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The Daunorubicin-Binding Protein of M_r 54,000 Is an Aldehyde Dehydrogenase and Is Down-regulated in Mouse Liver Tumors and in Tumor Cell Lines

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SUMMARY

A rabbit antiserum developed against purified rat liver daunorubicin-binding protein of M_r 54,000 (DNR-BP54) cross-reacted with a mouse protein of the same molecular weight. This protein was expressed in the liver and several other organs of mice. A series of tumors and cell lines tested for the presence of the protein were negative. By immunocytochemistry, we found that DNR-BP54 was abundantly expressed in the cytoplasm of normal hepatocytes but was expressed at much lower levels in urethane-induced mouse liver tumors. By immunoscreening of a mouse liver cDNA library, we cloned the cDNA coding for DNR-BP54 and we found that this protein is aldehyde dehydrogenase-2 (EC 1.2.1.3). This result was confirmed by the dehydrogenase activity found in pure preparations of DNR-BP54 from normal rat and mouse livers, assayed with acetaldehyde as substrate and NAD as cofactor. The enzyme activity was inhibited by daunorubicin. The inhibition was found to be competitive with respect to NAD.

The anthracycline antibiotics daunorubicin and doxorubicin are effective agents for cancer chemotherapy (1). DNA appears to be the main target for their cytotoxicity, through the impairment of DNA-dependent enzymes such as DNA and RNA polymerases and DNA topoisomerases (2, 3). However, the biochemical and pharmacological bases of their antitumor efficacy remain rather controversial (4), which led us to search for anthracycline-binding proteins as potential cellular targets of anthracycline action. Our effort resulted in the identification of DNR-BP54. We isolated this protein by affinity chromatography of the rat liver soluble fraction on daunorubicin-agarose columns; it was the only protein eluted from these columns and was characterized as a tetramer composed of four subunits of M_r 54,000 (5). This protein was found to be present also in mouse organs and to bind doxorubicin but not antitumor drugs of different chemical classes, such as vincristine or actinomycin D. Because differential expression of this protein in normal tissues and in tumors could modulate the biological activity and toxicity of anthracyclines, we evaluated DNR-BP54 levels in mouse organs and in several tumor cell lines, using a rabbit polyclonal antibody against the purified rat protein. Using the same antibody we were able to clone the gene coding for mouse DNR-BP54 protein, by immunoscreening of a mouse liver expression library, and to identify the gene from its partial nucleotide sequence. The protein is AHD-2 (EC 1.2.1.3).

Experimental Procedures

Antiserum preparation and purification. The rabbit antiserum against electrophoretically pure rat liver DNR-BP54 was prepared according to the procedure used by Bollum (6) for terminal transferase. The antibody was purified by ammonium sulfate precipitation, DEAE cellulose chromatography, and affinity chromatography on DNR-BP54-agarose. The affinity resin was prepared by coupling DNR-BP54 to *N*-hydroxysuccinimide-activated cross-linked agarose (Affi-Gel 10; Bio-Rad, Richmond, CA) (1 mg of protein/ml of settled gel) at pH 8.5.

Cells and tissues. The following tumor cell lines were tested for the presence of DNR-BP54: MethA fibrosarcoma, Ehrlich sarcoma, and YC8, EL4, and P388 leukemias (all grown in the ascitic form in mice); Yoshida hepatoma AH130 (grown in ascitic form in rats); and BALB/3T3 fibroblasts, B16 melanoma, P388 leukemia sensitive and resistant to doxorubicin (7, 8), Friend erythroleukemia (9), and virus-induced MS2 sarcoma and its variant MS2T (10) (all grown *in vitro*).

Different organs from a male C3H/He mouse (30 weeks of age) were used. Three normal adult livers from male AC3F$_1$ mice (44 weeks of age) and 13 hepatocellular tumors induced by urethane in male AC3F$_1$ mice (40 weeks of age) were examined (11). Animals were maintained and treated according to the Principles and Guidelines for the Use of Animals in Research of the Istituto Nazionale per lo Studio e la Cura dei Tumori.

Western blotting and immunocytochemistry. Cell and tissue extracts were obtained in 20 mM Tris·HCl, pH 8, 150 mM NaCl, 2 mM

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ABBREVIATIONS: DNR-BP54, daunorubicin-binding protein of M_r 54,000; AHD, aldehyde dehydrogenase; SDS, sodium dodecyl sulfate; kb, kilobase(s).

EDTA, 2 mM phenylmethylsulfonyl fluoride, 200 kallikrein-inactivating units/ml aprotinin (lysing buffer). Tissues and cells were homogenized in 4 volumes and 20 μl/10^6 cells of lysing buffer, respectively. Homogenates were centrifuged at 100,000 × *g* for 60 min and the supernatants were saved for further processing. Protein content of the soluble fraction was determined by the standard bicinchoninic acid method (Pierce, Rockford, IL). Equal amounts of protein (114 μg) were electrophoresed under denaturing conditions in 10% polyacrylamide gels (12) and then transferred to nitrocellulose membranes at 150 mA for 16 hr at 4° (13). The nitrocellulose was sequentially incubated with anti-DNR-BP54 IgG (2 μg/ml) and ^{125}I-Protein A (Amersham, Aylesbury, UK). The filters were then autoradiographed with Hyperfilm-MP (Amersham). The same procedure was used to detect DNR-BP54-producing clones.

Immunocytochemical procedures were performed on Bouin-fixed, paraffin-embedded sections of mouse liver tumors that had been induced in (A/J × C3H/He)F_2 female mice by a single urethane treatment (300 mg/kg, subcutaneously, at 1 week of age). Mice were killed at 65 weeks of age (11). Rabbit anti-DNR-BP54 antibody was used at 5 μg/ml. Control rabbit serum was diluted 1/1000. The antibody was counterlabeled by using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and was stained with 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO), followed by hematoxylin nuclear counterstaining.

RNA and DNA isolation and analysis. Total cellular RNA was prepared using the RNAzol-B kit (Biotecx Laboratories, Houston, TX). Twenty micrograms were used for Northern blot analysis, after electrophoresis through 1% agarose gels containing 6% formaldehyde. Mouse *LLRep3* (0.8-kb *Pst*I fragment from plasmid pLLRep3) (14) was used to check the Northern blots for the amount of RNA present in each lane (15). Quantitative analysis of the transcripts was performed by densitometric scanning of the autoradiographs.

Poly(A)$^+$ RNA was isolated according to the method of Aviv and Leder (16). DNA was digested with restriction endonucleases and separated by electrophoresis on 0.8% agarose gels. Gels were stained with ethidium bromide and photographed to check the quantity and quality of nucleic acids.

Hybridizations were performed in 50% formamide, 5× SSPE (1× SSPE is 0.15 M NaCl, 0.01 M sodium phosphate, 1 mM EDTA, pH 7.4), 1% SDS, 1× Denhardt's solution, 10% dextran sulfate, at 42°, using ^{32}P-labeled probes prepared by random primer synthesis (17). Filters were washed at a stringency of 2× SSC (1×SSC is 0.15 M NaCl, 0.015 M NaCitrate, pH 7.0), 0.1% SDS for 20 min at 65° (Southern blots) or 0.5× SSC 0.1% SDS for 15 min at 65° (Northern blots).

cDNA library and cDNA probes. The cDNA library was synthesized from the poly(A)$^+$ RNA of a normal C3H/He mouse liver (6 weeks of age) using the ZAP cDNA synthesis kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions, and the λUNI-ZAP XR vector (Stratagene). The resulting cDNA library contained 1.15 × 10^6 recombinants. Recombinant phages were plated at 6000 plaque-forming units/150-mm plate, on the XL1-Blue bacterial strain, and were screened with a PicoBlue immunoscreening kit (Stratagene). Briefly, phages were grown at 42° for 4 hr and then at 37° for 4 hr in the presence of a nitrocellulose filter soaked with 10 mM isopropyl β-D-thiogalactopyranoside. The filter was then removed, a second filter soaked with 10 mM isopropyl β-D-thiogalactopyranoside was placed with the phages, and the phages were grown overnight. The two replica filters were processed as were Western blotting filters. Phage clones showing a specific hybridization signal were purified by secondary screenings at low phage densities.

DNA sequence and analysis of recombinant clones. The pBluescriptSK(−) plasmids containing the inserts of interest were excised from the λUNI-ZAP XR vector according to the manufacturer's instructions (Stratagene). The inserts were either purified and used for hybridizations or sequenced by the dideoxy chain termination technique (18). Reactions were primed with T3 primer using the Sequenase kit (United States Biochemical Corp., Cleveland, OH) and [^{35}S]dATP (Amersham). Sequence comparisons and alignments were performed using Genetic Computer Group sequence analysis software (19). The plasmid DNAs were digested with *Eco*RI and *Xho*I to release the cDNA inserts, the digested DNAs were electrophoresed on 1% agarose gels, and Southern blots were prepared on nylon membranes (ICN, Costa Mesa, CA).

AHD assay. DNR-BP54 was isolated from normal Wistar rat and C57BL/GNCrl mouse livers as described (5). The enzyme assay was performed at 37° as described by Manthey *et al.* (20), except that 50 mM Tris·HCl, pH 8, was used instead of pyrophosphate buffer, to avoid anthracycline precipitation. The reaction was initiated by addition of the enzyme, and the appearance of NADH was monitored at 340 nm with a Kontron Uvikon 930 recording spectrophotometer.

Results

Expression of DNR-BP54 in mouse tissues and in tumor cells. The affinity-purified antibody prepared against the pure protein from rat liver cross-reacted with a mouse protein of identical molecular weight, as shown in Fig. 1, *top*. The protein was expressed at high levels in mouse liver, lung, testis, stomach, and intestine and at lower levels in thymus, pancreas, spleen, kidney, and skin and was absent from brain and salivary glands. The protein was also absent from heart, serum, and whole blood (data not shown).

Several mouse tumors and tumor cell lines were assayed for the expression of DNR-BP54. Surprisingly, none of the cells or ascitic tumors (see Experimental Procedures) tested expressed this protein in amounts detectable by Western blotting under our experimental conditions. Solid tumors B16, AH130, MS2, MS2T, MELC, and EL4, growing subcutaneously, were also negative (data not shown).

Immunohistochemical techniques were used to stain DNR-BP54 in liver sections from mice treated with urethane. The liver sections contained a total of seven hepatocellular tumors, with diameters ranging from 0.6 to 4.6 mm. In the surrounding normal liver, the anti-DNR-BP54 antibody strongly stained the cytoplasm of hepatocytes. However, the staining was absent in all of the examined liver tumor lesions (Fig. 2).

Immunoscreening of the cDNA library. We screened 42,000 recombinant phages using purified anti-DNR-BP54 IgG

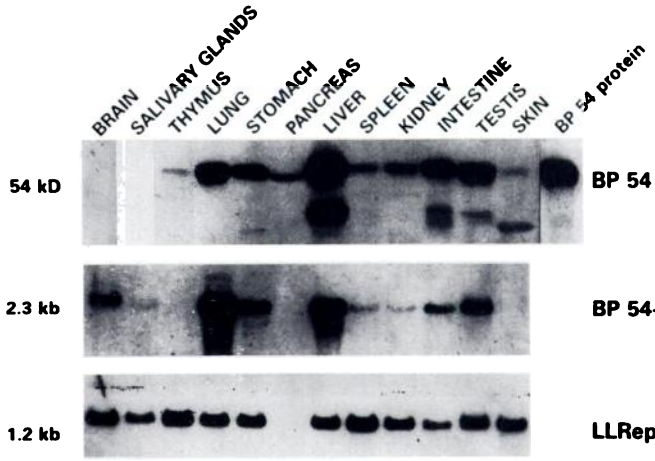

Fig. 1. *Top*, Western blot analysis of DNR-BP54 protein. For each sample, 114 μg of protein were separated by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel, transferred to nitrocellulose, and immunodecorated with anti-DNR-BP54 IgG and ^{125}I-Protein A. Pure rat DNR-BP54 (0.1 μg) was run as a standard. *Middle* and *bottom*, Northern blot analysis of BP54–1-related transcripts (*middle*) and *LLRep3* mRNA levels (*bottom*) in mouse organs. Twenty micrograms of RNA were electrophoresed on a 1% agarose gel and transferred to nylon membranes.

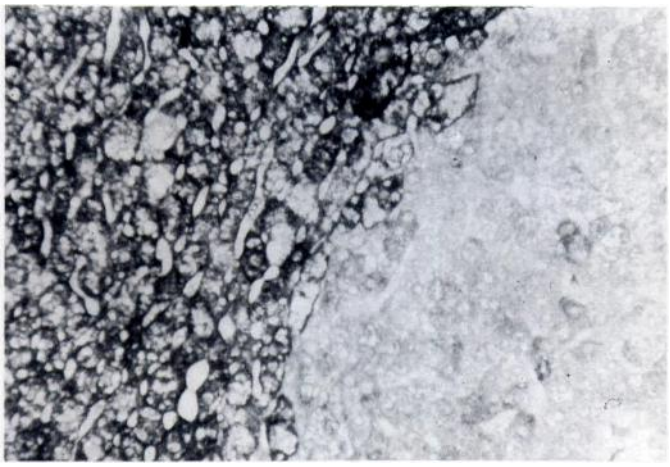

Fig. 2. DNR-BP54 immunostaining of a urethane-induced mouse liver tumor. Paraffin-embedded sections were treated with anti-DNR-BP54 antibody, counterlabeled with peroxidase-conjugated antibody, and stained with 3,3′-diaminobenzidine, followed by hematoxylin nuclear counterstaining. Strong DNR-BP54 staining is seen in all normal hepatocytes surrounding the tumor, whereas it is very weak in the tumor (original magnification, 312×).

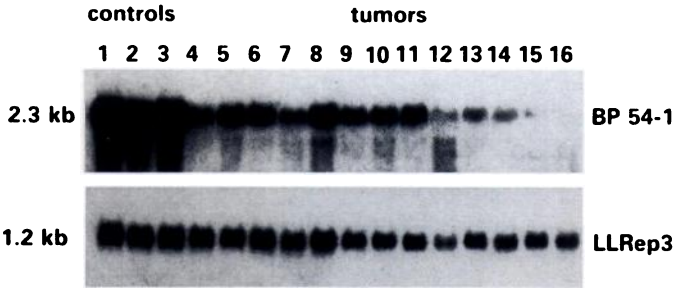

Fig. 3. Northern blot analysis of BP54–1-related transcripts (*top*) and *LLRep3* mRNA levels (*bottom*) in normal control livers (*lanes 1-3*) and in urethane-induced liver tumors of AC3F$_1$ male mice (*lanes 4-16*).

and detected eight clones, from five different plates, showing a hybridization signal. Five clones were purified and one (designated BP54–1 and containing a 1.8-kb insert) cross-hybridized with one of the seven other clones.

Northern blotting. Northern blot analysis of different mouse tissues showed that clone BP54–1 recognized a single 2.3-kb transcript and displayed a strong signal with normal adult liver and lung and a detectable signal with stomach and testis but a very weak signal with the other organs (Fig. 1, *middle*).

Fig. 3, *top*, shows the expression of clone BP54–1 in control adult liver (Fig. 3, *top*, *lanes 1-3*) and in urethane-induced hepatocellular tumors (Fig. 3, *top*, *lanes 4-16*). The BP54–1-related mRNA was present at 5–10-fold higher levels in normal adult liver, compared with hepatocellular tumors. Transcript levels of the *LLRep3* housekeeping gene were similar in each sample shown in Figs. 1 and 3, *bottom*, except for pancreas (where the mRNA was degraded), confirming that similar amounts of RNA were present in each lane.

Nucleotide sequence analysis. The sequence of clone BP54–1 was derived from two overlapping cDNA isolates, one of 1.8 kb and the other of 1.6 kb. A comparison of the partial nucleotide sequence of BP54–1 (396 base pairs) with the GenBank database demonstrated strong homology to the 5′ region of mouse, rat, and human AHD. The partial sequence of the BP54–1 clone completely matched the reported AHD-2 sequence (21). The only difference was a single T/C transition at nucleotide position 303, which led to a substitution of cysteine for arginine at amino acid position 87. Therefore, we concluded that clone BP54–1 represents the mouse AHD-2 gene.

AHD enzyme activity and inhibition by daunorubicin. The identification of DNR-BP54 as an AHD was confirmed by measuring the activity of fresh preparations of DNR-BP54 from both rat and mouse livers. The results are shown in Fig. 4. The proteins were electrophoretically pure and reduced NAD in the presence of saturating concentrations of acetaldehyde, at rates of the same order of magnitude as that reported for pure AHD-2 from mouse livers (22). Addition to the reaction mixture of 200 μM doxorubicin or daunorubicin caused a partial inhibition of the activity, which was slightly but reproducibly stronger for daunorubicin with both rat and mouse enzymes (Fig. 4). Reduction of the acetaldehyde concentration did not increase the inhibition by anthracyclines, at saturating 1 mM NAD concentration (data not shown).

Fig. 5A shows the competition of daunorubicin with NAD, using DNR-BP54 from mouse liver. From the double-reciprocal plot a K_m of 27 μM for NAD was calculated, in agreement with the reported value of 40 μM for mouse liver AHD-2 (22). The plot indicated a pure competitive mechanism of inhibition for the drug. The same data replotted in a $1/V$ versus daunorubicin concentration graph (Fig. 5B) gave a K_i of about 15 μM. No inhibition was observed at daunorubicin concentrations lower than 1 μM.

Discussion

In this work we showed that the antiserum raised against rat liver DNR-BP54 recognizes mouse AHD-2. Both the rat protein used for rabbit immunization and the affinity-purified mouse liver protein were confirmed to be AHDs by assays of their activity with acetaldehyde and NAD. Mouse DNR-BP54 had a specific activity comparable to that of pure AHD-2 (22); therefore, it could represent the mouse orthologue of rat DNR-BP54.

The immunocytochemical analysis showed that DNR-BP54

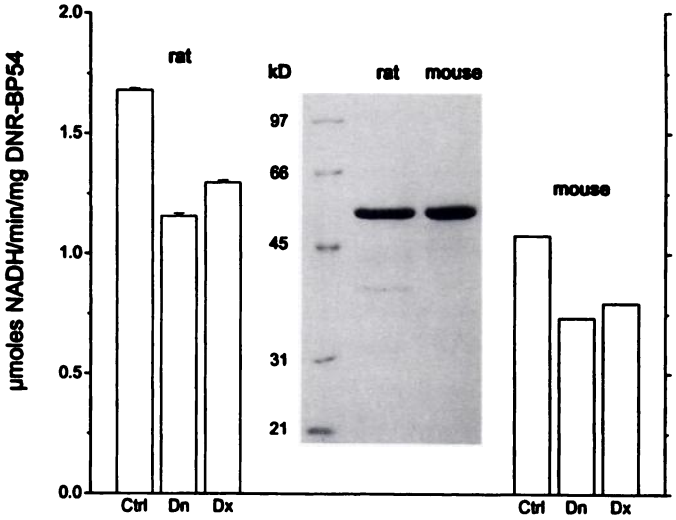

Fig. 4. SDS-polyacrylamidegel electrophoresis of mouse and rat DNR-BP54 and AHD activity of the proteins. DNR-BP54 (10 μg) was electrophoresed on a 10% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. The activity of the proteins was assayed as described in Experimental Procedures, using 50 mM acetaldehyde as substrate. *Ctrl*, control; *Dn*, 200 μM daunorubicin; *Dx*, 200 μM doxorubicin.

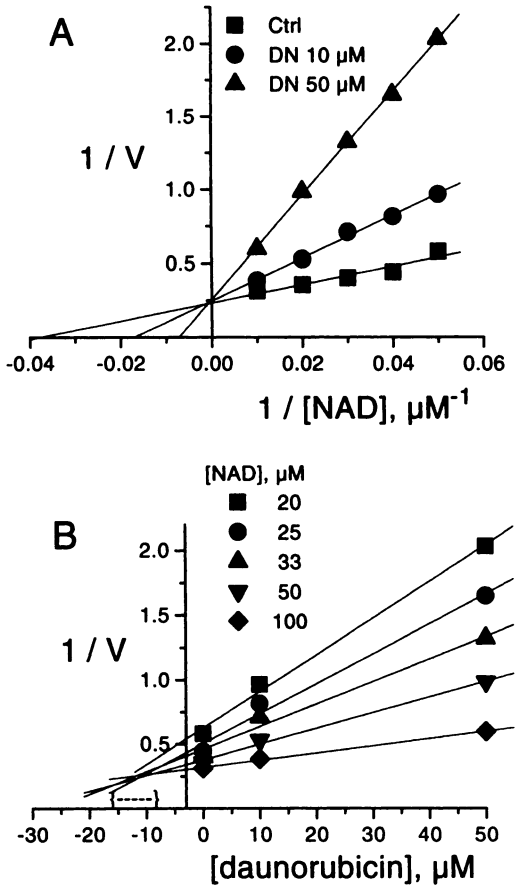

Fig. 5. Inhibition by daunorubicin of mouse DNR-BP54 AHD activity. The assays were performed at room temperature, as described in Experimental Procedures, with 10 mM acetaldehyde and the indicated daunorubicin concentrations. A, Lineweaver-Burk plots intersect the 1/*V* axis at a single point, indicating pure competitive inhibition. *Ctrl*, control; *DN*, daunorubicin. B, 1/*V* versus daunorubicin concentration plots cross at $x = -K_i$. *Dashed line*, range of K_i values.

was abundantly expressed in mouse liver and was localized in the cytoplasm of hepatocytes. DNR-BP54 expression was undetectable in hepatocellular tumors, even in those of microscopic size. Therefore, the suppression of DNR-BP54 expression could represent an early change in mouse liver tumor development. Our attempts to detect the protein in tumor cell systems were unsuccessful, suggesting that the expression of the protein could be a feature of normal but not tumor tissues. The absence of AHD, measured with aliphatic aldehydes, has been reported in several tumors (23, 24), and the tumor-specific AHDs appear to be immunologically unrelated to normal tissue enzymes, in addition to preferentially oxidizing aromatic aldehydes (25–27). Thus, our antibody directed against normal rat liver cytosolic AHD-2, which is the only soluble AHD described so far in this organ, should not cross-react with tumor-specific AHDs.

The antibody does react with M_r 54,000 polypeptides present in different amounts in the extracts of mouse organs. The tissue distribution reported herein for DNR-BP54 in mice appears to be somewhat different from that reported for mouse AHD-2, which has been shown, using assays of enzyme activity, to be mainly expressed in the liver and expressed at lower levels in the lung and testis (28). This might be due to a cross-reaction of our antibody with other normal tissue AHDs, especially AHD-5, which is found in all tissues (28), and AHD-8, which was recently reported in mouse stomach and liver (20, 29). In this respect the Northern blot data, showing higher levels of expression in liver, lung, and testis, appear to be in better agreement with the published enzyme activity distribution of AHD-2 in mice (28). The incomplete correspondence between Northern and Western data might also be due to other factors, such as mRNA degradation (especially in pancreas and skin), nonlinearity of Western blot responses, and different rates of mRNA and protein turnover in the different tissues.

Whether our findings are relevant to a better understanding of the mechanism of anthracycline antitumor action is still a matter of speculation. A drug-binding protein might influence the pharmacokinetics of the drug, e.g., by causing its accumulation or by favoring its elimination by acting as a transporter in an extrusion mechanism, or might determine its metabolic fate. Comparison of published information about anthracycline distribution in mice (30–32) and the expression of DNR-BP54 or AHD-2 did not reveal a simple relationship. The data indicated much higher drug levels in spleen than in intestine and kidney, whereas the reverse was true for DNR-BP54. Heart, though it has an area under the concentration-time curve value for doxorubicin similar to that of liver, did not express the protein. Most important are the similar distribution patterns for anthracyclines in different animals (33), compared with the variability of AHD expression, which is determined by racial (34) and genetic (35) factors and also by age (36), gender (37), and drug treatment (38). The antiserum against DNR-BP54 revealed important quantitative differences in the expression of the protein in mouse and rat organs.[1] In addition, several other factors, such as blood flow, DNA concentration (39), and expression and function of the M_r 170,000 glycoprotein extrusion pump (40), are involved in determining the tissue concentration of anthracyclines. Thus, a simple correlation between the expression of DNR-BP54 and the pharmacokinetic parameters of anthracyclines is not to be expected.

Tumor tissues do not express DNR-BP54, so a direct action of this protein in the mechanism of antitumor activity can be excluded. In normal tissues, however, drug binding to the protein would reduce drug interaction with the cytotoxicity targets, thus making normal cells less sensitive to the drugs. This hypothesis would account for a somewhat selective antitumor effect and is consistent with the high therapeutic index of doxorubicin but would require a high affinity of anthracyclines for the protein. This information is presently not available, because of the failure of several attempts to measure the K_d of the complex.

A biochemical consequence of anthracycline binding to AHD-2 is the inhibition of AHD activity. The inhibition was found to be competitive with respect to NAD, and the K_i of daunorubicin was estimated to be approximately 15 μM (Fig. 5). This indicates an interaction weaker than that with DNA but increases the number of the possible drug binding sites. Because the binding site for NAD is highly conserved, it is conceivable that anthracyclines could bind to other NAD-linked enzymes.

Another hypothesis can be suggested, however; AHDs are responsible for cyclophosphamide detoxification, in that they catalyze the oxidation of the drug metabolite aldophosphamide to inactive carboxyphosphamide. This prevents the formation of phosphoramide mustard, the active compound (20, 38). The lack of AHD activity in some tumors is the accepted mechanism

[1] P. Banti, C. Lanzi, and R. A. Gambetta, unpublished observations.

for selectivity of cyclophosphamide, and the induction of high levels of AHD is a mechanism of resistance to this drug (23, 41–43). A similar mechanism could be operative for anthracyclines. We have not yet been able to demonstrate this type of metabolic transformation for anthracyclines. However, the conversion of doxorubicin to aldehydic derivatives, which could be substrates for further oxidation by the AHDs, has been shown under particular experimental conditions. Interestingly, this pathway is available only to doxorubicin and not to daunorubicin (44). Thus, the differences in the therapeutic indices of different anthracylines, having the same inhibitory activities on cell proliferation (45–47), could be due to their different metabolic transformations by DNR-BP54. We are presently working to test this hypothesis and also to ascertain whether anthracyclines can bind to AHDs other than the soluble AHD-2.

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