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Lithocholic Acid, a Putative Tumor Promoter, Inhibits Mammalian DNA Polymerase β

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Lithocholic acid (LCA), one of the major components in secondary bile acids, promotes carcinogenesis in rat colon epithelial cells induced by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), which methylates DNA. Base-excision repair of DNA lesions caused by the DNA methylating agents requires DNA polymerase β (pol β). In the present study, we examined 17 kinds of bile acids with respect to inhibition of mammalian DNA polymerases *in vitro*. Among them, only LCA and its derivatives inhibited DNA polymerases, while other bile acids were not inhibitory. Among eukaryotic DNA polymerases α, β, δ, ε, and γ, pol β was the most sensitive to inhibition by LCA. The inhibition mode of pol β was non-competitive with respect to the DNA template-primer and was competitive with the substrate, dTTP, with the *Ki* value of 10 *μM*. Chemical structures at the C-7 and C-12 positions in the sterol skeleton are important for the inhibitory activity of LCA. This inhibition could contribute to the tumor-promoting activity of LCA.

Key words: Lithocholic acid — DNA polymerase β — Tumor promoter — Inhibitor — Colon carcinogenesis

Epidemiological studies have shown that dietary factors are important in the etiology of colon cancer. High intake of fat, which elevates secretion of fecal bile acids, correlates with high incidence of colon cancer.[1, 2)] This dietary habit in Western people results in a higher incidence of colon cancer and higher levels of fecal bile acids than in other peoples.[3)] Secondary bile acids, such as lithocholic acid (LCA), promote tumorigenesis induced in rats by a monoalkylating agent, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG).[4, 5)] LCA exerts its activity without further modification by the intestinal bacteria[6)] but the molecular mechanism of the cancer-promoting activity of this bile acid remains obscure. LCA is derived from a primary bile acid, chenodeoxycholic acid (CDCA), via deconjugation of the glycine or taurine group at C-24 and dehydroxylation at C-7 by the action of intestinal bacteria (Fig.1). In general, most of the primary, and part of the secondary bile acids and their salts are reabsorbed in the ileum, entering the enterohepatic circulation. However, LCA is poorly absorbed and is concentrated in colon, appearing at levels of up to 200 *μM* or more in feces.[7)]

Colon cancer is the result of the accumulation of mutations in a single colon epithelial cell.[8, 9)] Indeed, bile acids/salts have been reported as putative DNA-damaging agents. For instance, LCA produces DNA single-strand breaks in mouse L1210 cells.[10)] All of CDCA, LCA, glycolithocholic acid (GLCA) and taurolithocholic acid bind covalently to naked DNA *in vitro*.[11)] These studies have provided a possible mechanism for the tumor-initiating activity of bile acids, via formation of DNA adducts, but they may not explain the tumor-promoting activity of LCA.

Recently, Sobol *et al.*[12)] have demonstrated that mammalian DNA polymerase β (pol β) plays an essential role in the repair of damage caused by DNA-monomethylating agents such as MNNG or methyl methanesulfonate (MMS). In this context, we considered that mammalian DNA polymerases, especially pol β, might be a putative target of bile acids. Among five DNA polymerases in eukaryotic cells,[13)] pol α, δ, and ε are expressed in proliferating cells and participate in chromosomal DNA replication, whereas pol γ plays a role in mitochondrial DNA replication.[14)] Pol ε may also play a role in UV-induced

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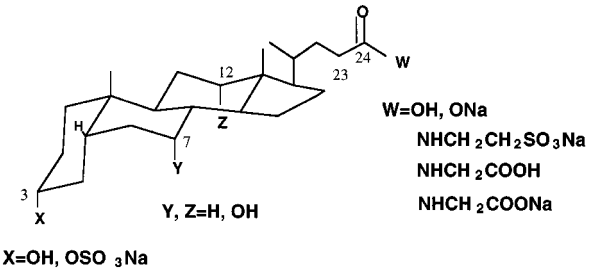
Abbreviations: pol α, DNA polymerase α; pol β, DNA polymerase β; pol γ, DNA polymerase γ; pol δ, DNA polymerase δ; pol ε, DNA polymerase ε; DTT, dithiothreitol; MMS, methyl methanesulfonate; DMSO, dimethyl sulfoxide; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; LCA, lithocholic acid; CA, cholic acid; NaC, sodium cholate; CDCA, chenodeoxycholic acid; NaCDC, sodium chenodeoxycholate; DCA, deoxycholic acid; NaDC, sodium deoxycholate; GLCA, glycolithocholic acid; NaTLC, sodium taurolithocholate; NaTUDC, sodium tauroursodeoxycholate; NaTC, sodium taurocholate; NaGC, sodium glycocholate; NaTCDC, sodium taurochenodeoxycholate; NaGCDC, sodium glycochenodeoxycholate; NaTDC, sodium taurodeoxycholate; NaGDC, sodium glycodeoxycholate; NaLCS, sulfolithocholic acid sodium salt; Na_2TLCS, sulfotaurolithocholic acid disodium salt; Na_2GLCS, sulfoglycolithocholic acid disodium salt.



	X (3α)	Y (7α)	Z (12α)	COW (24)
CA	OH	OH	OH	COOH
CDCA	OH	OH	H	COOH
DCA	OH	H	OH	COOH
LCA	OH	H	H	COOH

Fig. 1. Chemical structures of the bile acids/salts examined. The upper panel shows the general structure of bile acids. The lower panel shows the positions of hydroxyl groups and carboxyl group in the four major unconjugated bile acids. Cholic acid (CA) has three hydroxyl groups at X (C-3α), Y (C-7α), and Z (C-12α). Chenodeoxycholic acid (CDCA) has two hydroxyl groups at X and Y. Deoxycholic acid (DCA) has two hydroxyl groups at X and Z. Lithocholic acid (LCA) has a hydroxyl group at X alone. Taurine- or glycine-conjugated forms of bile acids have an amide linkage at C-24 shown as COW. The sulfated form of LCA has a sulfate group at X. Ursodeoxycholic acid has two hydroxyl groups at C-7β and X. Taurine-conjugated and glycine-conjugated (at C-24) forms of CA, CDCA, and DCA were compared with their deconjugated forms. Taurine-conjugated and glycine-conjugated (at C-24) forms of both LCA and sulfated LCA were also compared with their deconjugated forms.

nucleotide excision repair.[13, 15] In contrast to these replicative polymerases, the expression of pol β seems to be constitutive in most tissues, and is up-regulated by monomethylating agents.[12, 16, 17] Disruption of the *pol β* gene in mouse cells induces the defect of short-patch DNA synthesis in base-excision repair.[12] In the present study, we have shown that, among various bile acids, only LCA strongly inhibits pol β activity. The characteristic accumulation of LCA in the colon[7] also supports the possibility that LCA promotes carcinogenesis in colon by inhibiting pol β.

MATERIALS AND METHODS

Chemicals Seventeen kinds of bile acids and their sodium salts were products of Sigma Chemical Co. (St. Louis, MO). These bile acids differ from each other at the C-3, C-7, C-12, and C-24 positions, shown as X, Y, Z, and COW in Fig. 1. The compounds were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 m*M*, and diluted with distilled water just before use.

Enzymes We purified pol α from calf thymus by immunoaffinity-column chromatography as described previously.[18] Rat pol β was a gift from Dr. A. Matsukage, Nagoya, obtained from *Escherichia coli* carrying rat pol β-cDNA recombinant plasmid.[19] Pol γ was purified from bovine liver mitochondria.[20] Both pol δ and pol ε were from rat regenerating liver at 48 h after partial hepatectomy.[21]

DNA polymerase assays Pol α activity (0.2 unit) was assayed in a reaction mixture (25 μl) containing 40 m*M* potassium phosphate (pH 7.2), 200 μg/ml activated calf thymus DNA,[19] 8 m*M* $MgCl_2$, 4 m*M* dithiothreitol (DTT), 40 μ*M* [^{3}H]dTTP (240 cpm/pmol), 40 μ*M* each of unlabeled dATP, dGTP, and dCTP, and an aliquot of the enzyme solution. Pol β activity (0.2 unit) was assayed in the same reaction mixture as used for pol α (25 μl), except that it contained 100 m*M* KCl and 40 m*M* Tris-HCl (pH 8.8) in place of phosphate buffer. Pol γ activity (0.1 unit) was assayed in the same reaction mixture (25 μl) as used for pol α except that it contained 0.2 *M* KCl and 20 μ*M* [^{3}H]dTTP (500 cpm/pmol) as a labeled compound. Pol δ activity (0.1 unit) was assayed in a reaction mixture (25 μl) containing 40 m*M* HEPES-KOH (pH 6.5), 1 m*M* $MgCl_2$, 10 m*M* KCl, 2 m*M* DTT, 0.03% Triton X-100, 2% glycerol, 80 μg/ml bovine serum albumin, 50 μ*M* dATP, 20 μ*M* [^{3}H]dTTP (500 cpm/pmol), 45 μ*M* poly(dA-dT) co-polymer, and an aliquot of the enzyme solution. Pol ε activity (0.1 unit) was assayed in a reaction mixture (25 μl) containing 40 m*M* Tris-HCl (pH 6.7), 20 μg/ml poly(dA)-oligo$(dT)_{12-18}$, 8 m*M* $MgCl_2$, 1 m*M* DTT, 40 μ*M* [^{3}H]dTTP (240 cpm/pmol), and an aliquot of the enzyme.[21] In all cases, incubation was carried out at 37°C for 30 min and the acid-insoluble radioactivity was measured as described previously.[22] For kinetic analysis, the concentration of template-primer or [^{3}H]dTTP was variable.

Inhibition assay Inhibitory effects of bile acids/salts on pol α, β, and γ were measured using activated calf thymus DNA as a template-primer. Chemically synthesized template-primers were used in the cases of pol δ and pol ε. Various concentrations of bile acids were prepared in 5% DMSO, and a 5 μl aliquot was mixed with 5 μl of DNA polymerase preparation, followed by preincubation at 0°C for 20 min. The reaction was started by the addition of the reaction mixture (15 μl) to give the final concentrations of constituents as described above. Concentrations of the bile acids are indicated as final concentrations in the reaction mixtures.

RESULTS AND DISCUSSION

Importance of dehydroxylation at C-7 and C-12 for inhibitory activities of bile acids/salts on DNA polymerases Among 17 kinds of bile acids/salts, only LCA and its derivatives showed marked inhibition of DNA

Table I. Inhibitory Effects of 17 Kinds of Bile Acids/Salts against DNA Polymerases α, β, γ, δ, and ε at 100 *μM*

Bile acid		pol α	pol β	pol γ	pol δ	pol ε
Cholic acid	NaC	115 (%)[d]	90 (%)	87 (%)	107 (%)	108 (%)
	NaTC[a]	121	101	107	107	100
	NaGC[b]	85	110	93	108	95
Chenodeoxycholic acid	CDCA	90	127	102	105	102
	NaCDC	111	83	96	105	106
	NaTCDC[a]	117	102	109	114	94
	NaGCDC[b]	104	118	80	101	98
Deoxycholic acid	NaDC	99	105	95	128	93
	NaTDC[a]	119	110	104	108	108
	NaGDC[b]	90	114	95	116	100
Lithocholic acid	LCA	0	1	64	98	74
	GLCA[b]	27	19	68	111	84
	NaLCS[c]	31	46	63	113	44
	NaTLC[a]	7	13	62	117	88
	Na_2TLCS[a, c]	21	17	66	125	58
	Na_2GLCS[b, c]	12	15	81	121	58
Ursodeoxycholic acid	NaTUDC[a]	115	93	89	108	99

NaC, sodium cholate; NaTC, sodium taurocholate; NaGC, sodium glycocholate; CDCA, chenodeoxycholic acid; NaCDC, sodium chenodeoxycholate; NaTCDC, sodium taurochenodeoxycholate; NaGCDC, sodium glycochenodeoxycholate; NaDC, sodium deoxycholate; NaTDC, sodium taurodeoxycholate; NaGDC, sodium glycodeoxycholate; LCA, lithocholic acid; GLCA, glycolithocholic acid; NaLCS, sulfolithocholic acid sodium salt; NaTLC, sodium taurolithocholate; Na_2TLCS, sulfotaurolithocholic acid disodium salt; Na_2GLCS, sulfoglycolithocholic acid disodium salt; NaTUDC, sodium tauroursodeoxycholate.

a) C-24 taurine conjugate.
b) C-24 glycine conjugate.
c) C-3 sulfate.
d) Percent activity based on dTTP incorporation in the absence of the bile acids as 100%.

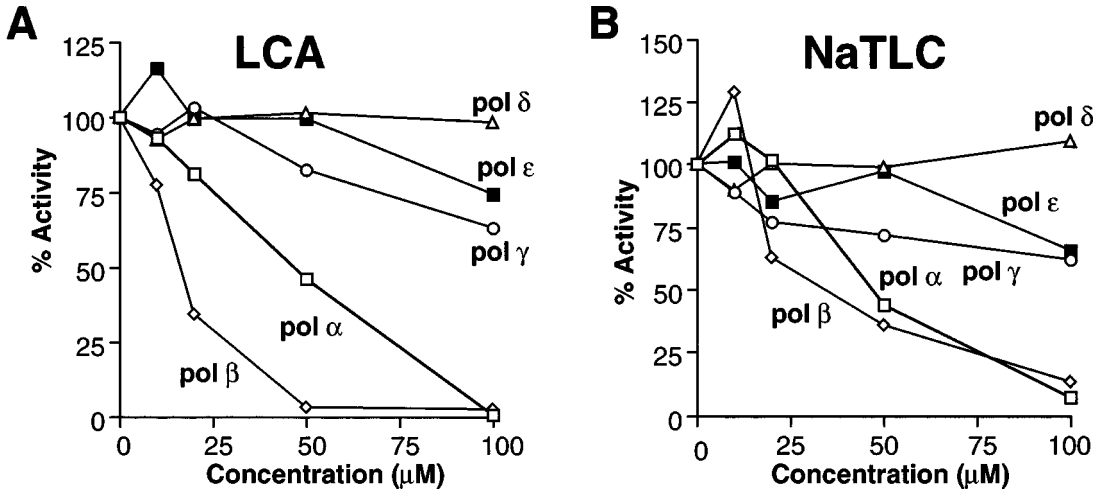


Fig. 2. Inhibition of DNA polymerases by LCA. Activities of pol α (□), β (◇), γ (○), δ (△), and ε (■) were measured in the presence of LCA (Fig. 2A) or NaTLC (Fig. 2B) at final concentrations of 10 *μM* to 100 *μM* as described in "Materials and Methods." Deoxy-TTP incorporation in the absence of each bile acid was taken as 100%.

polymerase activities at 100 *μM* (Table I). LCA lacks hydroxyl groups at C-7α and C-12α, in contrast to other bile acids (Fig. 1), and this peculiar structure may be important for its inhibitory activity. Among taurine-conjugated bile acids/salts, only sodium taurolithocholate (NaTLC) showed inhibition of pol β. Sodium tauroursodeoxycholate (NaTUDC), having a hydroxyl group at C-7β, showed no inhibition, although it lacks hydroxyl groups at C-7α and C-12α like LCA. Thus, dehydroxylation at C-7 (both α and β) and at C-12α may be essential for the inhibition. On the other hand, the structures at C-3 and C-24 may be less important, because LCA derivatives conjugated with glycine or taurine at these positions also showed the inhibition, though to a lesser extent. Previously, we showed that the sulfate group of sialic acid-containing glycolipid is indispensable for the inhibition of pol α.[23] In the cases of LCA and its derivatives, however, the absence of a sulfate group at C-3 did not negate, but rather enhanced the inhibitory effect on pol β (Table I).

Enzyme specificity in inhibition by LCA As shown in Fig. 2A, LCA inhibited the reaction of pol β most strongly (ID_{50}=15 *μM*), though it also inhibited pol α at higher concentrations (ID_{50} =50 mM). On the other hand, LCA inhibited pol γ and pol ε to a lesser extent, and did not inhibit pol δ. NaTLC, a sodium salt of taurine-conjugated LCA, inhibited pol β more weakly than LCA, but inhibited both pol α and pol γ as strongly as LCA (Fig. 2B).

Kinetic analysis of the inhibition of pol β by LCA Lineweaver-Burk plots showed that the inhibition mode of pol β by LCA was non-competitive with respect to template-primer, and competitive with dTTP substrate (Fig. 3, A and B). The *Ki* value was estimated at 10 *μM* from the Dixon plot (Fig. 3C). Competitive inhibition of pol β by LCA with substrate dTTP most probably indicates that LCA interacts with the dTTP-binding site of pol β, though the chemical structure of LCA is quite divergent from that of dTTP. A large divergence between the structures of competitive inhibitor and substrate is also observed in aphidicolin, a potent inhibitor of mammalian pol α, δ and ε[13]. Aphidicolin inhibits these three DNA replicative polymerases competitively with respect to dCTP (and/or dTTP)[24, 25] presumably by interacting with the side chains of amino acids within the highly conserved region I–II (dNTP-binding site).[13, 25–27] As a non-nucleotide type inhibitor, like aphidicolin, LCA could be useful to characterize the active site of pol β.

Conclusion The strong inhibition of pol β by LCA presented here, combined with recent studies indicating direct involvement of pol β in base-excision repair,[12] suggests that LCA may suppress the repair of DNA damage caused by monoalkylating agents via the inhibition of pol β. The inhibition of base-excision repair by LCA could be correlated with its tumor-promoting activity in rat colon cancer induced by MNNG.[4] DNA methylation can be repaired either by base-excision repair or via the O^6-methylguanine-DNA methyltransferase pathway.[28] Since O^6-methylguanine DNA adducts are rather minor among methylated DNA adducts,[29] the O^6-methylguanine-DNA methyltransferase pathway may not be as efficient as

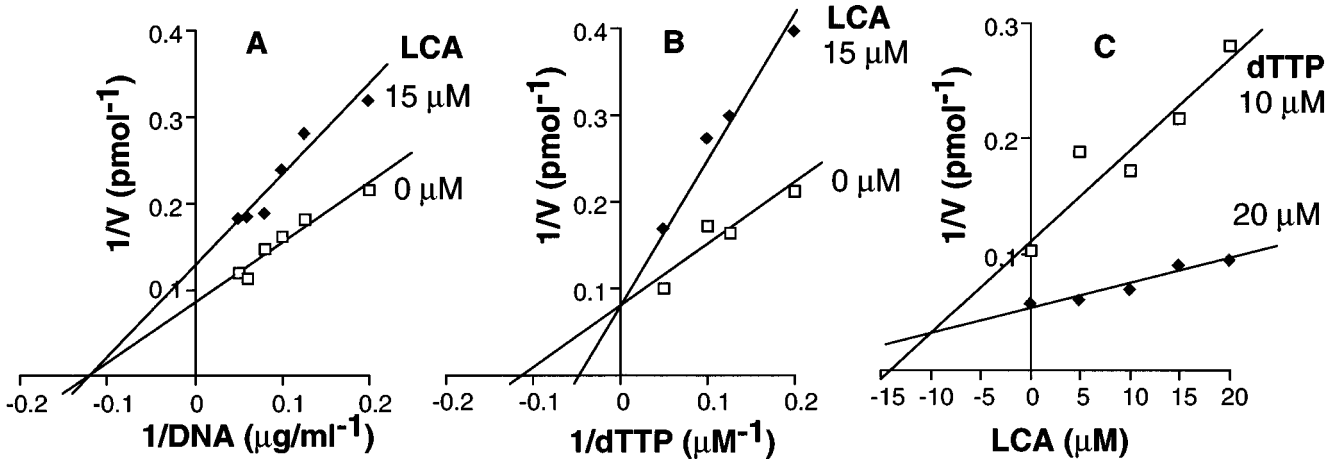


Fig. 3. Kinetic analysis of the inhibition of pol β by LCA. (A) Preincubation with pol β was done in the absence (□) or the presence of 37.5 *μM* (◆) LCA (final concentration, 15 *μM*). Pol β activity was then assayed as described in "Materials and Methods," using the indicated concentrations of activated calf thymus DNA as the template-primer. A Lineweaver-Burk plot of the data was prepared. (B) Pol β activity was assayed with the indicated concentrations of dTTP after preincubation of pol β in the absence (□) or presence of 37.5 *μM* (◆) LCA (final concentration, 15 *μM*). (C) Pol β activity was assayed with the indicated concentrations of LCA in 10 *μM* (□) and 20 *μM* (◆) dTTP. A Dixon plot of the data was prepared.

base-excision in the repair of methylated bases. We have found that a potent pol β inhibitor enhanced the mortality of human leukemic cells treated with a sublethal dose of MMS, but did not affect the mortality following UV irradiation.[30] Therefore, the high incidence of colon cancer in human populations with high concentrations of secondary bile acids could be explained in terms of the pol β inhibition by LCA. Another tumor promoter of colon cancer,[5] DCA, did not inihibit pol β. Therefore, DCA may act as a tumor promoter through a different mechanism. The interference with the repair pathway would cause cell death or enhance mutation incidence. Inhibition of DNA repair, in combination with endogenous or exogenous DNA damage, would enhance cytotoxicity and mutation frequency, which may result in carcinogenesis. For instance, xeroderma pigmentosum, an autosomal recessive disorder lacking the capacity of nucleotide-excision repair is accompanied with a high incidence of skin cancer when patients are exposed to UV.[31] Inhibitors of repair systems may act as a special class of tumor promoters, that would force plural mutations in a single cell after the initiation events, leading to the development of malignant cancer cells.

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