Metabolism of Daunorubicin by a Barbiturate-Sensitive Aldehyde Reductase from Rat Liver

By ANTHONY J. TURNER and PAULINE E. HICK

Department of Biochemistry, University of Leeds, 9 Hyde Terrace, Leeds LS2 9LS, U.K.

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A barbiturate-sensitive aldehyde reductase was purified to homogeneity from rat liver and shown to metabolize the cancer-chemotherapeutic antibiotic daunorubicin. This aldehyde reductase may have important roles in the metabolism of exogenous drugs as well as the aldehyde derivatives of the biogenic amines.

The anthracycline aminoglycosides daunorubicin and adriamycin have been demonstrated to be effective cancer-chemotherapeutic antibiotics (Middleman *et al.*, 1971; Connors, 1975) and are thought to act by intercalation within the DNA double helix, thereby inhibiting replication and transcription (Theologides *et al.*, 1968; Ward *et al.*, 1965). However, the use of these drugs is limited because of their cardiotoxicity, which is presumably due to their structural similarities to the digitalis glycosides. In order to overcome these problems, attempts are being made to design less toxic derivatives and also to understand the metabolic transformations that these drugs undergo *in vivo* (Connors, 1975; Bachur *et al.*, 1974).

In several species, the major metabolites of daunorubicin and adriamycin are their respective alcohol derivatives daunorubicinol and adriamycinol, which are formed by the action of an NADPH-dependent reductase (daunorubicin reductase) present in liver cytosol (Felsted *et al.*, 1974). These alcohol metabolites have been suggested to be important components in the intracellular actions of daunorubicin and adriamycin (Bachur, 1974).

Daunorubicin reductase has been purified from rat liver, and substrate-specificity studies have shown that the enzyme was capable of reducing a variety of 2-hydroxy aldehydes such as glyceraldehyde, in addition to daunorubicin and adriamycin (Felsted *et al.*, 1974). This observation suggested to us that daunorubicin reductase may be similar in properties to other well-characterized NADPH-dependent aldehyde and ketone reductases reported in the literature (Turner & Tipton, 1972a,b; Bosron & Prairie, 1972). The properties of such enzymes have been reviewed (Tipton *et al.*, 1976). The present paper demonstrates that daunorubicin reductase and a barbiturate-sensitive aldehyde reductase (alcohol-NADP oxidoreductase, EC 1.1.1.2) responsible for the metabolism of the aldehyde derivatives of the biogenic amines ('biogenic aldehydes') may be identical enzymes in rat liver.

Materials and Methods

Daunorubicin hydrochloride was a kind gift from Dr. S. Algeri, Mario Negri Institute for Pharmacological Research, Milan, Italy. Daunorubicin hydrochloride was also obtained from Calbiochem Ltd. (Hereford, U.K.) and identical results were obtained with both batches of drug. Sodium barbitone, sodium phenobarbitone and DL-glyceraldehyde were obtained from British Drug Houses Ltd., Poole, Dorset, U.K., and diphenylhydantoin was from Ralph N. Emanuel Ltd., Wembley, Middx., U.K. NADPH, NADH, indol-3-ylacetaldehyde and 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., and 4-hydroxyphenylglycolaldehyde was prepared as described previously (Turner & Tipton, 1972b).

NADP-Sepharose was prepared by the method of Mosbach *et al.* (1972) and 5'-AMP-Sepharose 4B was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Polyacrylamide-gel electrophoresis was carried out as described previously (Turner & Tipton, 1972a) and polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was carried out by the method of Weber & Osborn (1969), in 7.5% acrylamide/0.1% sodium dodecyl sulphate gels at pH 7.2. Rat liver alcohol dehydrogenase (EC 1.1.1.1) was isolated by affinity chromatography on 5'-AMP-Sepharose by the method of Andersson *et al.* (1974) and was assayed at pH 9.0 with ethanol as substrate as described by Bonnichsen (1963).

Daunorubicin reductase was purified from rat (Wistar albino) liver by the method of Felsted *et al.* (1974), except that the gel-filtration step was performed on Sephadex G-100 rather than Bio-Gel P200 and the enzyme was then subjected to affinity chromatography on a column (0.5 cm × 5 cm) of NADP-Sepharose to remove minor impurities (A. J. Turner & P. E. Hick, unpublished work).

Routine assays of daunorubicin reductase were

performed at 30°C in 200 mM-Tris/HCl buffer, pH 8.5, containing 0.1 mM-NADPH and 0.75 mM-daunorubicin. For kinetic studies, addition of enzyme was normally used to start the reaction. The reaction was monitored continuously by following the decrease in absorbance at 340 nm in a Gilford model 240 spectrophotometer coupled to a Servoscribe 8 in (20 cm) chart recorder. The reaction rate was linear for at least 5 min and the initial rate was proportional to enzyme concentration. Concentrations of daunorubicin were measured as described by Felsted *et al.* (1974).

A similar assay system was used to measure the reduction of glyceraldehyde or 4-hydroxyphenylglycolaldehyde, except that these aldehydes replaced daunorubicin in the reaction mixture. A unit of enzyme activity is defined as the amount that catalyses the oxidation of 1 μ mol of NADPH/min at 30°C.

Results and Discussion

When the product of the purification of daunorubicin reductase was subjected to polyacrylamide-gel electrophoresis, a single band of protein was observed that was coincident with enzyme activity detected on an identical gel run in parallel. Polyacrylamide-gel electrophoresis of daunorubicin reductase in the presence of sodium dodecyl sulphate also revealed a single band of protein. The final, apparently homogeneous, product was purified approx. 2500-fold over the high-speed-supernatant fraction. The enzyme was able to reduce daunorubicin and glyceraldehyde, and the respective Michaelis constants for these substrates were determined to be 0.05 mM and 1.3 mM at pH 8.5, in good agreement with the values reported by Felsted *et al.* (1974). In addition, we found that the biogenic aldehydes 4-hydroxyphenylglycolaldehyde (the aldehyde derivative of octopamine) and indolylacetaldehyde proved active as substrates for this enzyme.

To confirm that the reduction of these substrates is carried out by a single enzyme species, we have carried out mixed-substrate experiments as described by Dixon & Webb (1964). The use of mixtures of two substrates is a useful method for the detection of the presence of two enzymes of differing specificities. The initial velocities of reduction of the substrates daunorubicin and 4-hydroxyphenylglycolaldehyde were determined at concentrations close to their K_m values and compared with the initial velocities obtained when pairs of these substrates each present at their respective K_m values were used. The rate observed in the presence of both substrates together was 69% of the sum of the rates obtained for each aldehyde assayed separately, which is consistent with the involvement of a single enzyme.

The NADPH-dependent aldehyde reductases isolated from various tissues have been reported to be

Table 1. *Effects of drugs on the activity of daunorubicin reductase*

The effects of various drugs at a concentration of 1 mM were tested on the activity of rat liver daunorubicin reductase assayed with either daunorubicin or 4-hydroxyphenylglycolaldehyde as substrate. Assays were carried out as described in the Materials and Methods section and the reaction was started by the addition of enzyme. The observed rates are tabulated as percentages of the control value in the absence of drug.

Drug	Rate (% of control)	
	Daunorubicin	4-Hydroxyphenylglycolaldehyde
Sodium barbitone	<5	<5
Sodium phenobarbitone	5	8
Diphenylhydantoin	<5	<5
Homovanillic acid	65	59
Pyrazole	90	91

inhibited by a number of compounds, including anti-convulsant drugs and acidic metabolites of the biogenic amines (Erwin & Deitrich, 1973; Turner & Hick, 1975). The effects of a number of inhibitors on the reduction of daunorubicin and 4-hydroxyphenylglycolaldehyde by daunorubicin reductase are compared in Table 1, which shows that similar degrees of inhibition are observed in all cases. The classical alcohol dehydrogenase inhibitor pyrazole, however, had little effect on the activity of daunorubicin reductase. The kinetics of the inhibition of daunorubicin reduction by sodium barbitone and diphenylhydantoin were investigated in more detail and shown to be reversible and apparently non-competitive with respect to daunorubicin as substrate (Fig. 1). The inhibition constants (K_i values) obtained from Dixon (1953) plots were 18 μ M and 20 μ M respectively for barbitone and diphenylhydantoin at pH 8.5. Similar inhibition data have been reported for brain aldehyde reductases with aromatic aldehydes as substrates (Erwin & Deitrich, 1973). Further evidence supporting the identity of NADPH-dependent aldehyde reductase and daunorubicin reductase is the observation that both enzymes have molecular weights in the region of 30000–40000 (Turner & Tipton, 1972*b*; Bosron & Prairie, 1972; Felsted *et al.*, 1974).

Rat liver also contains a pyrazole-sensitive NAD-dependent alcohol dehydrogenase capable of reducing a variety of aldehydes and ketones, which was purified to a specific activity of 1.5 units/mg by the method of Andersson *et al.* (1974). This enzyme was unable to reduce daunorubicin or adriamycin in the presence of 0.1 mM-NADH or -NADPH at pH 7.2 and 8.5, thereby providing a further distinction between alcohol dehydrogenases and aldehyde

Fig. 1. Inhibition of daunorubicin reduction by sodium barbitone and diphenylhydantoin

(a) Double-reciprocal plot of the inhibition of daunorubicin reductase by barbitone with daunorubicin as the variable substrate. NADPH was kept constant at 0.1 mM and the concentrations of barbitone used were: Δ , 0; $\circ$, 0.01 mM; $\square$, 0.02 mM; $\blacktriangle$, 0.03 mM; $\bullet$, 0.04 mM. (b) Inhibition of daunorubicin reductase by diphenylhydantoin. Double-reciprocal plots of the inhibition of daunorubicin reductase by diphenylhydantoin with daunorubicin as the variable substrate. NADPH was kept constant at 0.1 mM and the concentrations of diphenylhydantoin used were: Δ , 0; $\circ$, 0.033 mM; $\square$, 0.067 mM; $\blacktriangle$, 0.1 mM.

reductases (for further discussion see Tipton *et al.*, 1976).

Thus, on the basis of apparent enzyme homogeneity, mixed-substrate criteria, inhibition data and molecular size, barbiturate-sensitive aldehyde reductase from rat liver appears to be identical with the reported daunorubicin reductase present in this tissue. In brain tissue, aldehyde reductase has been reported to exist in multiple forms (high- K_m and low- K_m types) (Turner & Tipton, 1972b; Ris & von Wartburg, 1973). Although the presence of possible isoenzymes of aldehyde reductase in rat liver has not been investigated, the enzyme isolated in the present work appears to be similar to the major form (high- K_m type) present in brain tissue. Whether reduction of daunorubicin is specific to one isoenzyme of aldehyde reductase remains to be established.

The conversion of daunorubicin into daunorubicinol involves the reduction of a ketone group. Aldehyde reductases have previously been reported to reduce some ketones, although the specificity is much more restricted than for aldehydes (Bosron & Prairie, 1972). The mechanisms for reduction of aldehydes and ketones by this enzyme may differ somewhat, since the apparent pH optimum for reduction of daunorubicin was approx. 8.5, whereas that for reduction of aldehydes was 7.0 or less (Felsted *et al.*, 1974; A. J. Turner, unpublished work). The differences in observed pH optima may also reflect the fact that it was not possible to assay with the substrates at saturating concentrations.

In conclusion, the reduction of the antibiotics daunorubicin and adriamycin appears to be carried out by a non-specific NADPH-dependent aldehyde reductase present in rat liver which is sensitive to inhibition by certain anti-convulsant drugs. The use of barbiturates to inhibit aldehyde reductase *in vivo* may allow the relative effectiveness and cardiotoxicity of daunorubicin and its major metabolite to be examined in more detail in various animal species. Barbiturate-sensitive aldehyde reductases appear to have important roles both in the metabolism of endogenous aldehydes, including the biogenic aldehydes, as well as in the metabolism of certain drugs. Indeed, the presence of exogenous drugs may modify the metabolism of the physiologically active biogenic aldehydes by competition for the enzyme.

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