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INHIBITION OF MOUSE GLUTATHIONE TRANSFERASES AND GLUTATHIONE PEROXIDASE II BY DICUMAROL AND OTHER LIGANDS

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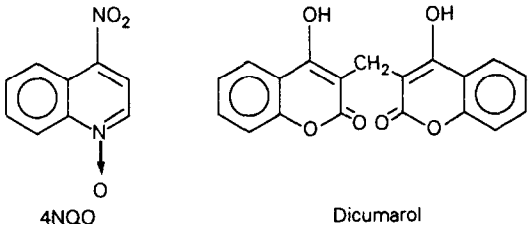
Abstract—Dicumarol, often used as a specific inhibitor of DT diaphorase (NAD(P)H:(quinone-acceptor) oxidoreductase; EC 1.6.99.2), was found to potently inhibit GSH transferases (EC 2.5.1.18). Dicumarol exhibited an IC_{50} of 11 μM in inhibiting the conjugation of 1-chloro-2,4-dinitrobenzene (50 μM) by GSH transferase GT-8.7, the major hepatic class mu isoenzyme of CD-1 mice. The activities of GT-8.7 and of the class pi isoenzyme, GT-9.0, toward a carcinogenic substrate, 4-nitroquinoline 1-oxide (100 μM), were inhibited by dicumarol with IC_{50} values of 14 and 9 μM, respectively. Dicumarol also affected GSH peroxidase II activity, inhibiting the reduction of cumene hydroperoxide by GT-10.6, the predominant class alpha GSH transferase of mouse liver, with an IC_{50} of 14 μM. GSH peroxidase I (EC 1.11.1.9) and GSH peroxidase II activities were resolved by chromatography of liver and testis cytosols. While inhibiting GSH peroxidase II with IC_{50} of 9–10 μM, dicumarol did not affect the activity of the selenoenzyme, GSH peroxidase I. Whereas several other non-substrate ligands were more potent inhibitors of 1-chloro-2,4-dinitrobenzene conjugation, dicumarol effectively inhibited GSH transferase and GSH peroxidase II activities in the range of dicumarol concentrations frequently used for detection of DT diaphorase action. These results indicate that physiological consequences resulting from the use of supramicromolar concentrations of dicumarol should not be interpreted in terms of DT diaphorase inhibition alone.

The glutathione (GSH†) transferases (EC 2.5.1.18) are a family of isoenzymes that catalyze the conjugation of numerous substrates with GSH and bind a variety of non-substrate ligands [1–3]. Recent studies in this laboratory have focused on the conjugative detoxication of a carcinogenic substrate, 4-nitroquinoline 1-oxide (4NQO), by GSH transferases which promote the displacement of the nitro group by GSH to form 4-(glutathion-*S*-yl)-quinoline 1-oxide [4–6]. According to a widely used system of classification, GSH transferase isoenzymes are divided on the basis of their catalytic, structural, and immunological properties into three classes (alpha, pi, and mu) [3]. Studies on GSH transferases purified from livers of CD-1 mice have shown that the conjugation of 4NQO is catalyzed primarily by class mu (Yb_1 subunit type) and class pi (Yf) isoenzymes [4]. Although potent inhibition by dicumarol is often considered an identifying characteristic of DT diaphorase (EC 1.6.99.2) [7], a structural resemblance between dicumarol and 4NQO (Scheme 1) suggested that dicumarol might also inhibit the enzymic conjugation of 4NQO with GSH. Another facet of the detoxicative role of GSH transferases is their GSH peroxidase activity toward organic hydroperoxides [8, 9]. In contrast to the selenoenzyme, GSH peroxidase I (EC 1.11.1.9), which catalyzes the reduction of both hydrogen peroxide and organic hydroperoxides, the GSH transferases lack activity toward hydrogen peroxide and have been designated GSH peroxidase II [8, 9]. Studies on mouse liver GSH transferases have shown that GSH peroxidase II activity is catalyzed primarily by a class alpha isoenzyme with Ya_3 subunits [3, 10, 11]. The present investigation explored the effectiveness of dicumarol and other non-substrate ligands as inhibitors of the GSH conjugation of the most widely used substrate, 1-chloro-2,4-dinitrobenzene (CDNB), and of 4NQO by murine class mu and class pi GSH transferase isoenzymes and as inhibitors of the reduction of an organic hydroperoxide, cumene hydroperoxide (CHP), by hepatic and testicular GSH peroxidase I and II fractions and by class alpha GSH transferases.



4NQO Dicumarol

Scheme 1. Chemical structures of 4NQO and dicumarol.

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† Abbreviations: GSH, glutathione; 4NQO, 4-nitroquinoline 1-oxide; CHP, cumene hydroperoxide; CDNB, 1-chloro-2,4-dinitrobenzene; and BSA, bovine serum albumin.

MATERIALS AND METHODS

Chemicals. Dicumarol, GSH, NADPH, CHP, H_2O_2, hematin, thyroxine, indocyanine green, estradiol, estrone, testosterone, bovine serum albumin (BSA), and yeast glutathione reductase were purchased from the Sigma Chemical Co., St. Louis, MO. CDNB and 4NQO were from Eastman Organic Chemicals, Rochester, NY, and the Aldrich Chemical Co., Milwaukee, WI, respectively. Lithocholate was obtained from Steraloids, Inc., Wilton, NH. DEAE CL-6B was from Pharmacia, Piscataway, NJ.

Animals. Male CD-1 mice, obtained from Charles River Breeding Laboratories, were 5 months old at the time of the experiment. They were housed as described previously and received Charles River pelleted mouse chow *ad lib.* The mice were killed by cervical dislocation. Organs were collected and processed immediately and stored at −70° until used for the preparation of cytosol fractions. These procedures have been described in detail elsewhere [12]. The purification and characterization of the murine GSH transferase isoenzymes have been described previously [10].

Measurement of enzyme activities. Enzyme activities were measured spectrophotometrically in a 1-mL assay system at 25°. GSH transferase activities toward CDNB and 4NQO were assayed as described by Habig *et al.* [13] and Stanley and Benson [4], respectively. GSH peroxidase activities were measured by a modification [14] of the method of Paglia and Valentine [15]. This is a coupled assay in which NADPH utilization by yeast glutathione reductase is measured. It was determined that 100 μM dicumarol caused only a 9% inhibition of the yeast glutathione reductase activity, which is present in excess in the assay system. GSH peroxidase assay systems contained sodium azide (11.25 mM) to eliminate interference from heme proteins present in cytosol fractions [14]. Rates were linear with time and with enzyme concentration under the assay conditions used. All activity measurements were corrected for the rates observed in the absence of enzyme. Protein concentrations were determined according to Lowry *et al.* [16] with BSA as a standard.

Separation of GSH peroxidases I and II. Tissues were homogenized in 0.25 M sucrose (3 mL/g tissue), and cytosol fractions were prepared as described previously [12] and supplemented with 2-mercaptoethanol at a final concentration of 7.2 mM. For each separation, a 1-mL portion of cytosol fraction was applied to a 0.6 × 7 cm column of DEAE Sepharose CL-6B equilibrated and developed at 4° with 10 mM Tris–HCl, pH 7.0, in 7.2 mM 2-mercaptoethanol (Buffer 1). After the elution of GSH peroxidase II activity in the first 4 mL of effluent, 4 mL of buffer containing 0.5 M KCl was used to elute GSH peroxidase I.

RESULTS

Inhibition of class mu and class pi murine GSH transferase isoenzymes. Figure 1 shows the effects of dicumarol on CDNB conjugation by GSH transferases GT-8.7 and GT-9.0, at a CDNB concentration of 50 μM. GT-8.7, which was inhibited by dicumarol with an IC_{50} of 11 μM under these conditions, is a class mu isoenzyme that accounts for most of the activity toward CDNB in liver cytosol from female CD-1 mice [10]. GT-9.0, a class pi GSH transferase that is the most abundant isoenzyme in livers of male mice [10, 17, 18], was less potently inhibited by dicumarol (Fig. 1). Previous studies showed that the conjugation of 4NQO with GSH is catalyzed primarily by GT-8.7, GT-8.8a and b, and GT-9.0 [4]. As suggested by its structural resemblance to 4NQO, dicumarol was a very effective inhibitor of 4NQO conjugation by these enzymes, with IC_{50} values ranging from 9 to 14 μM (Table 1). GT-9.0, which was most effectively inhibited by dicumarol, was found to have an exceptionally high K_m (0.5 mM) for 4NQO. Further kinetic studies on the inhibition of 4NQO conjugation by GT-8.8a, which has the highest specific activity toward 4NQO [4, 5], yielded a K_i of 4 μM for dicumarol. Increasing concentrations of dicumarol progressively increased the K_m for 4NQO without decreasing the V_{max}. The apparent K_m values were 32 μM in the absence of dicumarol and 133, 189, and 240 μM at dicumarol concentrations of 5, 10, and 20 μM, respectively. Table 1 shows the effects of dicumarol and several non-substrate ligands known to be highly potent inhibitors of GSH transferases [1, 3, 19, 20] on the conjugation of CDNB under standard assay conditions in which the CDNB concentration is 1 mM. Comparison of the IC_{50} values obtained for these ligands showed them to inhibit the conjugation of CDNB with varying but greater effectiveness than dicumarol.

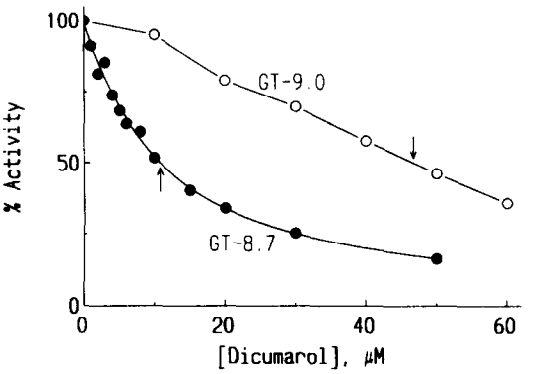

Fig. 1. Inhibition of the CDNB-conjugating activity of mouse glutathione transferase isoenzymes GT-8.7 and GT-9.0 by dicumarol. The assay systems contained 50 μM CDNB, 1 mM GSH, and sufficient quantities of enzyme to yield an activity of 6.6 nmol/min (GT-9.0) or 7.6 nmol/min (GT-8.7) in the absence of dicumarol. The arrows indicate the dicumarol concentrations corresponding to the IC_{50} values.

Inhibition of GSH peroxidase activities of class alpha GSH transferases. The GSH transferase-catalyzed reduction of organic hydroperoxide substrates such as CHP has been termed GSH peroxidase II activity, in distinction to the activity of the selenoenzyme, GSH peroxidase I, which reduces H_2O_2 as well as organic hydroperoxides

Table 1. Inhibition of murine class mu and class pi GSH transferases by dicumarol and other ligands

Inhibitor	Substrate	GT-8.7 mu	GT-8.8a mu	GT-8.8b Class mu	GT-9.3 mu	GT-9.0 pi
				IC_{50} (μM)		
Dicumarol	4NQO	14	14	14	ND*	9
Dicumarol	CDNB	71	63	63	>100†	42
Thyroxine	CDNB	3.3	1.5	1.1	>40‡	27
Hematin	CDNB	0.25	0.15	0.15	0.25	0.28
Lithocholate	CDNB	5.0	7.0	8.0	5.0	8.3
Indocyanine green	CDNB	0.08	0.09	0.05	0.39	8.9
				Specific activity (μmol/min/mg protein)		
None	4NQO	44	82	85	3	27
None	CDNB	95	86	109	22	69

The concentrations required for 50% inhibition of the GSH transferase activities (IC_{50}) were determined under standard assay conditions, with 100 μM 4NQO or 1 mM CDNB, as given in Materials and Methods. The specific activities have been published previously [4, 10].

* ND: not determined because of the low activity of GT-9.3 towards 4NQO.

† At 100 μM dicumarol 43% inhibition was observed.

‡ At 40 μM thyroxine 42% inhibition was observed.

Table 2. Inhibition of the GSH peroxidase activity of class alpha isoenzymes

Ligand	GT-10.6 Inhibition (%*)	GT-10.6 IC_{50} (μM)	GT-10.3 Inhibition (%*)	GT-10.3 IC_{50} (μM)
Dicumarol	62	14	48	56
Lithocholate	91	19	12	ND†
Indocyanine green	76	18	15	ND
Thyroxine	54	54	None‡	ND

The inhibition of CHP reduction by mouse class alpha GSH transferases GT-10.6 and GT-10.3 was examined under standard assay conditions. The specific activities of GT-10.6 and GT-10.3 toward CHP are 40.6 and 1.21 μmol/min/mg of protein, respectively [10].

* Percent inhibition was determined from measurements at 0 and 50 μM ligand.

† ND: not determined due to insufficient inhibition.

‡ Thyroxine activated GT-10.3, increasing activity by 19% at 20 μM and by 35% at 40 μM.

[8, 9]. Class alpha GSH transferases GT-10.3 and GT-10.6 from mouse liver exhibit GSH peroxidase II activity toward CHP [10]. GT-10.6 is a homodimer whereas GT-10.3 consists of two non-identical subunits which have been shown by HPLC analysis to be distinct from the GT-10.6 subunit [10]. Dicumarol substantially inhibited the GSH peroxidase activities of both of these isoenzymes (Table 2). Both the IC_{50} values and the percent inhibition at 50 μM ligand are shown in Table 2 since the relationship of dicumarol concentration to the reciprocal of the rate of catalysis is non-linear. Such non-linearity is also characteristic of the inhibition of DT diaphorase by dicumarol [7]. The GSH peroxidase activity of GT-10.6 was also sensitive to inhibition by lithocholate, indocyanine green, and thyroxine, whereas GT-10.3 was resistant to inhibition by these compounds. The observed activation of GT-10.3 by thyroxine is not unique. The activation of GSH transferases by other non-substrate ligands has been the subject of a recent report [21]. Although dicumarol potently inhibited the GSH peroxidase activity of GT-10.6, it was a much less effective inhibitor of the conjugation of CDNB by this isoenzyme, exhibiting an IC_{50} of approximately 100 μM under the standard assay conditions.

Several steroids were also tested as potential inhibitors of the GSH peroxidase activities of GT-10.3 and GT-10.6. Both estrone and testosterone were weak inhibitors of GT-10.3 ($IC_{50} > 100$ μM). GT-10.6 was also weakly inhibited by estrone ($IC_{50} > 100$ μM) but not by testosterone. Neither GT-10.3 nor GT-10.6 was inhibited by 100 μM estradiol or progestrone.

Effects of dicumarol on cytosolic GSH peroxidase I and II activities. GSH peroxidases I and II were examined in cytosol fractions of two organs from male CD-1 mice, specifically liver, which has the highest total GSH peroxidase activity, and testis, in which GSH peroxidase II activity predominates [22]. Chromatography of the pooled organ cytosols from three mice on DEAE-Sepharose CL-6B as described in Materials and Methods resolved the GSH peroxidase activity toward CHP into two fractions, as shown in Table 3. The first fraction, being devoid of activity toward H_2O_2, contained only GSH peroxidase II. The second fraction was active toward both CHP and H_2O_2, as expected for the selenoenzyme, GSH peroxidase I. Dicumarol inhibited hepatic GSH peroxidase II with an IC_{50} of 9 μM. This high sensitivity of hepatic GSH peroxidase II to inhibition by dicumarol is in accord with HPLC data showing that GT-10.6 is the predominant class alpha GSH transferase of mouse liver [10] and the observation (Table 2) that its GSH peroxidase

Table 3. Effects of dicumarol on GSH peroxidases I and II from mouse liver and testis

	Substrate	Organ	
		Liver	Testis
GSH peroxidase I			
Activity (μmol/min)	H_2O_2	5.87	0.103
	CHP	6.44	0.146
Inhibition by 100 μM dicumarol	CHP	0%	0%
GSH peroxidase II			
Activity (μmol/min)	H_2O_2	0.00	0.000
	CHP	5.60	0.186
Inhibition by 100 μM dicumarol	CHP	67%	62%
Dicumarol IC_{50}	CHP	9 μM	10 μM

activity is strongly inhibited by dicumarol. GT-10.3, which is less sensitive to inhibition by dicumarol (IC_{50}, 56 μM), is an inducible enzyme found in barely detectible amounts in livers of uninduced mice [10]. Despite the much lower cytosolic GSH peroxidase activities in testis than in liver, very similar results were obtained for the characteristics of inhibition by dicumarol, suggesting that GT-10.6 is the predominant form of GSH peroxidase II also in mouse testis. GSH peroxidase I activity was not inhibited by dicumarol.

DISCUSSION

The results show that dicumarol potently inhibits several major reactions catalyzed by GSH transferases. Thus, the conjugation of CDNB (50 μM) by the predominant class mu isoenzyme of mouse liver, GT-8.7, the conjugative inactivation of a carcinogenic substrate, 4NQO, by class mu and class pi isoenzymes, the GSH peroxidase activity of the constitutive class alpha isoenzyme, GT-10.6, and the GSH peroxidase II activities of liver and testis cytosol were all inhibited by dicumarol with IC_{50} values of 9–14 μM.

Dicumarol, the classical inhibitor of DT diaphorase [7] that exhibits an IC_{50} of 0.05 μM in the inhibition of this enzyme purified from rat liver [23], has been considered highly specific for DT diaphorase although it also inhibits UDP-glucuronosyltransferase activity toward the 3-hydroxy- and 3,6-quinol metabolites of benzo(*a*)pyrene with IC_{50} values of 0.2 to 3.5 μM [23]. As an identifying characteristic of DT diaphorase, sensitivity to inhibition by dicumarol has served as an indicator of the nature and extent of involvement of this enzyme in the metabolism of toxic and carcinogenic chemicals. Inhibition by dicumarol was a key factor in establishing the role of DT diaphorase in the nitroreductive activation of 4NQO to 4-hydroxyaminoquinoline-l-oxide, a proximate carcinogen [24, 25]. Several recent studies on the mutagenicity and cytoxicity of 4NQO and other organic and inorganic mutagens have used dicumarol-sensitivity to identify effects of DT diaphorase [26–30]. Although these studies typically employed dicumarol concentrations of 30–100 μM, dicumarol was shown to be maximally effective at 5 μM in increasing menadione toxicity to rat fibroblasts [26] and, in a bacterial mutagenicity assay system supplemented with pyridine nucleotides but not with GSH, dicumarol was maximally effective at submicromolar concentrations in inhibiting the reduction of 4NQO mutagenicity by liver cytosol [27, 28], leading to the conclusion that DT diaphorase was responsible for these dicumarol-sensitive effects [26–28]. Tsuda [29] found that dicumarol increases menadione toxicity and decreases 4NQO toxicity to hamster fibroblasts, but these effects were greatest at 100–300 μM dicumarol. Powis *et al.* [30] showed that three quinoneimines are substrates for DT diaphorase, observed that 30–100 μM dicumarol has diverse effects on the cytotoxicities of these quinoneimines, and concluded that the cellular effects of dicumarol were apparently not limited to the inhibition of DT diaphorase.

Our results indicate that the effects on mutagenicity and toxicity caused by the supramicromolar concentrations of dicumarol commonly used for identifying effects of DT diaphorase may also reflect the inhibition of GSH transferase and GSH peroxidase II activities. Our results do not suggest that reactions inhibited only at dicumarol concentrations higher than required for inhibition of DT diaphorase may, on that basis alone, be attributed to GSH transferases. Effects on other enzymes and on cellular uptake of xenobiotics may also occur and, since the GSH transferase isoenzymes differ in substrate specificity, distribution, and susceptibility to inhibition by dicumarol, their contributions to dicumarol-sensitive phenomena may be expected to vary greatly according to the test compound and the isoenzyme profile of the system under investigation.

The effects of dicumarol on 4NQO metabolism are particularly complex since DT diaphorase reduces 4NQO mutagenicity while also catalyzing the nitroreductive activation of 4NQO [24, 25, 27, 28]. Interpretation of the effects of dicumarol on 4NQO metabolism are further complicated both by the susceptibility of GSH transferase-catalyzed conjugation of 4NQO to inhibition by dicumarol and by our recent finding that, in mouse liver and

lung, the predominant 4NQO nitroreductase is not DT diaphorase but rather a dicumarol-resistant enzyme [5].

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