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Advances in Brief

Enhancement of Xanthine Dehydrogenase Mediated Mitomycin C Metabolism by Dicumarol[1]

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Abstract

These studies examined the effect of dicumarol on xanthine dehydrogenase (XDH), an enzyme recently shown to bioreduce mitomycin C. Dicumarol, which has previously been shown to inhibit xanthine oxidase (XO), inhibited both XDH and XO mediated conversion of xanthine to uric acid but potentiated the metabolism of mitomycin C by XDH and XO. Formation of 2,7-diaminomitosene following mitomycin C bioactivation by XDH was increased 3-fold aerobically and 4-fold hypoxically when 20 μM dicumarol was included in the reaction mixture. XO mediated metabolism of mitomycin C hypoxically was increased approximately 50% by the inclusion of dicumarol.

Introduction

In studies designed to elucidate the role of DT-diaphorase [NAD(P)H: (quinone acceptor) oxidoreductase, EC 1.6.99.2] in the activation and toxicity of quinone containing compounds, dicumarol has often been used to inhibit the enzyme. The differences in toxicity and mechanistic parameters between samples with and without dicumarol have been ascribed to the role of DT-diaphorase. The use of dicumarol to investigate the role of DT-diaphorase in the biological activity of the antitumor antibiotic MMC[3] has suggested a complicated role for DT-diaphorase in MMC toxicity (1–6). Previous studies by Keyes *et al.* have shown a potentiation of MMC toxicity hypoxically and inhibition of MMC toxicity aerobically by the addition of dicumarol using EMT6 cells (2). Keyes *et al.* (1) have also shown an increase in the formation of MMC reactive metabolites hypoxically in the presence of dicumarol using EMT6 cell sonicates. More recent work by Tomasz *et al.* (7) using [^{3}H]porfiromycin, a mitomycin antibiotic which responds to dicumarol in much the same way as MMC in EMT6 cells (3), have shown a change in the pattern of DNA adduct formation when dicumarol was added to the reaction mix with no change in the total incorporation of [^{3}H]porfiromycin (7). These data taken together have suggested that dicumarol does not modulate MMC activation solely through the inhibition of DT-diaphorase but that another enzyme capable of bioactivating MMC is somehow modulated by dicumarol (1, 3). Recently the enzyme xanthine dehydrogenase (EC 1.1.1.204) has been shown to bioreduce MMC (8, 9), and this study examines the effect of dicumarol on MMC metabolism by xanthine dehydrogenase.

Materials and Methods

Chemicals. Mitomycin C was provided by Bristol-Myers Squibb Company (Wallingford, CT). NADH (grade IV), NAD+ (grade III), 2-mercaptoethanol, phenylmethylsulfonyl fluoride, EDTA (disodium salt), dicumarol, reactive blue 2-Sepharose, Sephadex G-200, xanthine oxidase (grade IV from buttermilk), and xanthine were purchased from Sigma Chemical Co. (St. Louis, MO). 5-Chloro-2-pyridinol was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other reagents were of analytical grade.

Enzyme Preparation. Xanthine dehydrogenase was purified from EMT6 tumors grown in athymic nude mice according to the method of Suleiman and Stevens (10) as modified for EMT6 tissue by Gustafson and Pritsos (8).

Enzyme Assays. XDH activity was measured by monitoring the formation of uric acid at 293 nm and by measuring the xanthine dependent formation of NADH at 340 nm (11). XO activity was measured by monitoring the formation of uric acid at 293 nm in the absence of NAD+ (11) and by measuring oxygen consumption using a YSI Biological Oxygen Monitor (YSI, Inc., Yellow Springs, OH) equipped with a Clark electrode. The assay mixture contained 100 mM potassium phosphate (pH 7.8), 0.2 mM xanthine, and 0.4 mM NAD+ for XDH activity determination. XDH and XO activity is expressed as μmol uric acid formed/min. All enzyme assays were done at 25°C with the use of a Beckman DU-64 spectrophotometer.

HPLC Analysis of Mitomycin C Metabolites. High-pressure liquid chromatography analysis of mitomycin C and its metabolites was done by the method of Pan *et al.* (12) as modified by Siegel *et al.* (13). The reaction mixture contained 100 mM potassium phosphate (pH 6.0), 0.5 mM NADH, mitomycin C, and approximately 40 milliunits xanthine dehydrogenase or 200 milliunits xanthine oxidase and were kept at 37°C in a total volume of 1 ml for 45 min. 5-Chloro-2-pyridinol (10 μg/ml) was used as an internal standard. Hypoxic conditions were obtained by maintaining the reaction mixture under nitrogen gas throughout the incubation.

Statistical Analysis. Statistical analysis was performed using Student's *t* test. The level of significance was attributed to $P < 0.05$.

Results

Inhibition of XDH and XO Enzymatic Activity by Dicumarol. Inhibition of XDH and XO by dicumarol was measured and the results are shown in Fig. 1. Inhibition of both enzymes was assessed by measuring uric acid formation in the presence and absence of dicumarol as well as by monitoring NADH formation in the case of XDH and oxygen consumption in the case of XO. Inhibition of XDH catalyzed conversion of xanthine to uric acid by dicumarol was 27, 48, and 56% by 20, 40, and 60 μM dicumarol, respectively. When xanthine dependent formation of NADH was monitored as a measure of dicumarol inhibition of XDH, the values were 26, 38, and 47% for the same concentrations of dicumarol. Inhibition of XO mediated uric acid formation by 20, 40, and 60 μM dicumarol was 19, 27, and 35% and 3, 13, and 30% when oxygen consumption was used as a measure of XO activity. These data suggest that inhibition of XDH and XO catalyzed uric acid formation by dicumarol does not take place via a simple competitive interaction between xanthine and dicumarol as the electron transferring ability of the enzyme seems to be less affected by dicumarol than does uric acid formation.

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[3] The abbreviations used are: MMC, mitomycin C; XDH, xanthine dehydrogenase; XO, xanthine oxidase.

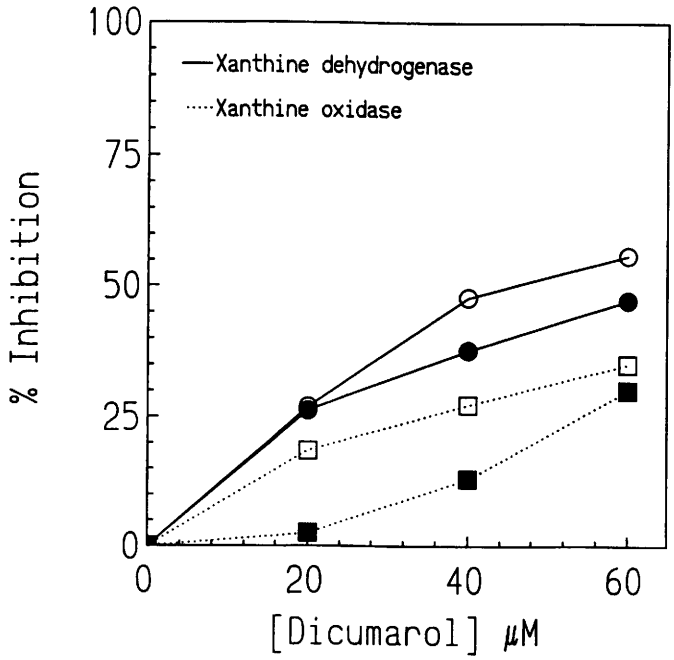

Fig. 1. Inhibition of xanthine dehydrogenase and xanthine oxidase by dicumarol. Values represent the mean of at least three independent determinations. The standard error was approximately 10% for each value. ○, □, activity as measured by monitoring uric acid formation. ●, ■, activity as measured by NADH formation or oxygen consumption.

Effect of Dicumarol on XDH Metabolism of MMC. Formation of 2,7-diaminomitosene following bioreduction of MMC by XDH was measured using high-pressure liquid chromatography and representative tracings are shown in Fig. 2. Values for metabolite formation are summarized in Table 1. The effect of 20 μM dicumarol on MMC metabolism by XDH under aerobic conditions are shown in Fig. 2, *A* and *B*, and under hypoxic conditions in Fig. 2, *C* and *D*. The effect of dicumarol in the absence of NADH is shown in Fig. 2*E*. The addition of dicumarol to the reaction mixture under aerobic conditions led to a 3-fold increase in MMC metabolite formation and under hypoxic conditions to a 4-fold increase. Addition of 20 μM dicumarol in the absence of NADH led to no metabolite formation, showing the NADH dependence of MMC metabolite formation in both the presence and the absence of dicumarol. XO catalyzed metabolism of MMC under hypoxic conditions is shown in Fig. 3 with and without the addition of 20 μM dicumarol. The addition of dicumarol led to a 50% increase in XO mediated metabolite formation. MMC consumption was also measured under these same conditions for both XDH and XO and these values parallel the increases observed in metabolite formation in the presence of dicumarol (Table 1).

Discussion

There have been considerable efforts made to elucidate the mechanism of action of mitomycin C and its analogues against solid tumors. Recent attention has focused on the role of DT-diaphorase and its two electron reduction of mitomycin C as a potentially important enzyme in mitomycin induced toxicity (1–7). A great majority of studies have used dicumarol, a DT-diaphorase inhibitor, as a means of determining the role of DT-diaphorase in a particular biological system. These studies on the whole, however, have provided data which are sometimes conflicting and at times inconsistent with the proposed mechanism for DT-diaphorase activated mitomycin C toxicity.

The addition of dicumarol to cells treated with mitomycin C was shown to decrease mitomycin C induced toxicity aerobically and increased toxicity hypoxically in EMT6 cells (2). Dicumarol, subsequently was shown to increase mitomycin C-induced toxicity both aerobically and hypoxically in L1210 cells, which contain no measurable DT-diaphorase (3). The effect of dicumarol on mitomycin C induced toxicity was also tested in L5178Y murine lymphoblasts and a resistant line, L5178Y/HBM10, which has a 24-fold increased DT-diaphorase activity over the parental L5178Y line (14). Dicumarol had no effect on mitomycin C induced toxicity in the L5178Y cells either aerobically or hypoxically. Dicumarol inhibition of DT-diaphorase in the L5178Y/HBM10 cell line (high in DT-diaphorase activity) decreased the sensitivity of these cells to MMC by only 29% and had no effect hypoxically (14). It appears from these studies that the effect of dicumarol on mitomycin C induced toxicity is dependent upon cell type and that its effect is not due only to the inhibition of DT-diaphorase.

Recent studies by Tomasz *et al.* (7) using [^{3}H]porfiromycin, a mitomycin C analogue, have looked at the formation of porfiromycin-DNA adducts in EMT6 cells. The addition of dicumarol under both aerobic and hypoxic conditions showed a decrease in the formation of three of the observed adducts. The formation of a fourth adduct, however, was significantly increased with the addition of dicumarol under both aerobic and hypoxic conditions. Small amounts of this adduct were formed hypoxically in the absence of dicumarol, but the addition of dicumarol resulted in a 4-fold increase of this adduct. Total incorporation of label remained unchanged by the addition of dicumarol thereby suggesting that although dicumarol changed the profile of porfiromycin-DNA adduct formation, the total amount of activated porfiromycin was unchanged (7). The authors concluded from their studies that adduct X must be produced by a dicumarol modulated enzyme other than DT-diaphorase.

We have recently shown that xanthine dehydrogenase can bioreduce MMC to alkylating species under both aerobic and hypoxic conditions and that the major metabolite formed is 2,7-diaminomitosene (8). This suggests that like DT-diaphorase, XDH can bioreduce MMC directly by two electrons. The

Table 1 *Effect of dicumarol on mitomycin C metabolism by xanthine dehydrogenase and xanthine oxidase*[a]

	MMC consumption[b]	Metabolite formation[c]
Xanthine dehydrogenase		
Aerobically		
200 μM MMC	34.6 ± 1.1	5.2 ± 0.1
200 μM MMC + 20 μM dicumarol	106.5 ± 3.6	15.3 ± 0.3
Hypoxically		
200 μM MMC	19.5 ± 1.8	16.2 ± 2.1
200 μM MMC + 20 μM dicumarol	75.7 ± 2.7	66.2 ± 2.6
500 μM MMC	71.5 ± 1.9	54.5 ± 4.4
500 μM MMC + 20 μM dicumarol	200.9 ± 7.2	191.8 ± 10.6
Xanthine oxidase		
500 μM MMC	16.4 ± 1.4	11.5 ± 0.3
500 μM MMC + 20 μM dicumarol	24.8 ± 3.5	18.5 ± 0.4

[a] Values represent the mean ± SE of at least three separate determinations.
[b] Values are in units of nmol MMC consumed/min/unit enzyme normalized to the internal standard. The addition of dicumarol led to a significant increase ($P < 0.05$) in all cases except for xanthine oxidase.
[c] Values are in units of area 2,7-diaminomitosene peak/area of internal standard/unit enzyme. The addition of dicumarol led to a significant increase ($P < 0.05$) in all cases.

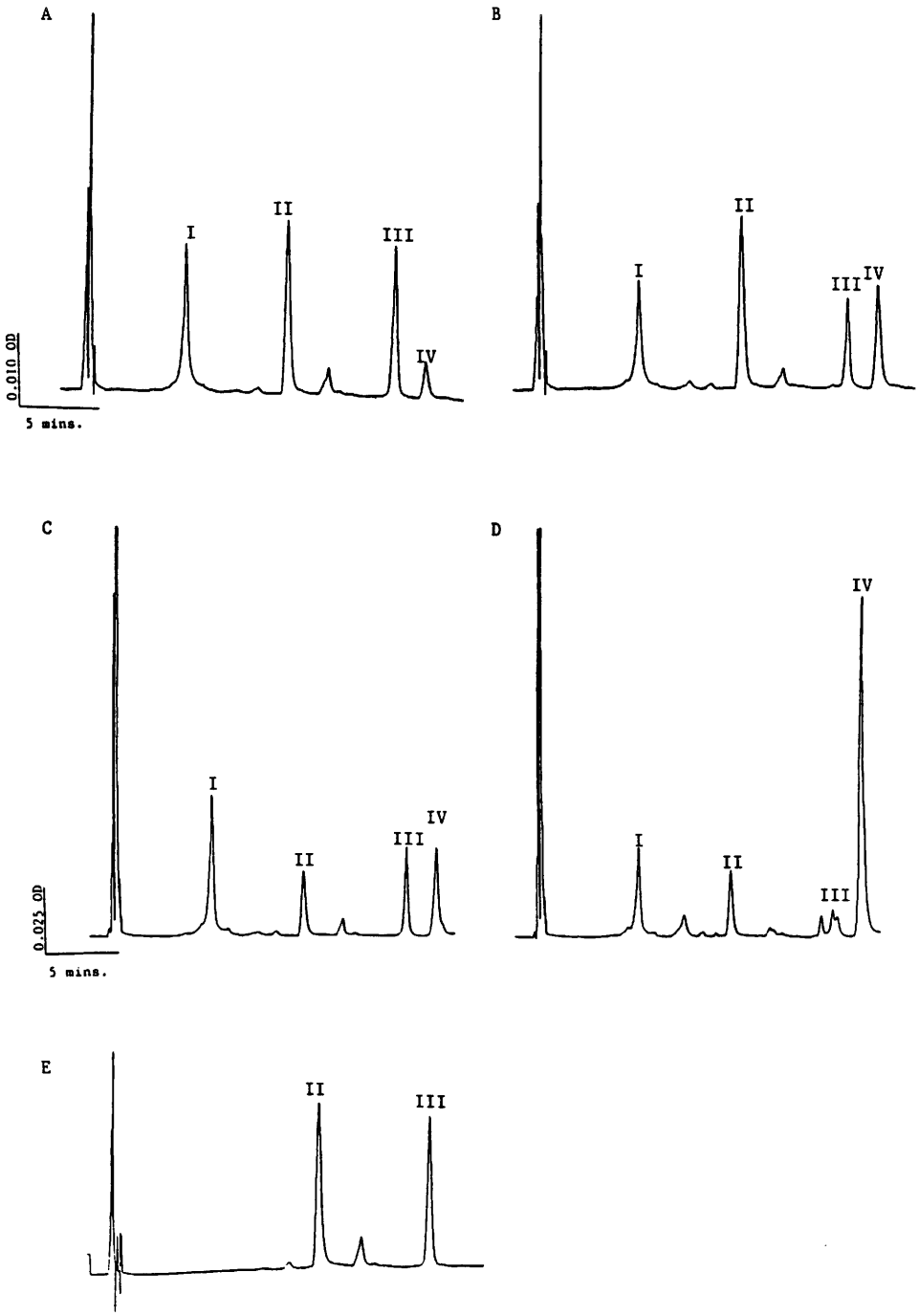

Fig. 2. Representative high-pressure liquid chromatography tracings of reaction mixtures of 200 μM mitomycin C, 500 μM NADH, and xanthine dehydrogenase with and without 20 μM dicumarol. *A*, aerobic; *B*, aerobic + 20 μM dicumarol; *C*, hypoxic; *D*, hypoxic + 20 μM dicumarol; *E*, hypoxic + 20 μM dicumarol without NADH. The reaction mixtures contained 0.1 M potassium phosphate (pH 6.0), 500 μM NADH, 200 μM MMC, and approximately 40 milliunits xanthine dehydrogenase, and the procedure was carried out at 37°C in a total volume of 1 ml for 45 min. *Peak I*, NADH; *Peak II*, 5-chloro-2-pyridinol; *Peak III*, mitomycin C; *Peak IV*, 2,7-diaminomitosene.

We have recently shown that xanthine dehydrogenase can bioreduce MMC to alkylating species under both aerobic and hypoxic conditions and that the major metabolite formed is 2,7-diaminomitosene (8). This suggests that like DT-diaphorase, XDH can bioreduce MMC directly by two electrons. The studies presented in this paper show that the addition of dicumarol significantly increased 2,7-diaminomitosene formation during the reaction between XDH and MMC both aerobically and hypoxically. A concomitant increase in NADH (Fig. 2) and MMC consumption (Table 1) accompanied the increase in 2,7-diaminomitosene formation. Dicumarol and XDH, without NADH in the reaction mixture (Fig. 2*E*), did not form MMC metabolites or lead to MMC consumption. These studies show that dicumarol is capable of modulating xanthine dehydrogenase activation of MMC through some interaction with the enzyme, presumably by enhancing its electron transferring capabilities.

The results of these studies therefore demonstrate that XDH activation of MMC is significantly increased 3–4-fold in the presence of dicumarol. These findings are significant in light of the previous studies in which dicumarol was used to assess the role of DT-diaphorase in MMC activation. This activation of

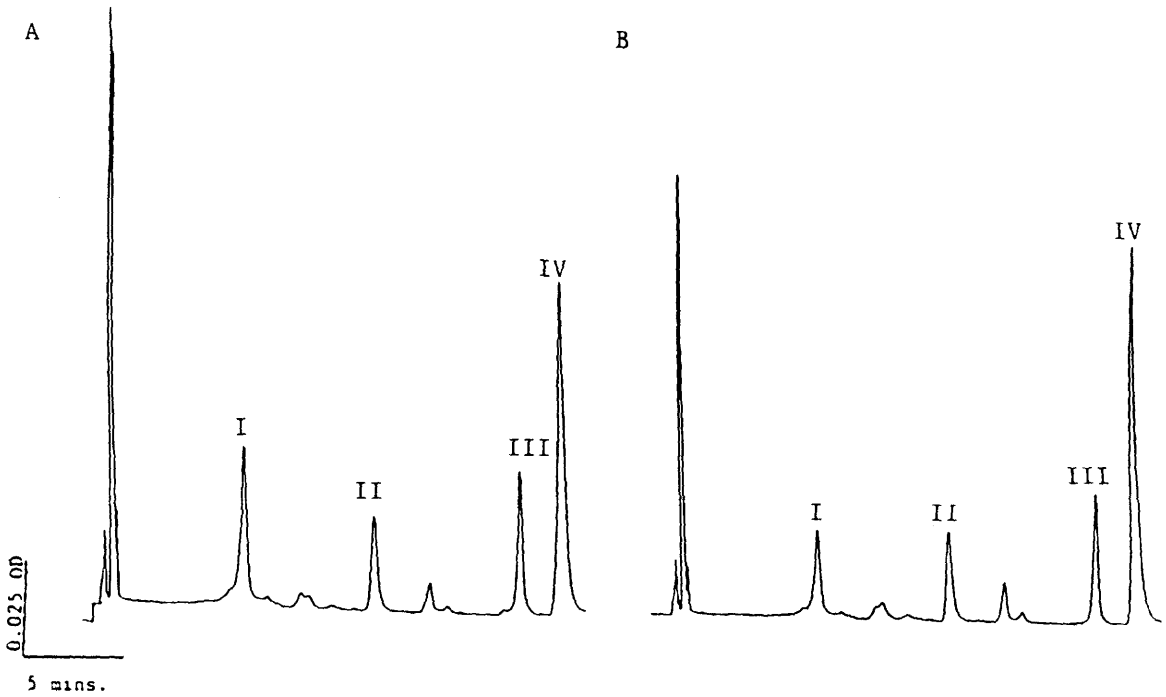

Fig. 3. Representative high-pressure liquid chromatography tracings of reaction mixtures of 500 μM mitomycin C, 500 μM NADH, and xanthine oxidase with and without 20 μM dicumarol. *A*, no dicumarol, *B*, with 20 μM dicumarol. The reaction mixtures contained 0.1 M potassium phosphate (pH 6.0), 500 μM NADH, 500 μM MMC, and approximately 200 milliunits xanthine oxidase, and the procedure was carried out at 37°C in a total volume of 1 ml for 45 min under hypoxic conditions. *Peak I*, NADH; *Peak II*, 5-chloro-2-pyridinol; *Peak III*, mitomycin C; *Peak IV*, 2,7-diaminomitosene.

XDH bioreduction of MMC could account for some inconsistencies observed in these various studies. These findings are consistent with the findings of Keyes *et al.* (1) who showed in EMT6 cells that MMC reactive metabolite formation increased dose dependently with the addition of dicumarol, despite having completely inhibited DT-diaphorase. These findings are also consistent with the studies by Tomasz *et al.* (7) in which dicumarol added to whole EMT6 cells modulated the DNA-porfiromycin adduct profile but did not appear to affect total incorporation of label onto the DNA. It is therefore apparent that dicumarol can modulate the activity of more than one enzyme involved in MMC activation and that caution must be used in attributing its effect to any one enzyme.

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