Biochemical Pharmacology, Vol. 38, No. 14, pp. 2285–2289, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

LACK OF COMPETITION BETWEEN CYTOCHROME *c* AND ANTHRAQUINONE TYPE DRUGS FOR THE REDUCTIVE SITES OF NADH DEHYDROGENASE

JOLANTA TARASIUK,* ARLETTE GARNIER-SUILLEROT†‡ and EDWARD BOROWSKI*

†Laboratoire de Chimie Bioinorganique (UA CNRS 198), UFR de Medecine et Biologie Humaine, 74 rue Marcel Cachin, 93012 Bobigny, France, and *Department of Pharmaceutical Technology and Biochemistry, Technical University of Gdansk, 80-952 Gdansk, Poland

(*Received* 28 *October* 1988; *accepted* 18 *January* 1989)

Abstract—We have shown that (i) the cytochrome *c* reductase activity of the commercial NADH dehydrogenase does not perturb its ability to catalyse the reduction of various antitumor compounds of the anthracycline class, (ii) the reduction of these compounds by NADH, catalysed by commercial NADH dehydrogenase, correlates with their reduction by NADH catalysed by microsomes. Moreover, our data strongly suggest that two catalytic sites are present, one for cytochrome *c* and one for quinone type compounds.

The anthracycline antibiotics Adriamycin® (doxorubicin hydrochloride)§ (Adr) and daunorubicin (Dr) are highly efficient antineoplastic agents; unfortunately their clinical use is limited due to clinical and histopathologic evidence of cardiotoxicity [1]. Although the mechanism of anthracycline cardiac toxicity remains incompletely understood, there are now numerous data suggesting that it is related to their ability to be reduced by an enzyme of the mitochondrial respiratory chain [2, 3], their subsequent reoxidation by molecular oxygen generating active oxygenated species largely responsible for cardiac injury. In a recent study, using beef heart submitochondrial preparation, Doroshow and Davies [4, 5] have clearly demonstrated that a component of mitochondrial complex I, probably the NADH dehydrogenase flavin, is the mitochondrial site of anthracycline reduction.

For some years we have been involved in the search for new derivatives exhibiting less cardiotoxic effects. We have thus synthesized compounds modified either at the level of the sugar or at the level of the dihydroxyanthraquinone moiety. We have shown that modified anthracyclines either with large groups at the sugar moiety [6–8] or complexed with metal ions [9–11] are more difficult to be reduced than daunorubicin or Adriamycin®. Their production of activated oxygen is thus limited.

Submitochondrial particles or microsomes are usually used to test the ability of anthracycline derivatives to generate activated oxygenated species and in a first step superoxide radicals [2, 12]. On the other hand it has been shown that a crude soluble NADH dehydrogenase preparation, from porcine heart mitochondria, can catalyse anthracycline reduction by NADH [13]. Unfortunately, probably due to extraction procedure, the commercially available enzyme appears to be damaged as evidenced by its cytochrome *c* reductase activity which is a non-physiological activity [14], and by loss of the physiological ubiquinone reductase (this inability has been referred to the fact that the dehydrogenase is essentially devoid of lipids).

However, the use of NADH dehydrogenase, available commercially, to determine the ability of anthracycline derivatives to generate superoxide radicals, is a very rapid and simple test as compared to that using submitochondrial particles or microsomes.

In this context we have undertaken a study to determine whether the commercially available NADH dehydrogenase could be used to this test. Our data show that (i) reduction of the drugs by NADH, catalysed by commercially available NADH dehydrogenase, correlates with their reduction by NADH catalysed by microsomes, (ii) the cytochrome *c* reductase activity of the enzyme does not perturb its ability to catalyse the reduction of anthracycline type drugs by NADH. Moreover, our data suggest that two catalytic sites are present: one for cytochrome *c* and one for quinone type compounds.

MATERIALS AND METHODS

Purified Adriamycin® (Adr) (Chart 1), 4′-epi-adriamycin (4′-Adr), aclacinomycin (Acla) were kindly provided by Laboratoires Roger Bellon and daunorubicin (Dr) by Laboratoires Rhône Poulenc. *N*-(1-deoxyfructos-1-yl)daunorubicin (Dr-10), *N*-[1-carboethoxy-propen-1-yl] daunorubicin (Dr-19), 5-imino-daunorubicin (5-IDr), dibenzyldaunorubicin (diB-Dr), ametantrone and novantrone were synthesized as indicated in previous work [6, 7, 8, 15–18]. Stock solutions were prepared just prior to use. Cytochrome *c* (type VI, from horse heart), NADH (grade III), NADH dehydrogenase (from porcine heart), superoxide dismutase (SOD), riboflavin 5′-phosphate (FMN) and *p*-chloromercuriphenylsulphonic acid (CPSA) were from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest quality available and deionized bidistilled

‡ To whom correspondence should be addressed.

§ Adriamycin is a registered trademark of Farmitalia Carlo Erba.

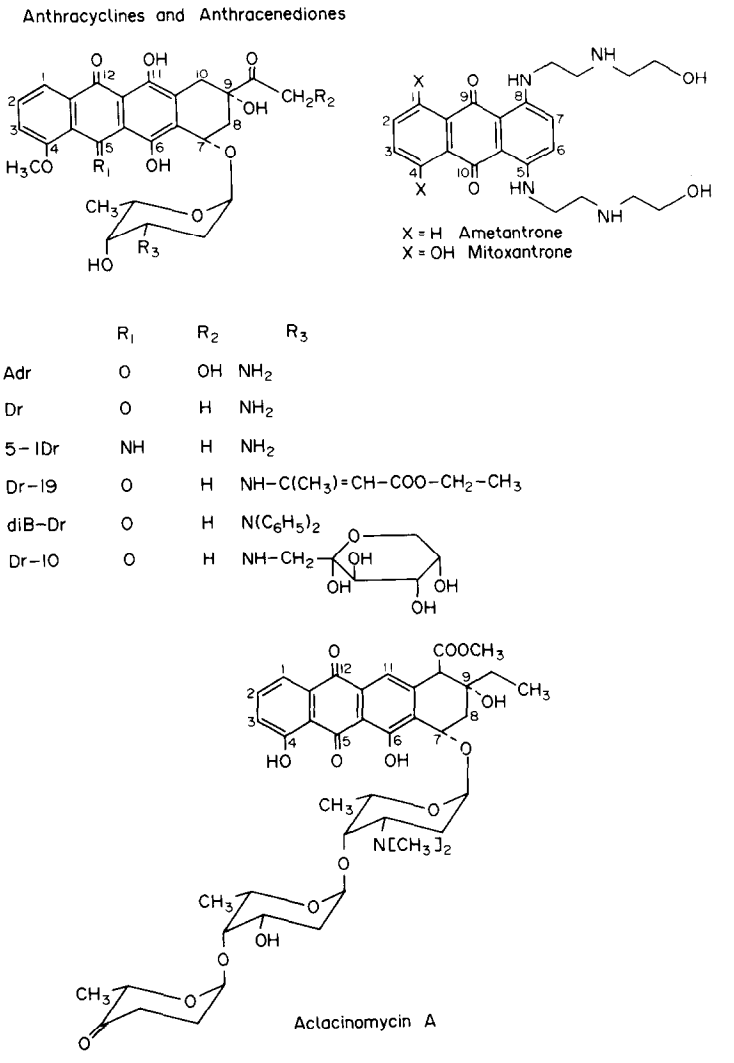

Chart 1.

water was used throughout the experiments. Absorption spectra were recorded on a Cary 219 spectrophotometer. The rate of oxygen consumption was determined at 25° using a YSI 5331 oxygen monitoring system. Microsomes were prepared from liver of male Sprague–Dawley rats as previously described [19, 20]. The final microsomal sediments were suspended in 0.15 M KCl–0.05 M Tris–HCl buffer, pH 7.4. Protein concentration was determined by the method of Lowry *et al.* [21].

NADH oxidation was measured at 340 nm using an extinction coefficient of 6200/M cm and cytochrome *c* reduction at 550 nm, using an extinction coefficient (reduced minus oxidized) equal to 19600/M cm.

RESULTS AND DISCUSSION

The reduction of various anthracycline derivatives by NADH, catalysed by NADH dehydrogenase, was compared with their reduction by NADH catalysed by microsomes. The rate of oxygen consumption or NADH oxidation was taken as 100% for daunorubicin and Table 1 shows the percentage of the rate in the case of the other derivatives. As can be seen results obtained for NADH dehydrogenase correlates more or less with those obtained with microsomes.

The NADH dehydrogenase catalysed reduction of the drug by NADH and their subsequent reoxidation by molecular oxygen, yielding superoxide radical, can be monitored spectroscopically at 550 nm by the reduction of cytochrome *c* by superoxide. This method is less time consuming than oxygen consumption measurements and is three times more sensitive than the spectroscopic determination at 340 nm of NADH oxidation.

A first set of experiments was performed in order to determine the kinetics of cytochrome *c* reduction. The variation of the optical density at 550 nm was thus followed as a function of time. Table 2 shows the data of a typical experiment in which 60 μl NADH dehydrogenase was added to a mixture containing 40 μM cyt c^{3+}, 0 or 100 μM drug and 83 μM

Table 1. Comparison of the rate of NADH oxidation and oxygen consumption by anthracyclines catalysed by NADH dehydrogenase or microsomes

	NADH dehydrogenase		Microsomes
	NADH[a] oxidation	Oxygen[b] consumption	Oxygen[c] consumption
Dr 0.1 M	100%	100%	100%
Adr	90	87	—
Acl	93	88	—
Dr-10	75	67	82
5-IDr	23	18	37
DiB-Dr	17	15	24
Dr-19	14	10	31
Nov	11	10	18
Ame	11	9	17
Control	5.9	0	22

Experimental conditions were as follows: HEPES buffer 0.05 M pH 7.4, 100 μM anthracycline and [a]200 μM NADH, 200 u/l NADH dehydrogenase, [b]200 μM NADH and 100 u/l NADH dehydrogenase, [c]100 μM NADH and 0.5 g/l microsomal proteins. Daunorubicin was taken as a standard. In the presence of NADH dehydrogenase the absolute rates of NADH oxidation and oxygen consumption corresponding to the 100% control were 11 μM/min and 25 μM/min, respectively. In the presence of microsomes, the absolute rate of oxygen consumption corresponding to the 100% control was 9 μM/min g. Standard deviation was about 5%.

NADH. The initial rate of cyt c^{3+} reduction has been determined. In the absence of drug, the formation of cyt c^{2+} is due to the reduction of cyt c^{3+} by NADH catalysed by the enzyme (E). With these conditions a mean value of 3.4 μM/min was obtained. The rate, hereafter labelled v_1 of cyt c^{2+} formation is proportional to the enzyme – cyt c^{3+} complex $[E - \text{cyt } c^{3+}]_1$ concentration, i.e. $v_1 = k_1[E - \text{cyt } c^{3+}]_1$, where k_1 is the kinetics constant for the catalysed reduction of cyt c^{3+} by NADH.

When the experiment is performed in the presence of drug (D), cyt c^{3+} is reduced either by NADH through the enzyme or by O_2^-. The rate, hereafter labelled v_2 of cyt c^{2+} formation, can thus be written

$$v_2 = k_1[E - \text{cyt } c^{3+}]_2 + k_2[\text{cyt } c^{3+}][O_2^-],$$

where k_2 is the kinetics constant for reduction of cyt^{3+} c by O_2^- and $[E - \text{cyt } c^{3+}]$ is the concentration of enzyme–cytochrome c complex in the presence of the drug. Taking into account that O_2^- concentration is proportional to the enzyme–drug complex $[E\text{–}D]$ concentration it follows that

$$v_2 = k_1[E - \text{cyt } c^{3+}]_2 + k_2'[\text{cyt } c^{3+}][E\text{–}D].$$

Similar experiments were performed in the presence of superoxide dismutase. In that case as O_2^- was eliminated by SOD and the reduction of cyt c^{3+} was due to NADH through the enzyme only. One should expect a kinetics $v_3 = k_1[E - \text{cyt } c^{3+}]_2$. The data reported in Table 2 show that v_1 and v_3 have the same values. We can infer that the concentration of enzyme–cytochrome c complex does not depend on the presence of the drug. This strongly suggests that cyt c^{3+} and drug do not compete for the reduction by the enzyme and that two different catalytic sites are present, one responsible for cytochrome c reduction and the other for drug reduction. The kinetics of reduction of cytochrome by O_2^- is thus the difference between the kinetics of reduction in the presence and in the absence of drug.

In a second set of experiments 100 μM cytochrome c and 100 μM drug were used. These compounds were in large excess in regards to NADH which was only 10 μM. The low NADH concentration was used in order to observe its complete oxidation. An enzyme amount of 125 u/l was used throughout these experiments. When the reaction was performed in the absence of drug the reaction stopped when exactly two moles cyt c^{3+} were reduced per mole NADH (Fig. 1). When the same experiment was

Table 2. Initial rate of cyt c^{3+} reduction by NADH catalysed by NADH dehydrogenase and initial rate of cyt c^{3+} reduction by O_2^- in the presence of anthracycline

Drug	[Cyt c^{2+}] (μM min^{-1})[a]		[Cyt c^{2+}] (μM min^{-1})[b]		[Cyt c^{2+}] (μM)[b] at the end of the reaction
	−SOD	+SOD	−SOD	+SOD	+SOD
None	3.4 (v_1)	3.4 (v_3)	12.2 (v_1)	12.2 (v_3)	18.3 ($c_2 = c_1$)
Dr	20.5 (v_2)	3.4	30.6 (v_2)	12.2	4.6 (c_3)
Adr	19.3	3.4	30.6	12.2	5.1
4′-Adr	19.3	3.4	—	—	—
Acla	16.5	3.4	24.4	12.3	4.3
Quinizarin	4	3.5	—	—	—
Dr-10	15.3	3.4	24.4	12.2	7.8
5-IDr	4.9	3.3	20.8	12.1	14.3
Dr-19	3.7	3.4	—	—	—
Nov	—	—	24.4	12.2	10.5

Experimental conditions were as follows: [a]40 μM cyt c^{3+}, 83 μM NADH, 60 u/l NADH dehydrogenase, 0 or 100 μM anthracycline, 0 or 50 μg/ml SOD, [b]100 μM cyt $^{3+}$, 10 μM NADH, 0 or 100 μM anthracycline, 125 u/l NADH dehydrogenase, 0 or 50 μg/ml SOD. Standard deviation was about 5%.

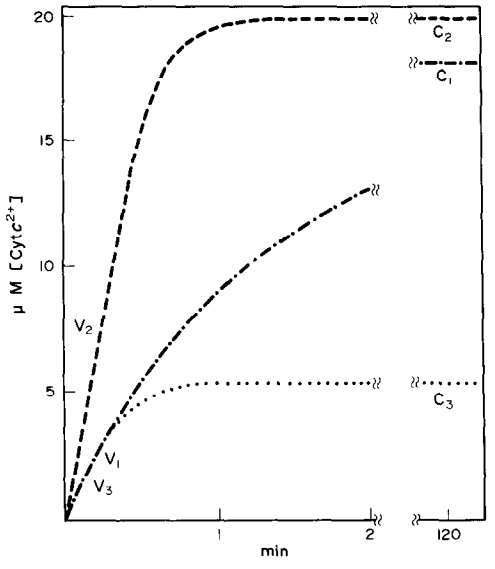

Fig. 1. Rate of cyt c^{3+} reduction by NADH catalysed by NADH dehydrogenase and rate of cyt c^{3+} reduction by O_2^- in the presence of anthracycline. Experimental conditions were as follows: (1) 100 μM cyt c^{3+}, 10 μM NADH, 125 u/l NADH dehydrogenase (—·—); (2) 100 μM cyt c^{3+}, 10 μM NADH, 125 u/l NADH dehydrogenase, 100 μM anthracycline (– – –); (3) 100 μM cyt c^{3+}, 10 μM NADH, 125 u/l NADH dehydrogenase, 100 μM anthracycline 125 u/l NADH dehydrogenase, 50 μg/ml SOD (·····). v_1, v_2 and v_3 are the initial rate; c_1, c_2 and c_3 are the concentrations of cyt c^{3+} at the end of the reaction.

Table 3. Concentrations of enzyme or FMN required to reduce 1.1 μM cyt c^{3+} per minute. Experimental conditions: 150 μM NADH, 80 μM cyt c^{3+}

	Enzyme	Multiplicating factor
HEPES 7.2	5 u/l 0.21 μM	1
HEPES 7.2	FMN 100 μM	476
pH 3.2	FMN 2.5 μM	12
HEPES 7.2	CPSA treated enzyme 125 μM	590

performed in the presence of drug, the kinetics of reduction of cytochrome *c* was increased more or less, depending on the type of the drug, as previously shown. The reaction stopped when two moles cytochrome *c* were reduced per mole NADH. When the experiment was performed in the presence of drug and SOD, the kinetics of reduction of cyt c^{3+} during the first 30 sec was the same as that observed in the absence of drug but the quantity of cyt c^{3+} reduced was lower (Fig. 1). The concentrations of cyt c^{3+} reduced once the reaction has stopped and the kinetics of reduction are reported in Table 2.

Thus, the concentration of cyt c^{3+} reduced by the enzyme depends on the drug present (nature and concentration). The difference between the concentration of cyt c^{3+} reduced in the presence of drug with and without SOD gives the concentration of cyt c^{3+} reduced by O_2^-. As can be seen there is a correlation between v_2–v_3 and c_2–c_3.

The above-described experiments raised the possibilities of two different pathways for the reduction of drug and cyt c^{3+}. To test whether this hypothesis could be valuable we tried to determine if the two types of electrons transferring groups, i.e. FMN and the four Fe—S clusters were equally required for the reduction either of cyt c^{3+} or of the drug. In this context two types of experiments were performed.

In a first set of experiments we tested the ability of FMN to catalyse the reduction of cyt c^{3+} by NADH. We observed that in HEPES buffer FMN was able to catalyse the reduction of cyt c^{3+} by NADH. In a typical experiment, in the presence of 150 μM NADH, 80 μM cyt *c* and 100 μM FMN, 1.1 μM cytochrome *c* were reduced per minute. In the same conditions of concentrations 5 u/l (i.e. 0.21 μM) NADH dehydrogenase produce the same effect (Table 3). We observed that the ability of FMN to catalyse the reduction of cyt c^{3+} by NADH was strongly pH dependent and that at pH 3.2 only 2.5 μM FMN were required to reduce the same amount of cyt c^{3+}. Similar experiments were performed in the presence of drug: FMN was not able to catalyse the reduction of drug by NADH.

NADH dehydrogenase available from Sigma company is prepared according to Mahler's method [22]. This yields a low molecular weight with grossly modified enzyme properties. Assuming one mole FMN per mole of enzyme, molecular weight of 70,000 was estimated and we used this value to estimate the enzyme concentration in our solution.

In a second set of experiments we used the enzyme which was incubated in the presence of *p*-chloromercuriphenylsulphonic acid (CPSA). This acid has the properties to inhibit the iron cluster. A 50-fold excess of CPSA was added to the enzyme. When the enzyme 10 μM was added to 80 μM cyt c^{3+} a slow reduction of cyt c^{3+} occurred but the reaction stopped when 10 μM of cyt c^{3+} had been reduced (i.e. about 1 mole cyt c^{3+} per mole enzyme). This can be taken as an indication that the inhibition of iron centre by CPSA gave rise to the reduction of FMN. The subsequent addition of NADH yielded a further reduction of cyt c^{3+}. In the presence of 150 μM NADH and 80 μM cyt c^{3+}, about 125 μM CPSA-treated enzyme were necessary to catalyse the reduction of 1.1 μM cyt c^{3+}.

All these data have been gathered up in Table 3. As can be seen at pH 7.4, the activity of the CPSA-treated enzyme compares with that of FMN. This is not true at acidic pH value where the activity of the acid-treated enzyme completely vanished. As it was observed in the case of FMN the acid-treated enzyme did not catalyse the reduction of the drug by NADH.

Our data show that the cytochrome *c* reductase activity exhibited by the commercially available NADH dehydrogenase does not prevent the enzyme catalysing the reduction of anthracycline by NADH in a way that compares with that observed for microsome. Moreover our data suggest that contrary to what is observed for cytochrome *c* the drugs are not reduced at the FMN site.

Acknowledgements—This work was supported by grants from University Paris Nord, CNRS and Institut Curie. We are indebted to Laboratoires Rhône Poulenc and Roger Bellon for supplying us with daunorubicin, Adriamycin®, 4'-epiadriamycin and aclacinomycin.

REFERENCES

1. V. J. Ferrans, *Cancer Treat. Rep.* **62**, 955 (1978).
2. W. S. Thayer, *Chem. Biol. Interact.* **19**, 265 (1977).
3. K. J. A. Davies, J. H. Doroshow and P. Hochstein, *FEBS Lett.* **153**, 227 (1983).
4. K. J. A. Davies and J. H. Doroshow, *J. Biol. Chem.* **261**, 3060 (1986).
5. J. H. Doroshow and K. J. A. Davies, *J. Biol. Chem.* **261**, 3068 (1986).
6. B. Stefanska, P. Kolodziejczyk, L. Falkowski and E. Borowski, *Proc. 14th Int. Symp. Chemistry of Natural Products*, Vol 1, p. 387. Poznan, Poland (1984).
7. B. Stefanska, Z. Mazerska, L. Falkowski and E. Borowski, *J. Antibiot.* **37**, 1213 (1984).
8. B. Stefanska, E. Borowski, L. Falkowski and S. Martelli, *Pol. J. Chem.* **60**, 525 (1986).
9. H. Beraldo, A. Garnier-Suillerot, L. Tosi and F. Lavelle, *Biochemistry* **24**, 284 (1985).
10. M. M. L. Fiallo and A. Garnier-Suillerot, *Biochemistry* **25**, 924 (1986).
11. A. Garnier-Suillerot, in *Anthracycline and Anthracenedione-based Anticancer Agents* (Ed. J. W. Lown), p. 129. Elsevier, Amsterdam (1988).
12. J. H. Doroshow, *Cancer Res.* **43**, 460 (1983).
13. J. H. Doroshow, *Cancer Res.* **43**, 4543 (1983).
14. Y. Hatefi and D. L. Stiggall, *The Enzymes* (Ed. P. D. Boyer), Vol 13, p. 175. Academic Press, New York (1976).
15. G. L. Tong, D. H. Henry and E. M. Acton, *J. Med. Chem.* **22**, 36 (1979).
16. G. L. Tong, H. Y. Hu and D. W. Henry, *J. Med. Chem.* **22**, 912 (1979).
17. K. C. Murdock, R. G. Child, P. F. Fabio, R. B. Augier, R. B. Wallace, F. E. Durr and R. V. Citarella, *J. Med. Chem.* **22**, 1024 (1978).
18. R. Zee-Cheng and C. C. Cheng, *J. Med. Chem.* **22**, 291 (1978).
19. A. Martonosi, *J. Biol. Chem.* **243**, 71 (1968).
20. T. Omura and S. Takesue, *J. Biochem. Tokyo* **67**, 249 (1970).
21. O. H. Lowry, N. J. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
22. H. Mahler, *Meth. Enzymol.*, Vol. 11, p. 688. Academic Press, New York (1955).