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EVIDENCE FOR COVALENT BINDING OF ADRIAMYCIN TO RAT LIVER MICROSOMAL PROTEINS

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Adriamycin (NSC-123,127) is an anticancer drug of the anthracycline type widely used in the clinical practice for the treatment of different malignancies (1). Intercalation with DNA has been suggested as having a role in the mechanism of action of this drug (2,3,4). Recently, reports by Sinha (5) and Lucacchini et al. (6) suggested a covalent interaction of Adriamycin (AM) with DNA and proteins. These studies, however, were not conclusive since they were carried out by spectrophotometric and chromatographic procedures. In the present study a covalent interaction between AM and microsomal proteins was demonstrated in a more "classical" covalent binding study with the ^{14}C-labelled compound.

Materials and Methods.

Chemicals. 14-^{14}C-AM (specific activity, 28.2 mCi/mmol) was obtained from Farmitalia, Nerviano, Milan, Italy. The radiochemical purity of labelled AM was checked by high pressure liquid chromatography according to the procedure described by Israel et al. (7). The compound was found to be more than 99% pure. The following reagents were used: NADPH, Boehringer, Mannheim, West Germany; superoxide dismutase (SOD), Istituto Farmacologico Serono, Rome, Italy. All other chemicals and solvents were of the purest grade commercially available.

Preparation of microsomes. CD-COBS rats (200-220 g) from Charles River Italy (Calco, Como, Italy) were used for the preparation of liver microsomes. The animals were killed by decapitation and the livers were excised, pooled and washed with 0.05M phosphate buffer pH 7.4. The livers were minced and homogenized at 0°C in 4 volumes of cold 0.05M phosphate buffer pH 7.4 using a Potter-Helvehjem homogenizer with a Teflon pestle. Microsomes were prepared according to Kato and Takayanaghi (8). The protein concentration was determined by the method of Lowry et al. (9), with albumin fraction V as the standard.

Covalent binding to microsomal proteins. The incubation mixture in a final volume of 2 ml contained 5 mg of NADPH, 0.05M phosphate buffer pH 7.4, magnesium chloride 5mM, potassium chloride 0.15M and 20 mg of microsomal protein. Labelled AM was added in 10 µl of acetone to obtain a final substrate concentration of 2µM. Incubation was at 37°C for 1 hour in subdued light. The reaction was stopped by the addition of 5 ml of ethanol. The samples were centrifuged, the supernatant was discarded and the precipitate was extracted three times with 5 ml volumes of the following solvent sequence: 70% ethanol, three times with ethanol, boiling ethanol, benzene-ethanol (1:4, v/v), twice with acetone-chloroform (4:1, v/v), acetone-ethanol (5:1, v/v), diethylether-ethanol

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Table 1. Irreversible protein binding of 14-^{14}C-AM catalyzed by rat liver microsomes.

Experimental condition	AM metabolites irreversibly bound to 1 mg of microsomal proteins (pmol ± S.E.)	% bound radioactivity/total radioactivity incubated
Acetonitrile denatured microsomes + AM + NADPH	2.25 ± 0.01	1.1
Microsomes + AM - NADPH	1.61 ± 0.03	0.8
Microsomes + AM + NADPH	8.86 ± 0.36[a]	4.4
Microsomes + AM - NADPH + SOD (100 μg)	2.07 ± 0.18	1.0
Microsomes + AM + NADPH + SOD (100 μg)	8.98 ± 0.08[b]	4.5

Each value is the mean ± S.E. of three determinations.

[a] $p<0.01$ compared with acetonitrile denatured microsomes + AM + NADPH and microsomes + AM - NADPH.

[b] $p<0.01$ compared with microsomes + AM - NADPH + SOD (100 μg).

(5:1, v/v), twice with ethylacetate-ethanol (5:1, v/v), ethanol and ethanol-water (2:3, v/v). All the extraction supernatants were tested for radioactivity. After the last cycle of extraction no further radioactivity could be removed from the proteins. This exhaustive extraction thus removed the original AM as well as all metabolites reversibly bound to the proteins. Protein loss was very low during the procedure. The precipitate was dissolved in 0.5 ml of Soluene^{R}-350, Packard, Downers Grove, Illinois, U.S.A., and transferred into a counting vial containing 10 ml of a PPO-POPOP solution in toluene. Radioactivity was measured in a Nuclear Chicago Isocap 300 liquid scintillation counter. The values were corrected for quenching by the external standardization method.

Results and Discussion.

In the present study to demonstrate the chemical reactivity of AM towards cellular macromolecules microsomes were chosen as both activating system and target. Initial studies with DNA would have been difficult to interpret because of interference due to intercalated drug and/or metabolites. The radioactive material used, ^{14}C-AM, was preferred to the tritiated compound in order to avoid possible isotope exchange effects. It can be assumed that the full metabolism of a certain drug can never be completely characterized; in fact, together with major pathways, minor ones can be present too. The problem of an isotope exchange effect in the evaluation of irreversible binding, which in general is very low compared to the dose, would thus have been of primary importance.

Table 1 shows the results of experiments where AM was incubated for 1 hour in the presence of microsomes. AM covalent binding was found to require microsomal activation and NADPH. In fact, when incubations were carried out either with denatured microsomes or without NADPH, a significantly lower amount ($p<0.01$) of bound radioactivity was found. This table also sets out the irreversibly bound radioactivity as a percentage of total radioactivity incubated. The high percentage (4.4%) offers the best demonstration that binding cannot be ascribed to impurities of the labelled material.

It has been suggested that AM is biotransformed to semiquinone radical species during a reduction process catalyzed by NADPH (10). Semiquinone radicals can stimulate the formation of superoxides and may bind irreversibly to biological macromolecules. The possible implication of superoxide radicals in AM covalent binding to microsomal proteins was studied in experiments where SOD was added to the incubation mixture. SOD had no effect on AM irreversible binding to microsomes.

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