 cells are usually resistant to apoptosis induction, probably because of the expression of *bcr-abl*, the hybrid gene characteristic of the Philadelphia chromosome (t 9;22). However, we have previously shown that amsacrine and, to a lesser extent, doxorubicin, could induce apoptosis in the doxorubicin-resistant variant of this cell line. In order to elucidate the role of *bcr-abl* in triggering apoptosis, we investigated the effect of the topoisomerase II inhibitors doxorubicin, amsacrine, and etoposide on the expression of several genes that may be related to apoptosis induction in both cell lines. This was done using a technique of reverse transcription–polymerase chain reaction coupled with HPLC of the amplified fragments to obtain semiquantitative evaluations. We showed that amsacrine, at pharmacologically relevant concentrations, was able to decrease the expression of *bcr-abl* down to 20% of the basal value in the doxorubicin-resistant variant only, whereas doxorubicin and etoposide were unable to do so. No effect of these drugs was seen on the expression of the normal *abl* gene. In addition, there was an effect of amsacrine on the expression of *bcl-*x_L in the resistant cell line only, but at concentrations higher than the IC_{50} of this drug. Our results emphasize the role of *bcr-abl* in protecting cells from apoptosis and the possible involvement of specific topoisomerase II inhibitors in overcoming resistance to apoptosis. BIOCHEM PHARMACOL **60**;12:1823–1828, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. topoisomerase II; *bcr-abl* gene; *bcl-*x_L gene; apoptosis; amsacrine; chronic myeloid leukemia

DNA topoisomerase II is a nuclear enzyme involved in DNA replication and transcription and in chromatin organization [1]. Drugs such as anthracyclines, acridines, or epipodophyllotoxins are called topoisomerase II poisons because they are able to stabilize the DNA–topoisomerase II cleavable complex at specific sites. Stabilization of a cleavable complex and the subsequent formation of DNA double-strand breaks have been shown to be correlated to drug cytotoxicity and/or antiproliferative activity. Although the formation of topoisomerase II–DNA complexes requires some sequence specificity that may vary as a function of the drug stabilizing the complex [2], it is not known whether it occurs all over the genome or at the level of specific genes, some of which are involved in cell death or proliferation. It was reported that there was no direct correlation between the number of DNA double-strand breaks and the cytotoxicity of various topoisomerase II–interacting drugs [3], suggesting that cytotoxic effects result from the stabilization of specific sites rather than of random sites on the DNA. Riou *et al.* [4] were the first to show the existence of a specific site for amsacrine-induced DNA breaks in the promoter P2 of the c-*myc* gene in N417 cells. We confirmed [5] that, in K562 leukemia cells, doxorubicin and amsacrine could stabilize the cleavable complex at least at one specific site, located within the promoter P2 of the c-*myc* gene.

It was previously demonstrated that K562 cells exhibit an apoptosis-resistant phenotype upon the action of most antiproliferative agents. Such a survival advantage has been associated in tumor cells with the overexpression of the antiapoptotic protein BCL-2 [6] or of the hybrid protein tyrosine kinase encoded by the *bcr-abl* gene resulting from the t(9;22) translocation characteristic of this cell line [7]. In fact, apoptosis sensitivity of K562 cells could be restored by *bcr-abl* antisense oligonucleotides [8]. In addition, Belloc *et al.* [9] showed that induction of resistance to apoptosis in other leukemia cell lines was accompanied by *bcr-abl* translocation and overexpression. In a prior study, we demonstrated that amsacrine, and to a lesser extent doxorubicin, were able to induce apoptosis in the doxorubicin-resistant variant of K562 cells, but not in wild-type K562 cells at similarly cytotoxic concentrations. We thus wanted to know whether the ability to undergo apoptosis could be associated with a specific effect of these drugs on the expression of the *bcr-abl* or the *bcl-*x_L genes.

We studied here the effect of amsacrine, doxorubicin, and etoposide on the expression of the *bcr-abl*, *abl*, and

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bcl-x_L genes by the method of RT–PCR* coupled with HPLC. The expression of the normal *abl* allele was also evaluated as a control. We show that apoptosis induction in doxorubicin-resistant K562 cells is associated with the decrease in *bcr-abl* and *bcl*-x_L expression occurring upon the action of amsacrine and, only for *bcr-abl*, of doxorubicin.

MATERIALS AND METHODS

Drugs and Chemicals

Doxorubicin hydrochloride (Adriblastine®) was obtained from Pharmacia & Upjohn, amsacrine (Amsidine®) from Parke-Davis, and etoposide from Novartis. All other chemicals were of reagent grade.

Cell Lines and Culture Conditions

The human myelogenous leukemia cell line K562 [10] and its doxorubicin-resistant variant K562doxR [11] were maintained in RPMI-1640 medium containing 10% fetal bovine serum and antibiotic mixture. The K562doxR resistant subline was continuously grown in the presence of 0.2 μg/mL of doxorubicin, which was discontinued one week before each experiment.

Cytotoxicity

For the sensitive line, 1000 cells were seeded in each well of 96-well plates. Two days later, incubations with doxorubicin, amsacrine, and etoposide were performed for 2 hr at appropriate concentrations. At the end of the incubations, the medium was removed and, after centrifugation, the cells were washed with buffered saline and allowed to grow for 2 days in fresh medium. For the resistant line, which has a different growth pattern, the seeding was of 2000 cells, the delay before drug incubation was 3 days, and the regrowth in fresh medium also 3 days. At this time, the surviving cells were estimated by the MTT (3,4,5-trimethylthiazol-2,5-diphenyltetrazolium bromide) assay [12].

RNA Extraction and RT–PCR

Total cellular RNAs were isolated using the guanidine isothiocyanate method as described by Glisin *et al.* [13]. The conditions of reverse transcription were as follows: 1 × buffer (KCl 50 mM, Tris–HCl 10 mM, pH 8.3), $MgCl_2$ 1.2 mM, dNTP 1 mM each, RNase inhibitor 40 U, random hexamers 2.5 μM, and reverse transcriptase (MuLV) 100 U in a volume of 50 μL. Reverse transcription was performed at 42° for 15 min and inactivation of the enzyme was carried out at 99° for 5 min. The primers used for PCR were the following:

bcr-abl, sense: 5′-GAAGAAGTGTTTCAGAAGCT TCTCC-3′;
bcr-abl, antisense: 5′-GACCCGGAGCTTTTCACCTT TAGTT-3′;
abl, sense: 5′-GCCGCTCGTTGGGAACTCCAAGG-3′;
abl, antisense: 5′-GACCGGAGCTTTTCACCTT TAGTT-3′;
bcl-x_L, sense: 5′-GGACTGAGGCCCCAGAAGGG-3′;
bcl-x_L, antisense: 5′-GGGTCGAGGTGTAGTGGGGT-3′;
β-actin, sense: 5′-GAGAAGATGACCCAGATCATGT -3′;
β-actin, antisense: 5′-CAGAGGCGTACAGGGATAG CAC-3′.

For all the genes, PCR was performed on 5 μL of cDNA. A typical 50-μL reaction mixture contained 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1.5 μM of each primer, and 2.5 U of Taq polymerase (all reagents obtained from Perkin Elmer). The initial heating step was performed at 94° for 3 min, 65° for 1 min, and 72° for 1 min, and the subsequent 30 cycles were performed at 94° for 1.5 min, 65° for 1 min, and 72° for 1 min. PCR was conducted in a thermocycler (model Gene Amp PCR system 2400, Perkin Elmer).

HPLC

The amplification products of the gene of interest (20 μL) were mixed with those of β-actin (10 μL) and injected onto a high-performance liquid chromatograph (ThermoQuest) equipped with an ion-exchange column (TSK DEAE-NPR, particle size 2.5 μm, Perkin Elmer). The DNA fragments were detected with a UV detector (UV 1000, ThermoQuest). The column was equilibrated with the starting eluent (25 mM Tris–HCl in 1 M NaCl) for 30 min at a flow rate of 1 mL/min at room temperature. A gradient was then run through the column, with a progressive increase in the proportion of NaCl in 25 mM Tris–HCl (from 250 mM to 1 M) as described by the manufacturer. Between runs, the column was washed and regenerated with 0.2 M NaOH. Peaks were integrated with an automatic integrator (LCI 100, Perkin Elmer).

Western Blotting

After a 2-hr exposure with amsacrine or doxorubicin, 10^7 cells were incubated with isotonic buffer (Tris 40 mM, pH 8). After centrifugation, the pellet was resuspended and incubated in ice-cold hypotonic buffer (Tris 5 mM, pH 8, $MgCl_2$ 6 mM, and aprotinin 0.05 U) for 15 min. Cells were then sonicated and 250 μL of hypertonic buffer was added (Tris 80 mM, pH 8, $MgCl_2$ 6 mM, DNase I 20 U, and aprotinin 0.05 U). After a 30-min incubation at room temperature, DNA was precipitated and, after centrifugation, proteins were quantified in the supernatant using the Bradford assay. Protein analysis was performed by Western blotting after electrophoresis as described by Laemmli [14]. The BCR-ABL protein was revealed with a rabbit polyclonal antibody (Oncogene Products, PC01) at a 1:200

* *Abbreviation:* RT–PCR, reverse transcription–polymerase chain reaction.

TABLE 1. Cytotoxicity of amsacrine, doxorubicin, and etoposide on K562 and K562doxR cells

	K562 cells	K652doxR cells
Amsacrine	1.5 ± 0.1	111 ± 13
Doxorubicin	1.3 ± 0.09	30.0 ± 5.1
Etoposide	1.3 ± 0.12	37.1 ± 1.4

Cytotoxicity was estimated as IC_{50}, i.e. the drug concentration (μM) providing a 50% decrease in cell survival, evaluated as described in Materials and Methods. Results are means ± SD of three independent determinations.

dilution. The secondary antibody (diluted at 1:4000) was coupled with peroxidase, and revelation was performed with the ECL$^+$ system (Amersham).

DNA Fragmentation Assay

Apoptosis was evaluated by DNA fragmentation, which was quantified by the filter elution method described by Fan *et al.* [15] with some modifications, as already described [5]. Briefly, cells were labeled with [methyl-^{3}H]thymidine (Amersham; 0.5 μCi/mL of medium) for 1.5 to 2 doubling times. After a chase period of 20 hr in fresh label-free medium, 1–2 × 10^6 cells in each flask were treated with different drug concentrations for 2 hr at 37°. The cells were centrifuged, washed with prewarmed buffered saline solution, and reincubated in fresh medium for 48 hr. After lysis of the cells and treatment of the filter, radioactivity on the filter and in the different fractions was counted.

RESULTS

Cytotoxicity of Amsacrine, Doxorubicin, and Etoposide in K562 and K562doxR Cells

The cytotoxicity of the three drugs in both cell lines was evaluated as their IC_{50} determined as described in Materials and Methods. Table 1 presents these IC_{50} values. The evaluation of gene expression was studied as a function of these IC_{50} values in order to work on both cell lines with similarly cytotoxic concentrations and compensate for the differences in P-glycoprotein activity in the sensitive and the resistant cell line.

Evaluation of Apoptosis Produced by Etoposide

We had already shown that amsacrine and doxorubicin produced very few apoptotic cells in the K562 cell line at pharmacologically relevant concentrations, while in K562doxR cells, amsacrine and, to a lesser extent, doxorubicin, induced apoptosis at IC_{50} [5]. Here, we investigated whether a non-intercalating drug such as etoposide could also specifically induce apoptosis in the resistant cell line. K562 and K562doxR were exposed to etoposide for 2 hr and apoptosis was evaluated 48 hr after treatment (Fig. 1). In sensitive cells, there was no apoptosis at the IC_{50} value (1.3 μM) while at 10 μM, etoposide induced a slight DNA

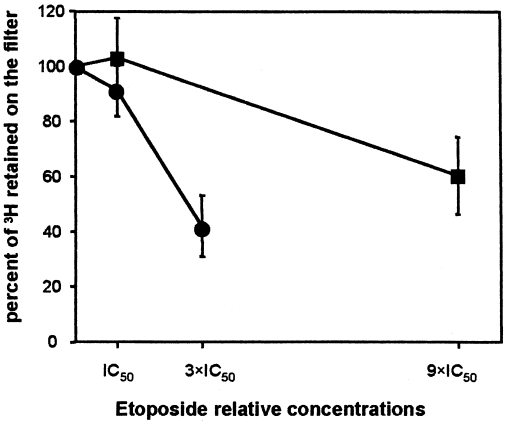

FIG. 1. Quantification of apoptosis induced by etoposide in K562 cells (■) and in K562doxR cells (●). Values are means ± SD of three independent experiments.

fragmentation. In the resistant line, we obtained the same result as in the sensitive one: no apoptosis at the IC_{50} value (37.1 μM) and some DNA fragmentation at high concentration (3 × IC_{50}).

Expression of bcr-abl *and* abl

We evaluated the effects of 2-hr exposures to amsacrine, doxorubicin, and etoposide on *bcr-abl* and *abl* gene expression, as assessed by RT–PCR coupled with HPLC, by comparison with a housekeeping gene, β-actin. Amsacrine and doxorubicin, at about their IC_{50}, produced a small decrease (at most 20%) in *bcr-abl* and *abl* gene expressions in the K562 cell line (Fig. 2). In the K562doxR cell line, amsacrine concentration dependently induced an important decrease in *bcr-abl* gene expression of about 80% of the initial value, while doxorubicin produced a smaller decrease in *bcr-abl* gene expression (60% of the initial value at the IC_{50} of the drug) (Fig. 3). The decrease in specific *bcr-abl* mRNA levels was accompanied by a decrease in the levels of the hybrid protein BCR-ABL as evaluated by Western blotting (data not shown). By contrast, there was no modification in *abl* gene expression with the two drugs. Etoposide exerted no effect on the expression of *bcr-abl* and *abl* genes in any of the cell lines.

Expression of bcl-x_L

We evaluated the expression of the *bcl-*x_L gene in the two cell lines under the same conditions as the *bcr-abl* and *abl* genes. In K562 cells, amsacrine produced a slight decrease in *bcl-*x_L expression, down to 40% of the initial value, but only at high concentration (3 × IC_{50}), while doxorubicin and etoposide produced no decrease in *bcl-*x_L gene expression (Fig. 2). In K562doxR cells, amsacrine induced an important decrease in *bcl-*x_L, down to 20% of the initial value, but this diminution was not concentration-dependent and occurred only beyond the IC_{50}, while doxorubicin and etoposide produced no specific decrease in this cell line

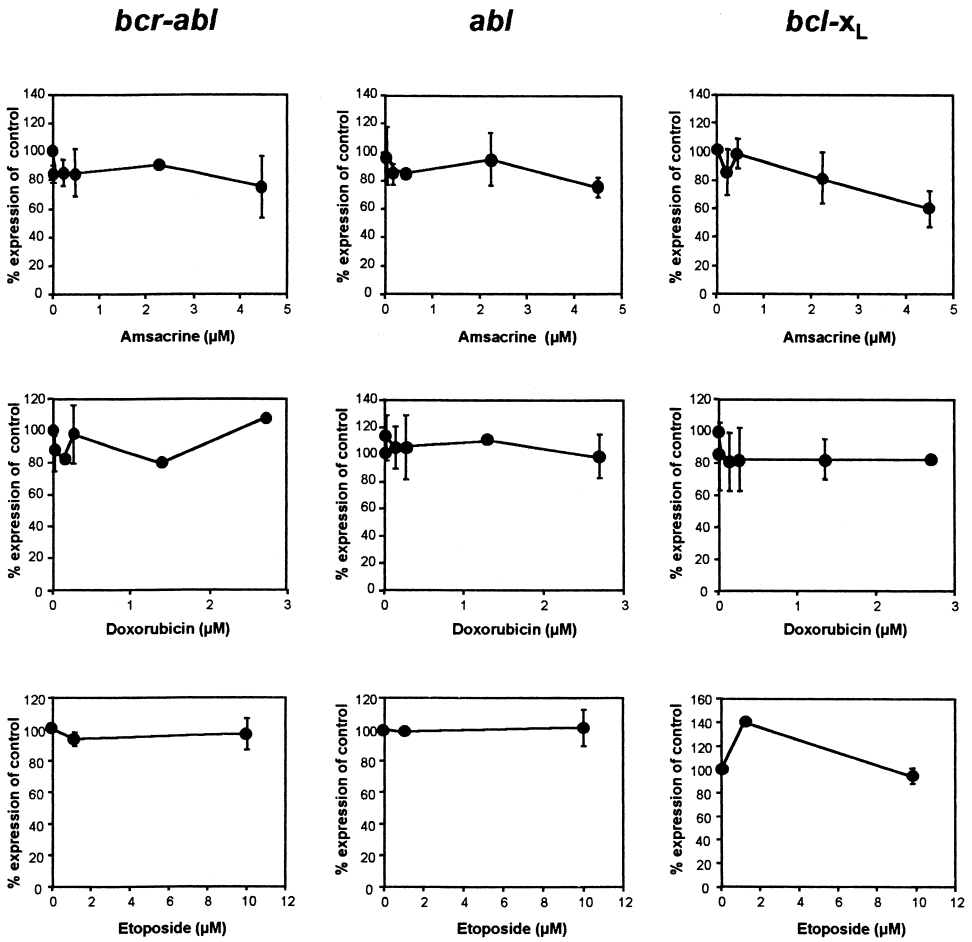

FIG. 2. Effects of amsacrine, doxorubicin, and etoposide on the expression of *bcr-abl*, *abl*, and *bcl*-x_L genes in K562 cells exposed for 2 hr to various drug concentrations. These concentrations were chosen on both sides of their IC_{50}s. Gene expression was quantified by RT–PCR coupled with HPLC relative to the expression of β-actin. Results are means ± SD of three independent experiments.

(Fig. 3). There was a good correlation between *bcr-abl* and *bcl*-x_L expression in amsacrine-treated K562doxR cells (Fig. 4).

DISCUSSION

We had already shown that amsacrine and, to a lesser extent, doxorubicin, were able to induce apoptosis in K562doxR cells but not in K562 wild-type cells at similarly cytotoxic concentrations [5]. In the literature, it was postulated that the K562 line could not undergo apoptosis because it expressed the hybrid *bcr-abl* gene. Our results show that amsacrine and doxorubicin produced a decrease in *bcr-abl* gene expression that was correlated with apoptosis induction. The amsacrine-induced diminution of the *bcr-abl* mRNA level was correlated with a decrease in protein expression, while such was not the case for doxorubicin. Our results suggest that the diminution of *bcr-abl* expression was responsible for the reinduction of the apoptosis pathway. Belloc *et al.* [9] have observed, in other leukemia cell lines able to undergo apoptosis, that the selection of daunorubicin-resistant cells was accompanied both by induction of resistance to apoptosis and by expression of the *bcr-abl* hybrid gene, whereas the original lines did not display the t(9;22) translocation.

Benito *et al.* [16] reported that the BCL-2 protein was weakly expressed in K562 cells, while the *bcl-2* gene was expressed at a high level; in fact, this was due to the overexpression of *bcl*-x_L, a gene closely related to *bcl-2*. In this work, we have shown that amsacrine induced an important decrease in *bcl*-x_L gene expression in the resistant cell line only, and this occurred at higher drug concentrations than the decrease in *bcr-abl* expression. Therefore, amsacrine seems to be able to reinduce the apoptosis pathway in K562doxR via a diminution of both *bcr-abl* and *bcl*-x_L gene expressions. These two genes appear to display a concerted effect on the trigger of apoptosis: a scheme was proposed by Datta *et al.* [17] where BCR-ABL, in response to interleukin-3, activates the AKT protein, which is in turn able to phosphorylate the BAD protein. BAD becomes unable to bind BCL-X_L, which is then active for the inhibition of apoptosis. The effect of amsacrine on the expression of these two genes in the K562doxR cell line is difficult to assign to a direct effect (targeting a topoisomerase II–induced cleavage site in the promoter of the *bcr* gene, for instance) or to an indirect effect (via a transcription factor common to *bcr-abl* and *bcl*-x_L).

In order to see whether the effects observed with amsacrine on gene expression were or were not common to all

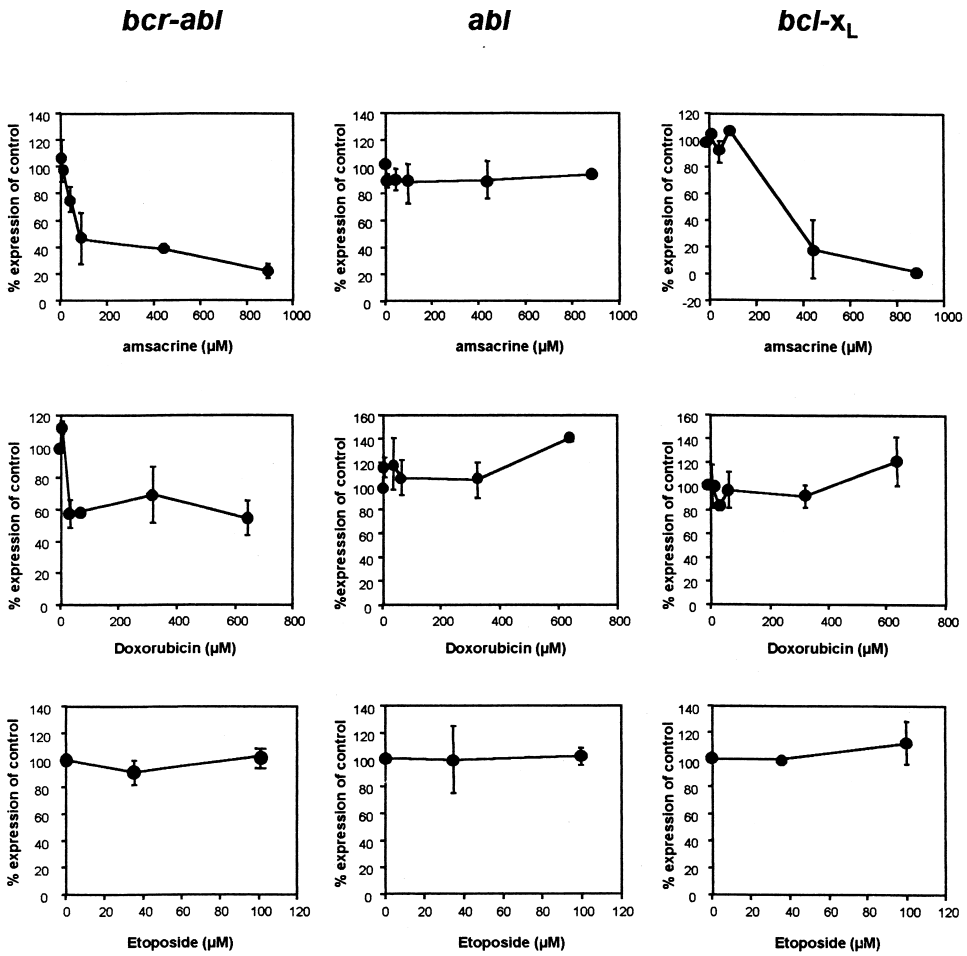

FIG. 3. Effects of amsacrine, doxorubicin, and etoposide on the expression of *bcr-abl*, *abl*, and *bcl*-x$_L$ genes in K562doxR cells exposed for 2 hr to various drug concentrations. Same legend as Fig. 2.

topoisomerase II poisons, we have evaluated the ability of etoposide to induce apoptosis and to decrease *bcr-abl* and *bcl*-x$_L$ gene expression. Whereas amsacrine and doxorubicin were able to induce apoptosis at their IC$_{50}$ in the resistant cell line [5], etoposide was unable to do so and DNA fragmentation was only detected far above the IC$_{50}$ value. Ritke *et al.* [18] also showed that etoposide could induce apoptosis in K562 cells at 100 μM. No modification of *bcr-abl* and *bcl*-x$_L$ gene expression was observed in the two cell lines, even at the high concentrations where apoptosis occurs. It therefore appears that this minimal etoposide-induced apoptosis in our lines is independent of *bcr-abl* and *bcl*-x$_L$ gene expression. In the HL60 cell line, it has been shown that etoposide induced a death pathway dependent on DNase II and not on classical endonucleases [19], suggesting that events occurring prior to enzyme activation are different for etoposide and other topoisomerase II poisons.

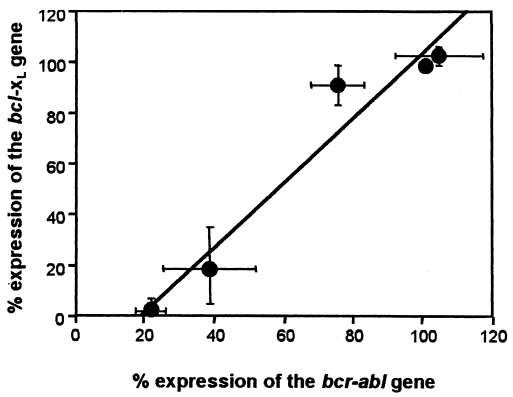

FIG. 4. Linear regression between *bcr-abl* and *bcl*-x$_L$ gene expressions in K562doxR cells treated with amsacrine. r = 0.97, $P < 0.01$.

The mechanism by which amsacrine and, to a lesser extent, doxorubicin are able to down-regulate *bcr-abl* and *bcl*-x$_L$ remains unknown. It was suggested by Pommier *et al.* [20] that the down-regulation of c-*myc* they observed with amsacrine, and not with etoposide, could be due to the presence of a consensus sequence for topoisomerase II–induced cleavage, as defined earlier [21]. In the promoter region of c-*myc*, there is a sequence of 18 nucleotides with a 16/18 matching with this consensus sequence. We have, therefore, attempted to detect such sequences in the *bcr-abl* and the *bcl*-x$_L$ genes as obtained from Genbank (accession numbers U07000 and D30746, respectively). We indeed

found a sequence of 18 nucleotides in both genes, with a 16/18 matching for *bcr-abl* and a 15/18 matching for *bcl-*x_L. This is in agreement with the hypothesis of a direct action of amsacrine and doxorubicin on gene expression via the stabilization of topoisomerase II–DNA complexes at specific sites. There was such a sequence in the *abl* gene (Genbank accession number M14752) studied under the same conditions, the best matching being of 12/18 nucleotides.

The fact that only doxorubicin-resistant cells are able to undergo apoptosis at cytotoxic exposures of amsacrine and doxorubicin remains puzzling. This may be related either to the regulations involved during *MDR*1 gene overexpression or to phenomena accompanying the acquisition of drug resistance, independently of the *MDR*1 gene. It is well known that numerous events occur during the process of selection of drug-resistant cells; we have shown that MDR cells are able to tolerate relatively high intracellular amounts of doxorubicin that may be sequestrated in compartments not allowing drug–DNA interaction [22, 23]. One can hypothesize that cytoplasmic drug may be present, in MDR cells, at sufficient levels to trigger apoptosis, which is not the case in sensitive cells where no drug sequestration occurs in cytoplasmic organelles. In that case, the effect of topoisomerase II inhibitors on *bcr-abl* or *bcl-*x_L expression would be independent of the formation of cleavable complexes on specific sites present in its sequence.

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