BENZYLIDENE CYCLOPENTANONE DERIVATIVES AS INHIBITORS OF RAT LIVER GLUTATHIONE S-TRANSFERASE ACTIVITIES

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ABSTRACT

The effect of the curcumin analogues, 2,6-bis-(4-hydroxy-3-methoxy benzylidene) cyclopentanone (B1) and two of its derivatives on μ class glutathione S-transferases (GSTs) from phenobarbital-induced and uninduced rat liver cytosol has been studied to elucidate their anti-inflammatory activity. GST activity was monitored spectrophotometrically using 1,2-dichloro-4-nitrobenzene. B1 was the most potent inhibitor of GSTs, both in uninduced and in phenobarbital-induced rat liver cytosol. These inhibitory properties might be explained as part of the anti-inflammatory activity of benzylidene cyclopentanone derivatives (B1 and B12).

Keywords: *curcumin; benzylidene cyclopentanone; inhibitory potency; glutathione S-transferases mesoporous*

INTRODUCTION

Non-steroid anti-inflammatory drugs (NSAIDs) are active through inhibition of glutathione S-transferases (GSTs). Curcumin is an established NSAID [1], but there is potential for improved products with higher potency and stability based on synthetic analogues. Curcumin [*bis*-(4-hydroxy-3-methoxy phenyl)-1,6-diene-3,5-dione] (Figure 1) is a yellow dye derived from the rhizome of *Curcuma longa* L [2] and is relatively unstable in phosphate buffer at pH 7.4 [3]. However, the stability of curcumin can be improved by lowering the pH or by adding glutathione, N-acetyl L-cysteine, ascorbic acid, rat liver microsomes or rat liver cytosol [4]. Photosensitivity of curcumin has been reported by Tonnesen *et al*. [5], and Dahl *et al*. [6].

Glutathione S-transferases are a family of multifunctional isoenzymes that catalyze the conjugation of glutathione (GSH) with electrophilic compounds, thereby neutralizing their electrophilic sites and rendering the products more water-soluble. This results in the protection of cellular macromolecules from xenobiotics [7]. Another activity of GSTs is the conversion of leukotriene A_4 to leukotriene C_4 [8]. Leukotrienes are oxidation products of arachidonic acid metabolism *via* the lipoxygenase pathway [9].

Inhibition of GSTs affects a large number of endogenous processes. For example, the anti-inflammatory drug sulfasalazine inhibits formation of leukotriene C_4, by inhibiting both leukotriene C synthase and several GST isoenzymes [10]. Another anti-inflammatory drug, indomethacin, is a moderate inhibitor of α, μ, and π GSTs [11; 12]. Curcumin strongly inhibits leukotriene B_4 formation in rat peritoneal polymorphonuclear neutrophils [13] due to inhibition of lipoxygenase and cyclooxygenase activity [14] and also inhibition of α, μ, and π GST activity [4; 15; 16] as measured with 1-chloro-2,4-dinitrobenzene (CDNB). During inflammation, GSTs are also involved in the formation of inflammation mediators such as prostaglandin [17] and leukotriene [8].

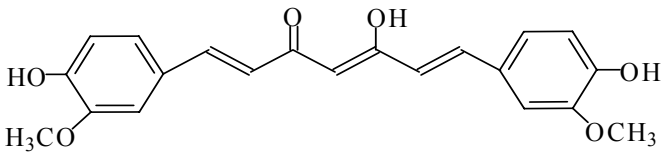
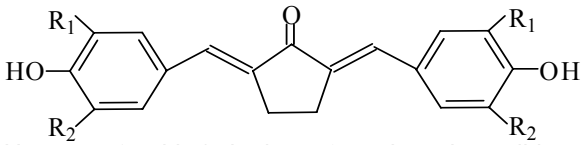


Fig 1 Curcumin [*bis*-(4-hydroxy-3-methoxy phenyl)-1,6-diene-3,5-dione]



R_1 = -H, R_2 = $-OCH_3$: 2,5-*bis*-(4-hydroxy-3-methoxy benzylidene) cyclo-pentanone = B_1
R_1 = $-CH_3$, R_2 = $-CH_3$: 2,5-*bis*-(4-hydroxy-3,5-dimethyl benzylidene) cyclo-pentanone = B_{11}
R_1 = $-C_2H_5$, R_2 = $-C_2H_5$: 2,5-*bis*-(4-hydroxy-3,5-diethyl benzylidene) cyclo-pentanone = B_{12}

Fig 2 Structures analogues of curcumin

In a preliminary study, analogues of curcumin, such as 2,5-*bis*-(4-hydroxy-3-methoxy benzylidene) cyclopentanone were about 6 times more potent than curcumin in inhibiting rat liver cytosolic GSTs. In these experiments, 1,2-dichloro-4-nitrobenzene (DCNB) was used as a specific substrate for μ class GSTs. Benzylidene cyclopentanone (Figure 2) is a known anti-inflammatory compound [18] and so we synthesized 2,5-*bis*-(4-hydroxy-3-methoxy benzylidene) cyclopentanone and its derivatives by changing the acetyl acetone group in the centre of the curcumin molecule with a cyclopentanone group [19]. This paper presents the results of inhibition studies of the compounds on GST activity.

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EXPERIMENTAL SECTION

Materials

1,2-Dichloro-4-nitrobenzene (CDNB) was obtained from Aldrich Chemie (Beerse, Belgium), reduced glutathione (GSH), and bovine serum albumin were purchased from Sigma Chemical Co. (St Louis, MO). All solvents and buffer components used were obtained from E. Merck (Darmstadt, Germany). Curcumin was synthesized according to Pabon [20]. 2,5-*bis*-(4-hydroxy-3-methoxy benzylidene) cyclopentanone (B_1), 2,5-*bis*-(4-hydroxy-3,5-dimethyl benzylidene) cyclopentanone (B_{11}) and 2,5-*bis*-(4-hydroxy-3,5-diethyl benzylidene) cyclopentanone (B_{12}) (Figure 2) were kindly given by Dr. Sardjiman, from the Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia. Stock solutions of curcumin and its analogues were prepared at 10 mM in dimethylsulfoxide (DMSO). These were diluted in ethanol before use to give the appropriate working concentrations.

Inhibition Studies

Male Wistar rats (210-230 g) from the laboratory of Pharmacology and Toxicology, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia, were used to obtain GSTs following treatment with or without phenobarbital. GSTs were isolated from uninduced and phenobarbital-induced rat liver cytosol [4] and then stored at – 80 ^oC until use. Protein concentrations were determined using a bovine serum albumin standard [21].

GST activity was measured spectrophotometrically using DCNB [22]. The reaction mixtures contained 17.5 μL of liver cytosol from either rats pretreated with phenobarbital, or from untreated rats, along with 10.0 μL of 50 mM ethanolic DCNB, and 75 μL of 50 mM GSH. The mixtures was made up to 750 μL using 0.1 M potassium phosphate buffer pH 7.5. In inhibition studies, 7.5 μL samples of inhibitors in ethanol were preincubated for 4 min before GSH and DCNB were added. In the kinetic studies, the following concentration of DCNB in the final incubation mixtures were used: 1; 0.67; 0.50; 0.40; 0.33; and 0.285 mM. All of the concentrations of inhibitor studied were kept lower than 50 μM in the final incubation mixture due to their low water solubility. All treatments were replicated 4 times.

Analysis

IC_{50} values (concentration of inhibitor resulting in a 50 % reduction of GST-activity) were obtained from regression analysis of concentration of inhibitor *vs* % of inhibition at fixed concentrations of DCNB (0.67 mM) or GSH (5 mM). The values of V_{max}, K_m, K_i and the type of inhibition were determined according to [23] and [24].

RESULTS AND DISCUSSION

Inhibitory effects were strongly correlated with concentration of curcumin and the analogues B_1 and B_{12} ($p > 0.001$; $r > 0.94$) for GSTs from both uninduced (GST-N) and phenobarbital-induced (GST-PB) rat liver cytosol. For B_{11}, there was no strong correlation between concentration and inhibitory effect. However, there was some inhibitory activity of B_{11} on both GSTs from uninduced and phenobarbital-induced rat liver. At an inhibitor concentration of 10 μM, the relative inhibitory effects were 53.6% (for curcumin), 79.9% (for B_1), 25.6% (for B_{11}), and 26.9% (for B_{12}) relative to controls.

The IC_{50} values of curcumin, B_1 and B_{12} were 9.6, 2.7 and 43.5 μM (for uninduced GST), while

corresponding IC_{50} values for phenobarbital-induced rat liver were 12.2, 2.5 and 54.7 μM respectively. These results showed that compound B_1 was the most potent inhibitor of GST activity.

In the presence of curcumin and B_1, all V_{max} values decreased compared to controls, while K_m values increased (Tables I and II). For compound B_{12}, the V_{max} values also decreased in the presence of inhibitor, but the K_m value decreased only slightly upon the addition of a low concentration of B_{12}. The K_m value increased when a higher concentration of B_{12} (Table III) was added.

Inhibition of GSTs by curcumin and analogues B_1 and B_{12} have been shown here for the first time. Other plant phenols [25] and polyphenols [26] have been shown to be inhibitors of GSTs and they share a phenolic structure with the three compounds showing inhibitory activity here. From a therapeutic point of view, inhibition of GST activity is important as these enzymes are involved in drug resistance and in the biosynthesis of prostaglandin and leucotriene from arachidonic acid [8]. Modulation of GST-activity can be used for controlling such compounds [27]. The use of a GST inhibitor, together with a cytostatic drug, is a strategy for increasing therapeutic efficiency of the latter, as some tumors show increasing GSH concentration and GST activity [28]. A role of GST in the resistance to alkylating agents has also been shown by the use of GST inhibitors. Inhibition of GST would thus be potentially beneficial in the treatment of tumors [29]. Tew *et al*. [30] used ethacrynic acid and piriprost as GST inhibitors to enhance the cytotoxic action of alkylating agents in drug resistance and sensitive cell lines. Further, ethacrinic acid treatment of patients with advanced cancer inhibited cellular GST-activity and increased the plasma levels of the alkylating agent thiotepa [31]. Another inhibitor of GST, indomethacin, also acts as an anti-inflammatory drug and potentiates the cytotoxicity of chlorambucil in CHO cells resistance to nitrogen mustards [11].

Table 1 V_{max}, K_m values in DCNB-GSH conjugation catalyzed by GST-N and GST-PB in the presence of curcumin

GST	Curcumin (μM)	GSH constant at 5 mM; DCNB concentration range 0.29-0.67 mM		*Curcumin* (μM)	DCNB constant at 0.67 mM; GSH concentration range 0.2 – 1.0 mM	
		V_{max} (nanomol/min/mg)	K_m (mM)		V_{max} (nanomol/min/mg)	K_m (mM)
GST-N	0	41.75	0.65	0	20.15	0.31
	5	19.10	0.64	5	10.04	0.54
	10	18.90	0.99	10	7.81	0.69
GST-PB	0	72.94	0.43	0	42.25	0.29
	5	56.69	1.36	5	21.70	0.60
	10	68.48	2.38	10	16.50	0.50

Table 2 V_{max}, K_m values in DCNB-GSH conjugation catalyzed by GST-N and GST-PB in the presence of analogue B_1

GST	B_1	GSH constant at 5 mM; DCNB concentration range 0.29-0.67 mM		B_1	DCNB constant at 0.67 mM; GSH concentration range 0.2 – 1.0 mM	
	(μM)	V_{max} (nanomol/min/mg)	K_m (mM)	(μM)	V_{max} (nanomol/min/mg)	K_m (mM)
GST-N	0	41.75	0.65	0	20.15	0.31
	2	23.54	0.90	2	9.40	0.40
	3	16.85	0.77	3	6.93	0.39
GST-PB	0	72.94	0.43	0	42.25	0.29
	1	86.09	1.38	1	28,32	0.78
	2	50.16	1.26	2	13,74	0.37

Table 3 V_{max}, K_m values in DCNB-GSH conjugation catalyzed by GST-N and GST-PB in the presence of analogue B_{12}

GST	B_{12}	GSH constant at 5 mM; DCNB concentration range 0.29-0.67 mM		B_{12}	DCNB constant at 0.67 mM; GSH concentration range 0.2 – 1.0 mM	
	(μM)	V_{max} (nanomol/min/mg)	K_m (mM)	(μM)	V_{max} (nanomol/min/mg)	K_m (mM)
GST-N	0	41.75	0.65	0	20.15	0.31
	20	23.27	0.41	20	16.39	0.31
	40	26.84	0.85	40	11.98	0.52
GST-PB	Experiments for K_1 were not performed because the IC_{50} could not be obtained					

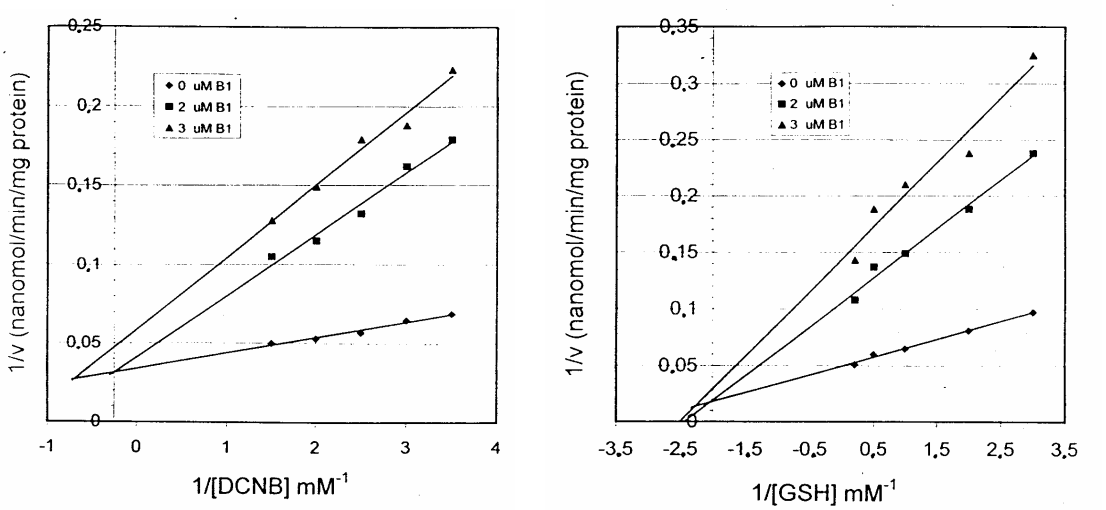

Fig 3 Lineweaver-Burk plot Showing mixed-type inhibition of uninduced rat liver GST's toward DCNB by 2 (■) or 3 μM (▲) 2,5-bis-(4-hydroxy-3-methoxy benzylidine) cyclopentanone (left) of non-competitive inhibiton towards gluthathione (right). Control (◆). The values are the average of four incubations.

In the results presented here (Fig 3), curcumin, B_1 and B_{12} showed a mixed type inhibition [23] with respect to DCNB. Curcumin at low concentrations, showed non-competitive inhibition of uninduced GSTs, while at low concentrations of B_1, competitive inhibition was observed for phenobarbital-induced GSTs. In comparison, with respect to GSH, curcumin showed a mixed type inhibition both on uninduced and phenobarbital-induced GSTs. Both B_1 and B_{12} showed non-competitive inhibition on uninduced GSTs, while mixed type inhibition was observed for phenobarbital-induced GSTs.

CONCLUSION

From the results, it can be concluded that B_1 is the most potent inhibitor of GSTs both from uninduced and phenobarbital-induced rat liver cytosol, with DCNB representing a substrate for the μ class GSTs. These inhibitory properties might be part of the anti-inflammatory activity of curcumin and benzylidene cyclopentanone derivatives (B_1 dan B_{12}).

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