# REDUCTIVE CLEAVAGE OF ANTHRACYCLINE GLYCOSIDES BY MICROSOMAL NADPH-CYTOCHROME C REDUCTASE

Sir:

In the course of the studies on the metabolism of a new antitumor anthracycline antibiotic, aclacinomycin A[1], we found that the enzyme in rat liver microsomes which catalyzed the reductive cleavage at the C-7 position of anthracycline glycosides (Fig. 1) was identical with the microsomal NADPH-cytochrome C reductase (EC 1.6.2.4).

The enzyme responsible for the reductive cleavage of anthracycline glycosides was extracted and purified from the microsomal fraction of rat liver (Wistar strain) by the method of OMURA *et al.*[2] Washed microsomes were digested with trypsin (Mochida Pharm. Co.) at 0.014%~0.016% at 0°C for 16 hours. By means of this treatment more than 90% of the enzyme activity was solubilized. The trypsin-digested microsomal supernatant was obtained by centrifugation at 100,000 × *g* for 90 minutes and lyophilized. It was applied to a Sephadex G-100 column (1.8 × 65 cm) which was equilibrated with 10 mM potassium phosphate buffer, pH 7.5 and eluted with the same buffer. The reductase fractions were combined and applied to a DEAE-cellulose column (1.4 × 15 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.5. The column was washed with 50 mM potassium phosphate buffer, pH 7.5, containing 0.08 M potassium chloride and the enzyme was eluted with a linear gradient of potassium chloride from 0.1 to 0.35 M in 50 mM potassium phosphate buffer, pH 7.5. Throughout the purification procedures the ratio of specific activities of NADPH-cytochrome C reductase to anthracycline C-7 reductase remained constant (3.57~3.70) and both activities appeared in the same fractions. The yield of the enzyme activity from the trypsin-digest was 30~40%. The purified enzyme preparation showed a single band on disc gel electrophoresis, and possessed absorption maxima at 455, 380 and 275 nm. Details of the purification and properties of the

Fig. 1. Enzymic reductive cleavage of aclacinomycin A (R=$R_2$ for Aclacinomycin A in Table 1)

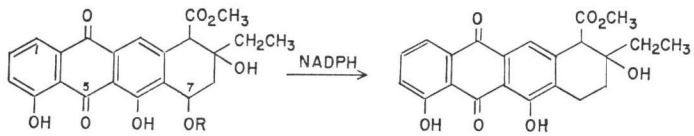
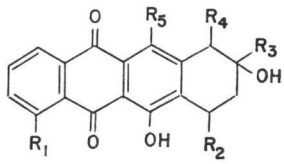



Table 1. Structures of anthracycline antibiotics and their aglycones



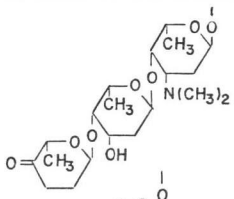
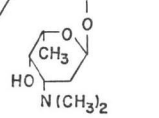
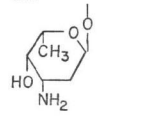
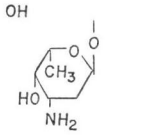
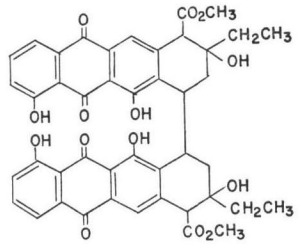
| Compounds | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|
| Aclacinomycin A | OH |  | $CH_2CH_3$ | $CO_2CH_3$ | H |
| 1-Deoxypyrromycin | OH |  | $CH_2CH_3$ | $CO_2CH_3$ | H |
| Aklavinone | OH | OH | $CH_2CH_3$ | $CO_2CH_3$ | H |
| Adriamycin | $OCH_3$ |  | $COCH_2OH$ | H | OH |
| Adriamycinone | $OCH_3$ | OH | $COCH_2OH$ | H | OH |
| Daunomycin | $OCH_3$ |  | $COCH_3$ | H | OH |
| Daunomycinone | $OCH_3$ | OH | $COCH_3$ | H | OH |
| Dihydrodaunomycinone | $OCH_3$ | OH | $CHOHCH_3$ | H | OH |

Fig. 2. Structure of MA144 E1



enzyme will be reported elsewhere.

Under aerobic conditions, the enzyme reduced cytochrome C, 2,6-dichlorophenol indophenol and potassium ferricyanide in the presence of NADPH. It also exhibited the NADPH oxidase activity in the presence of vitamin $K_3$ or adriamycin. These properties of the enzyme were similar to those reported by OMURA *et al.*[2], SATO *et al.*[3] and HANDA *et al.*[4] for cytochrome C reductase. ASBELL *et al.*[5] reported that the enzyme which catalyzed the C-7 reductive cleavage of daunomycin and adriamycin was located on the microsomal membrane and the reaction proceeded under anaerobic conditions requiring NADPH as a sole electron donor, moreover HANDA *et al.*[4] reported that under aerobic conditions quinone-containing antitumor compounds, such as adriamycin, *etc*, stimulated the NADPH oxidation by partially purified microsomal NADPH-cytochrome C reductase.

The action of the enzyme to catalyze the reductive cleavage of anthracycline antibiotics and their aglycones (Table 1) was studied under anaerobic conditions in the presence of NADPH by procedures described in Table 2. The substrate specificity of this enzyme was broad and the enzyme reduced not only the glycosidic bond of anthracycline glycosides but also the C-7 hydroxyl group of aglycones such as aklavinone, adriamycinone, daunomycinone and dihydrodaunomycinone. The reaction products were 7-deoxyaglycones such as 7-deoxyaklavinone, 7-deoxyadriamycinone, 7-deoxydaunomycinone, and 7-deoxydihydrodaunomycinone, respectively. Reductive cleavage by the enzyme proceeded essentially under anaerobic conditions and the optimum pH was 8.0. The reaction was completely inhibited by *p*-mercuribenzoate. NADH did not serve as an electron donor. It was also interesting to note that a reductively condensed aglycone dimer (MA144 E1 in Fig. 2) was also

Table 2. Reduction of anthracycline antibiotics and their aglycones by purified rat liver NADPH-cytochrome C reductase

| Substrates | Products ($\times 10^{-8}$ moles) | |
|---|---|---|
| Aclacinomycin A | 7-Deoxyaklavinone | 0.24 |
| | *MA144 E1 | 0.40 |
| 1-Deoxypyrromycin | 7-Deoxyaklavinone | 0.29 |
| | *MA144 E1 | 0.34 |
| Aklavinone | 7-Deoxyaklavinone | 0.83 |
| | *MA144 E1 | 1.16 |
| Adriamycin | 7-Deoxyadriamycinone | 3.05 |
| Adriamycinone | 〃 | 4.75 |
| Daunomycin | 7-Deoxydaunomycinone | 6.69 |
| Daunomycinone | 〃 | 2.69 |
| Dihydro-daunomycinone | 7-Deoxydihydro-daunomycinone | 2.02 |

* Structures are shown in Fig. 2

Antibiotics were dissolved in 5 mM acetic acid and their aglycones in 10 mM N,N-dimethylformamide. The reaction mixture (containing 78 mM of Tris-HCl, pH 8.0, 0.1 $\mu$moles of substrate and 2.5 $\mu$g of purified enzyme in a total volume of 0.9 ml) was placed in the main vessel of a Thunberg tube and NADPH solution (0.2 $\mu$moles in 0.1 ml) was placed in the side vessel. The gaseous phase was exchanged with nitrogen gas.

The reaction was started by the addition of NADPH to the main vessel and the tube was incubated at 37°C for 30 minutes. After stopping the reaction by addition of 2 ml of a chloroform - methanol mixture (1: 1), the chloroform layer was evaporated to dryness, dissolved in 0.1 ml of the chloroform - methanol mixture (1: 1) and a 10~20 $\mu$l of the samples was subjected to silicic acid thin-layer chromatography using the following solvent systems: chloroform - methanol, 50: 1, for aklavinone, 7-deoxyaklavinone, MA144 E1, daunomycinone and dihydrodaunomycinone, 20: 1 for aclacinomycin A, 10: 1 for 7-deoxydaunomycinone, 7-deoxyadriamycinone and 7-deoxydihydrodaunomycinone, and 5: 1 for 1-deoxypyrromycin; chloroform - methanol - acetic acid, 80: 20: 4, for adriamycin and daunomycin; chloroform - methanol - benzene, 7: 3: 3, for adriamycinone and 7-deoxyadriamycinone. The concentration of residual substrates and reaction products were determined spectrophotometrically at 470 nm for adriamycin, daunomycin and their aglycones, and at 430 nm for aclacinomycin A, 1-deoxypyrromycin and their aglycones. The structures and identification of the reaction products were confirmed by Rf values on silicic acid thin-layer chromatography, cochromatography with authentic samples, and by pmr and mass spectra.

produced as one of the reaction products of this enzyme with aclacinomycin A, 1-deoxypyrromycin and aklavinone as substrates (Table 2).

These results indicate that the anthracycline glycoside C-7 reductase which plays an important role in the metabolism of these antibiotics *in vivo* is similar to or identical with the microsomal NADPH-cytochrome C reductase.

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## References

1) Oki, T.; Y. Matsuzawa, Y. Yoshimoto, K. Numata, I. Kitamura, S. Hori, A. Takamatsu, H. Umezawa, M. Ishizuka, H. Naganawa, H. Suda, M. Hamada & T. Takeuchi: New antitumor antibiotics, aclacinomycins A and B. J. Antibiotics 28: 830~834, 1975
2) Omura, T. & S. Tatesue: A new method for simultaneous purification of cytochrome $b_5$ and NADPH-cytochrome C reductase from rat liver microsomes. J. Biochem. 67: 249~257, 1970
3) Nishibayashi-Yamashita, H. & R. Sato: Vitamin $K_3$-dependent NADPH oxidase of liver microsomes. Purification, properties and identity with microsomal NADPH-cytochrome C reductase. J. Biochem. 67: 199~210, 1970
4) Handa, H. & S. Sato: Stimulation of microsomal NADPH-oxidation by quinone containing anticancer chemicals. Gann 67: 523~528, 1976
5) Asbell, M. A.; E. Schwartzbach, F. J. Bullock & D. W. Yesair: Daunomycin and adriamycin metabolism *via* reductive glycosidic cleavage. J. Pharmacol. Exp. Ther. 182: 63~69, 1972