main bile acid component in these mice. LCA serves as a weak agonist for FXR, while it strongly antagonizes CDCA-mediated FXR activation.[36] Thus, Sult2a1 expression in LCA-fed mice might be regulated by complex networks with negative FXR signaling and positive PXR/CAR signaling through CDCA and LCA, respectively. Furthermore, PCN-mediated increases in Sult2a protein levels were attenuated by CDCA feeding in wild-type mice. This result also suggests the possibility of CDCA-mediated negative FXR signaling and the potential for cross-talk between FXR and PXR in the regulation of Sult2a expression.

It was reported that CDCA-mediated FXR activation stimulates reporter CAT expression directed *via* the rat SULT2A1 promoter from −366 to +38 in HepG2 cells.[19] An FXR binding site, designated IR0, in this promoter region was also demonstrated in this study. Furthermore, PXR, CAR and VDR were found to bind to this element and enhance reporter activity. The IR0 element has also been detected in the mouse Sult2a1 promoter, but not in the human SULT2A1 promoter. Our results using FXR-null mice and HepG2 cells suggest that the mouse and human SULT2As are negatively regulated by FXR signaling. Two possible mechanisms for negative transcriptional regulation of target genes by FXR activation have been proposed. One is an indirect mechanism *via* FXR-mediated induction of negative transcription factor such as SHP. The other is a direct mechanism *via* FXR binding to the negative response element of the target gene. Further studies are needed to understand whether FXR regulates SULT2A expression directly or indirectly.

SULT2A seems to facilitate the excretion of toxic bile acids such as LCA from body because human and mouse SULT2A predominantly catalyze LCA sulfation, but not primary bile acid, CA and CDCA sulfation.[6] Under physiological conditions, the main components of mouse hepatic bile acids, muricholic acid and cholic acid, are effectively conjugated with amino acids but not sulfated and excreted into bile and about 95% of the biliary amino-conjugated bile acids are reabsorbed in the intestine. On the other hand, under cholestasis, toxic bile acids such as LCA accumulate in liver and liver injury is accelelated. Thus, the nuclear receptor-mediated enhancement of bile acid sulfation that stimulates the excretion of toxic bile acids into feces might be an adaptive response to protect from bile acid-induced toxicity. Indeed, feeding of the primary bile acid CDCA suppresses hepatic Sult2a expression in wild-type mice, whereas LCA feeding increases its expression. Rodent CYP7A1, the rate-limiting enzyme in bile acid synthesis, is positively and negatively regulated by LXR signaling and FXR/SHP signaling respectively. These play key roles in bile acid and cholesterol homeostasis. SULT2A, involved in the bile acid excretion, seems to also be positively and negatively regulated by several bile acids through a complex nuclear receptor network to maintain bile acid homeostasis.

The hepatic bile acid components and concentration were analyzed and correlated with the hepatic Sult2a expression profile. Hepatic bile acid concentrations were significantly different between wild-type mice and FXR-null mice after feeding bile acids. Furthermore, unlike in vitro LCA treatment, CDCA was detected as the main bile acid component in livers of LCA-fed mice, suggesting hepatic accumulation of the LCA metabolite, CDCA in vivo. Hepatic bile acids are critical determinants for hepatic gene expression regulated by nuclear receptors such as PXR, CAR and FXR. The analyses of hepatic bile acids are thus important in order to compare wild-type mice and null mice treated with bile acids.

In the present study, we have demonstrated that SULT2A-mediated LCA sulfation is negatively regulated by CDCA-mediated FXR activation. Further studies are needed to elucidate the precise mechanism for this suppression and to determine the physiological significance of this response.

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Role for enhanced faecal excretion of bile acid in hydroxysteroid sulfotransferase-mediated protection against lithocholic acid-induced liver toxicity

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Abstract

The efficient clearance of toxic bile acids such as lithocholic acid (LCA) requires drug-metabolizing enzymes. We therefore assessed the influence of pregnenolone 16α-carbonitrile (PCN) treatment on LCA-induced hepatotoxicity and disposition of LCA metabolites using female farnesoid X receptor (FXR)-null and wild-type mice. Marked decreases in serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, and hepatic tauroLCA (TLCA) concentrations were found in LCA-fed wild-type mice co-treated with PCN. Whereas induction of Cyp3a and hydroxysteroid sulfotransferase (Sult2a) proteins was observed in FXR-null and wild-type mice, clear increases in biliary 3α-sulfated TLCA but not total 6α-hydroxy LCA (taurohyodeoxycholic acid and hyodeoxycholic acid) were only observed in PCN-treated wild-type mice. Biliary 3α-sulfated TLCA output rate was increased 7.2-fold, but accounts for only 4.2% of total bile acid output rate in LCA and PCN-co-treated wild-type mice. Total 3α-sulfated LCA (LCA and TLCA) was, however, the most abundant bile acid component in faeces suggesting that efficient faecal excretion of biliary 3α-sulfated TLCA through escape from enterohepatic circulation. FXR-null mice, which have constitutively high levels of the Sult2a protein, were fed a diet supplemented with 1% LCA and 0.4% dehydroepiandrosterone (DHEA), a typical Sult2a substrate/inhibitor. The faecal total 3α-sulfated bile acid excretion was reduced to 62% of FXR-null mice fed only the LCA diet. Hepatic TLCA concentration and serum AST activity were significantly higher in FXR-null mice fed DHEA and LCA diet than in FXR-null mice fed the LCA diet or DHEA diet. These results suggest that hepatic formation of 3α-sulfated TLCA is a crucial factor for protection against LCA-induced hepatotoxicity.

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Introduction

Bile acids, the major products of cholesterol catabolism in the liver, are critical for the absorption of dietary fat and vitamins in the intestine and the regulation of the enzymes and transporters involved in the lipid homeostasis through activation of nuclear receptors such as farnesoid X receptor (FXR), pregnane X receptor (PXR) and constitutive androstane receptor (CAR) (Eloranta and Kullak-Ublick 2005; Handschin and Meyer 2005). Under physiological conditions, approximately 5% of the bile acids are excreted in the faeces while the remaining 95% are recirculated back to the liver through enterohepatic circulation (Hofmann 1994). Hepatic bile acid levels are modulated by a balance between hepatic uptake, biosynthesis and efflux of bile acids regulated by FXR (Sinal et al. 2000). FXR-null mice, in which bile acid homeostasis is disrupted, are susceptible to cholic acid (CA)-induced toxicity. Transporter-mediated bile acid excretion, which is regulated by FXR, is a critical factor for protection against CA-induced toxicity (Zollner et al. 2003; Miyata et al. 2005; Zollner et al. 2005). On the other hand, female FXR-null mice, expressing constitutively higher levels of hydroxysteroid sulfotransferase (Sult2a), are resistant to LCA-induced toxicity, suggesting that Sult2a is involved in protection against LCA-induced toxicity (Kitada et al. 2003).

Lithocholic acid (LCA) is a hydrophobic secondary bile acid formed in the large intestine by bacterial 7α-dehydroxylation of chenodeoxycholic acid (CDCA). In humans, elevated levels of LCA are found in patients with chronic cholestatic liver disease (Fischer et al. 1996). LCA administration to experimental animals is known to cause hepatotoxicity, however, species differences in the susceptibility to LCA-induced toxicity have been reported (Palmer 1976; Hofmann 2004); rabbit and rhesus monkey are the most susceptible, rodents are intermediate whereas humans and chimpanzee are resistant.

Certain steroids protect against chemically induced toxicity through acceleration of the their metabolism. The synthetic steroid, pregnenolone 16α-carbonitrile (PCN) was found to prevent LCA-induced hepatotoxicity in rodents (Selye 1972). PCN, a prototypical agonist of the nuclear receptor PXR in rodents (Kliewer et al. 1998), is a potent inducer of various drug-metabolizing enzymes and transporters including CYP3A, CYP2B, hydroxysteroid sulfotransferase (SULT2A) and OATP2. Thus, these proteins are thought to play critical roles in PCN-mediated protection against LCA-induced toxicity. Indeed, PXR-null mice were used to demonstrate that PXR signalling prevents LCA-induced toxicity (Staudinger et al. 2001; Xie et al. 2001). Recent studies using CAR-null and PXR-null mice revealed that LCA-induced toxicity is protected through CAR as well as PXR activation (Saini et al. 2004; Zhang et al. 2004; Uppal et al. 2005; Wagner et al. 2005).

LCA metabolism through hydroxylation and sulfation reactions, alter the hydrophilicity of the molecule to facilitate its excretion from body. In mice, LCA hydroxylation and sulfation catalysed by Cyp3a and Sult2a, are enhanced by PXR and CAR activation (Sonoda et al. 2002; Assem et al. 2004; Echchgadda et al. 2004). These results suggest that Cyp3a and Sult2a play central roles in the nuclear receptor mediated protective mechanisms in mice. Although a large number of studies have been carried out on the protective mechanisms of LCA-induced toxicity through nuclear receptor signalling, little is known about the role of LCA metabolism, pharmacokinetics and excretion pathways

in LCA toxicity. These latter data are necessary in order to extrapolate properly animal model studies to humans.

In the present study, to explore the protective mechanisms for LCA-induced hepatotoxicity, LCA metabolism and disposition were assessed in FXR-null and wild-type mice treated with PCN. Furthermore, the influence of DHEA, a typical SULT2A substrate/inhibitor on LCA-induced toxicity was examined. Our results indicate that the increase in the production of hepatic Sult2a-mediated 3α-sulfated TLCA and efficient faecal excretion contributes to the protection against LCA-induced toxicity.

Materials and methods

Materials

Taurochenodeoxycholic acid (TCDCA), urusodeoxycholic acid (UDCA), deoxycholic acid (DCA), cholic acid (CA), chenodeoxycholic acid (CDCA), taurolithocholic acid (TLCA), lithocholic acid (LCA), 3α-sulfated TLCA, 3α-sulfated LCA, dehydroepiandrosterone (DHEA), choloylglycine hydrolase and pregnenolone 16α-carbonitrile (PCN) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Hyodeoxycholic acid (HDCA), taurohyodeoxycholic acid (THDCA), muricholic acid (MCA) and murideoxycholic acid (MDCA) were purchased from Steraloids, Inc. (Newport, RI, USA). The HPLC columns, Chemcosorb 5-ODS-H (6.0 × 150 mm) were purchased from Chemco Scientific Co. (Tokyo, Japan) and L-column ODS (2.1 × 150 mm) from Chemical Evaluation and Research Institute (Tokyo, Japan). Enzymepak 3α-HSD column was obtained from Jasco (Tokyo, Japan).

Animal treatment and sample collection

FXR-null mice (Sinal et al. 2000) and the wild-type mice (C57BL/6N) were housed under standard 12 hr light/12 hr dark cycle. Prior to the administration of special diets, mice were fed standard rodent chow (CE-2) (Clea, Tokyo, Japan) and water *ad libitum* for acclimation. Age matched groups of 8–12-week-old animals were used for all experiments and were allowed access to water ad libitum. FXR-null mice and wild-type mice were fed a control diet supplemented with 1% LCA or 1% LCA and 0.4% DHEA for 9 days and co-treated with PCN (100 mg kg^{-1}, in corn oil) or vehicle for last 4 days. Bile, blood, faeces and tissue samples were taken for biochemical assays after 9 days of feeding special diets. Each mouse was placed in metabolic cages for the last one day to collect faeces and urine. Biliary excretion was monitored in mice anaesthetized with ethyl ether as previously described (Miyata et al. 2005). After ligation of the common bile duct, the gall bladder was cannulated with a polyethylene tube (PE-10) with an internal diameter of 0.28 mm. The cannula was ligated into the gall bladder to obtain bile samples. After a 5 min equilibration period, bile was collected for 30 min. All experiments were performed in accordance with Guidelines for Animal Experiments of Tohoku University.

Serum AST and ALP activities

Serum aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activities were determined by the POP-TOOS method using a commercial kit, Transaminase CII-B-test

Wako (Wako, Osaka, Japan). Bile, liver and serum 3α-hydroxy bile acid concentrations were estimated by an enzyme-colorimetric method using the Total bile acid-test kit from Wako (Wako, Osaka, Japan).

Content of bile acid components in liver, bile and faeces

Hepatic, biliary and faecal 3α-hydroxy bile acid contents were measured by HPLC as previously described (Kitada et al. 2003). A portion (100 µl) of liver homogenate was mixed with 1 ml ethanol containing 2 nmol of androstandiol and treated at 85°C for 1 min, and then centrifuged at 1000 *g* for 5 min. After the supernatant was isolated, the precipitate was extracted twice with 1 ml ethanol and the combined extracts were dried and redissolved in 200 µl methanol. Faeces was homogenized in 50% of tertiary-butanol and centrifuged at 1000 *g* for 5 min. The supernatants were diluted with methanol. To analyse biliary 3α-OH bile acid components, biliary bile acids were deconjugated by choloylglycine hydrolase. Bile (2 µl) was diluted 50-fold with 100 mM of sodium acetate buffer (pH 5.6) and was treated with choloylglycine hydrolase (5 units) from *Clostridium perfringens* for 12 h at 37°C. The reaction mixture was diluted with methanol. HPLC analyses were performed with a Jasco intelligent model PU-980 pump (Jasco, Tokyo, Japan), Waters M-45 pump (Waters, Milford, MA, USA) and FP-920S fluorescence detector (Jasco, Tokyo, Japan). Bile acids were separated at 35°C with an L-column ODS (2.1 × 150 mm) (Chemical Evaluation and Research Institute). The eluates were mixed with an NAD^+ solution prior to introduction of 3α-hydroxysteroid dehydrogenase immobilized on an Enzymepak 3α-HSD column. The NADH produced was measured by fluorescence using an excitation wavelength of 365 nm and an emission wavelength of 470 nm. The separation was started at a flow rate of 0.5 ml min^{-1} with a 60 min linear gradient of solution A/solution B mixture (25:75) to solution A/solution B mixture (55:45), and then continued with solution A/solution B mixture (55:45) for 25 min. (Solution A; 10 mM phosphate buffer (pH 7.2)/acetonitrile (60:40); solution B; 30 mM phosphate buffer (pH 7.2)/acetonitrile (80:20).) The eluates were passed through a 3α-HSD column after mixing with solution C (1:1). (Solution C; 10 mM phosphate buffer (pH 7.2), 1 mM EDTA, 0.05% 2-mercaptoethanol, and 0.3 mM NAD^+.)

Measurement of 3α-sulfated bile acid

Total 3α-sulfated bile acid concentrations were determined by an enzyme-colorimetric method using UBASTEC-AUTO (Daiichi Pure Chemical, Tokyo, Japan) (Tazuke et al. 1994; Kato et al. 1996). The LC/electrospray ionization-MS method was employed for the detection of 3α-sulfated bile acids. The LC/MS system consisted of Quattro Ultima (Micromass, Manchester, UK) and HP1100 system (Agilent Technologies, Palo Alto, CA, USA). Bile acid extracts were separated with an L-column ODS (2.1 × 150 mm) (Chemical Evaluation and Research Institute) with a gradient mobile phase of acetonitrile containing formic acid (solution A) and 50 mM ammonium-acetate buffer (solution B). The separation was started at a flow rate of 0.4 ml min^{-1} with a 5 min linear gradient of solution A/solution B mixture (2:98) to solution A/solution B mixture (95:5), and then continued with solution A/solution B mixture (95:5) for 1 min. The MS conditions were as follows: negative ion mode; capillary volt, 3.0 kV; cone volt, 50 V. Detection was performed in the selected ion monitoring mode. The [M–H] ions, *m/z* 455.2 and 562.2 were monitored for quantification of 3α-sulfated LCA and 3α-sulfated TLCA, respectively.

Western blot analysis

Cytosolic proteins (3 μg/lane) or microsomal proteins (2 μg/lane) were loaded onto a 10.5 or 8.0% polyacrylamide gel, respectively, isolated and transferred to nitrocellulose filters. The filters were immunostained with a polyclonal antibody prepared against the purified recombinant rat SULT2A1 protein (1:1000 dilution) that reacts with mouse Sult2a (Shimada et al. 2001) and a polyclonal antibody against rat CYP3A2 (1:1000 dilution) that cross-reacts with mouse Cyp3a. These antibodies do not react with other family isoforms. The sheets were stained with horseradish peroxidase conjugated goat anti-rabbit IgG and 3,3′-diaminobenzidine tetrahydrochloride. The stained sheets were scanned with a GT-8700 spectrophotometer (Epson, Suwa, Japan) and their intensities measured by use of the NIH image quant (version 1.59) software (Bethesda, MD, USA).

LCA 6α-hydroxylase activities

A typical incubation mixture consisted of 0.1 M phosphate buffer (pH 7.4), 4.8 mM $MgCl_2$, 0.32 mM $NADP^+$, 2.4 mM glucose 6-phosphate, 0.26 U ml^{-1} glucose 6-phosphate dehydrogenese, 0.25 μM LCA, and 250 μg microsomal protein in a final volume of 500 μl. The mixture was incubated for 20 min at 37°C. The incubation was terminated by addition of 1 ml of ethyl acetate. LCA 6α-hydroxylation (HDCA) was determined by HPLC.

Estimation of faecal/biliary ratios of bile acid excretion

Faecal bile acid excretion amount per day was measured in faeces collected for 24 h. Biliary bile acid excretion amount per day was estimated by multiplying biliary bile acid amount in bile collected for 30 min by 48. Faecal/biliary ratios of bile acid excretion were calculated by dividing faecal bile acid excretion amounts per day by biliary bile acid excretion amounts per day.

Statistical analysis

All values are expressed as the means ± standard deviation. All data were analysed by an unpaired Student's *t*-test or a Dunnett's multiple comparisons test for significant differences between the mean values of each group.

Results

Influence of PCN treatment on LCA-induced hepatotoxicity and hepatic bile acid concentrations

To understand the mechanism by which PCN protects against LCA-induced toxicity, FXR-null mice and wild-type mice were fed a diet supplemented with 1% LCA for 9 days and co-treated with PCN (100 mg kg^{-1}, in corn oil) or vehicle for the last 4 days. Marked decreases in levels of the diagnostic markers for liver damage, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were found in the wild-type mice co-treated with PCN as compared with vehicle treated mice (Figure 1). FXR-null mice were resistant to LCA-induced hepatotoxicity. Low levels of these activities were observed in both groups of FXR-null mice.

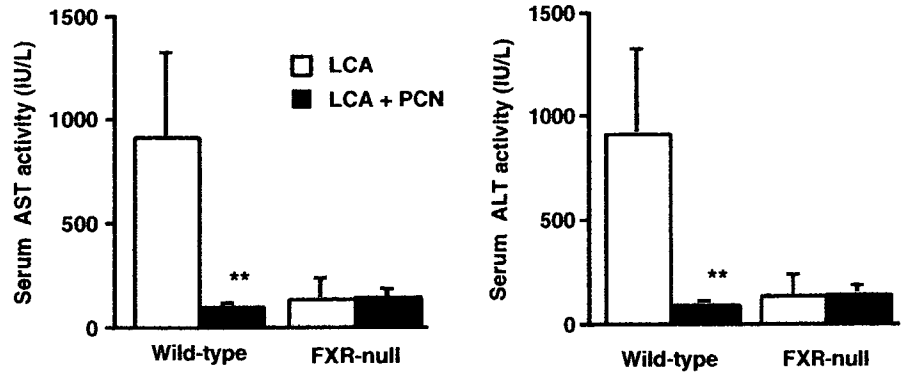

Figure 1. Influence of PCN treatment on serum AST and ALT activities in LCA-fed mice. Sera were isolated from wild-type and FXR-null mice fed a 1% LCA diet for 9 days and injected with PCN (100 mg kg^{-1}, intraperitoneally) or vehicle (corn oil) the last 4 days. Data are shown as the mean ± standard deviation ($n = 5$). **Significantly different from corresponding control group ($p < 0.01$).

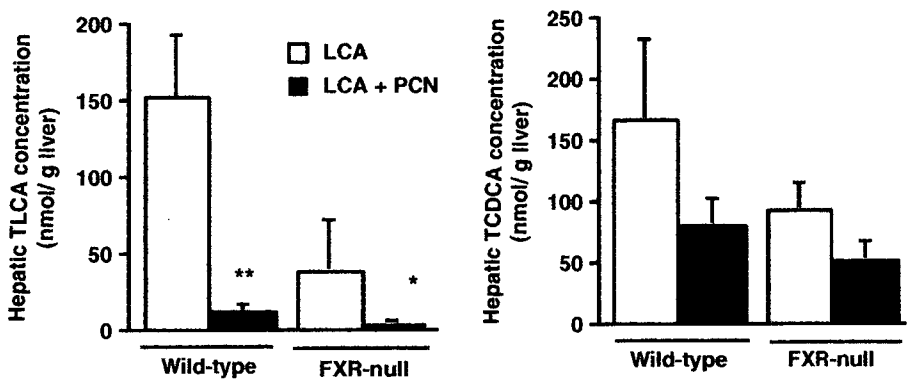

Figure 2. Influence of PCN treatment on hepatic bile acid concentrations. Livers were isolated from wild-type and FXR-null mice fed a 1% LCA diet for 9 days and injected with PCN (100 mg kg^{-1}, intraperitoneally) or vehicle (corn oil) the last 4 days. Hepatic TLCA and TCDCA concentrations were measured by HPLC. Data are shown as the mean ± standard deviation ($n = 5$). Significantly different from the corresponding control group (*$p < 0.05$, **$p < 0.01$).

TLCA and TCDCA were detected as the hepatic major bile acid components (Figure 2). Marked decreases in hepatic TLCA concentrations were observed in FXR-null and the wild-type mice co-treated with PCN compared with the vehicle-treated mice. Hepatic TCDCA concentrations were also low in PCN-treated wild-type and FXR-null mice. A clear correlation was observed between AST activity and hepatic TLCA concentrations in the wild-type mice (data not shown).

Influence of PCN treatment on hepatic contents of Sult2a and Cyp3a proteins and LCA 6α-hydroxylase activity

Hepatic contents of Sult2a protein were higher in LCA-fed FXR-null mice than in the wild-type mice (Figure 3A, B). Significant increases in Sult2a protein contents were

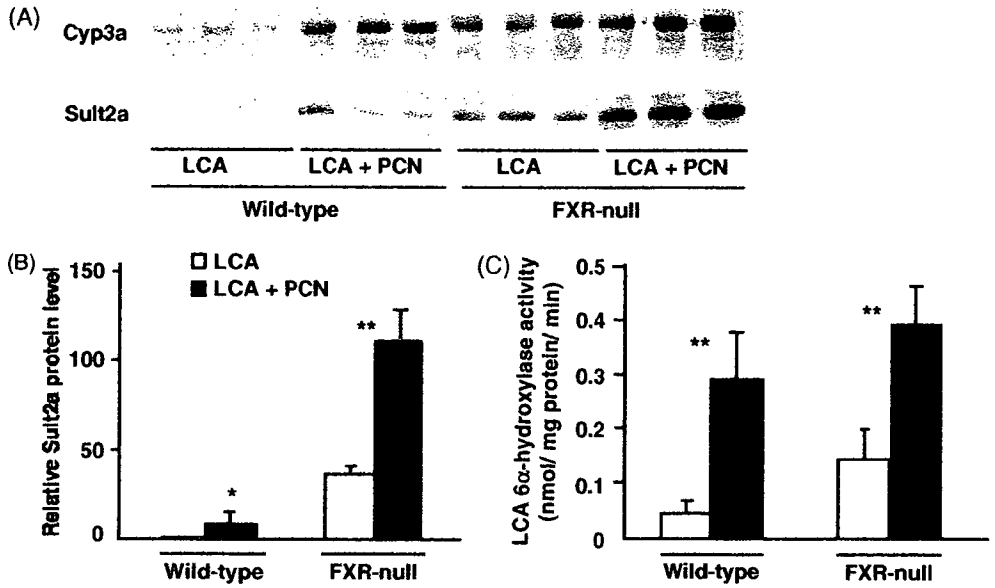

Figure 3. Influence of PCN treatment in hepatic Sult2a and Cyp3a protein contents and LCA 6α-hydroxylase activity. (A) Immunoblot analysis of hepatic Sult2a and Cyp3a. Livers were isolated from wild-type and FXR-null mice fed a 1% LCA diet for 9 days and injected with PCN (100 mg kg^{-1}, intraperitoneally) or vehicle (corn oil) the last 4 days. Three representative samples were shown. Cytosolic proteins (10 μg) and microsomal proteins (2 μg) were loaded. (B) Quantification of hepatic Sult2a protein content. (C) Hepatic microsomal LCA 6α-hydroxylase activity. Data are shown as the mean ± standard deviation ($n = 5$). Significantly different from the corresponding control group ($^*p < 0.05$, $^{**}p < 0.01$).

observed in the wild-type and FXR-null mice co-treated with PCN, although Sult2a protein levels were higher in FXR-null mice than in PCN-treated wild-type mice. Hepatic levels of Cyp3a proteins were also increased with PCN treatment in both wild-type and FXR-null mice. Cyp3a protein levels were higher in wild-type mice treated with PCN than in FXR-null mice treated with the vehicle. Consistent with hepatic Cyp3a protein levels, microsomal LCA 6α-hydroxylation was increased 6.1-fold in PCN-treated wild-type and 2.7-fold in FXR-null mice (Figure 3C).

Output rates of biliary bile acids

Bile acid output rates (biliary 3α-hydroxy bile acid excretion rate) were determined to estimate the biliary bile acid excretion capacity. No significant difference in bile acid output rates was observed in wild-type and FXR-null mice with or without PCN treatment. CDCA, MDCA, HDCA, LCA, UDCA, DCA, CA and MCA were detected in bile treated with choloylglycine hydrolase. The main bile acid components, CDCA, MDCA, HDCA, LCA and UDCA were quantified (Table I). Total CDCA (TCDCA and CDCA) was detected as the most abundant biliary 3α-OH bile acid components. In spite of LCA feeding, biliary total LCA levels were less than 10% of the total bile acids in each group. Although microsomal LCA 6α-hydroxylase activities were markedly increased in both wild-type and FXR-null mice after treatment with PCN, biliary total 6α-hydroxy LCA (THDCA and

Table I. Biliary bile acid output rate in wild-type and FXR-null mice.

	Biliary bile acid output rate (nmol/min/100 g BW) (%)			
	Wild-type		FXR-null	
Metabolite	LCA	LCA + PCN	LCA	LCA + PCN
Total CDCA	75.0 ± 54.1 (48.7)	128.0 ± 27.6 (75.1)	127.8 ± 43.6 (50.5)	120.5 ± 57.0 (73.2)
Total MDCA	46.6 ± 28.4 (30.3)	9.1 ± 2.9* (5.3)	44.0 ± 29.2 (17.4)	4.9 ± 2.0* (3.0)
Total LCA	14.8 ± 10.9 (9.6)	10.5 ± 7.6 (6.2)	20.4 ± 11.2 (8.1)	3.8 ± 2.2* (2.3)
Total HDCA	7.9 ± 3.4 (5.1)	6.5 ± 2.7 (3.8)	8.2 ± 4.6 (3.2)	5.7 ± 2.7 (3.5)
Sulfated TLCA	1.0 ± 1.3 (0.6)	7.2 ± 5.0** (4.2)	15.2 ± 11.9 (6.0)	25.2 ± 5.6 (15.3)
Sulfated LCA	ND	ND	ND	ND

Total CDCA, CDCA and TCDCA; Total MDCA, MDCA and TMDCA; Total LCA, LCA and TLCA; Total HDCA, HDCA and THDCA. Values are mean ± standard deviation ($n = 5$). *, $p < 0.05$ vs LCA group; **, $p < 0.01$ vs LCA group. Values in parentheses were expressed as a percentage of total main bile acids (total CDCA, total MDCA, total LCA, total HDCA, total UDCA, and sulfated TLCA). ND, not detected.

HDCA) contents were not increased compared with vehicle-treated mice. On the other hand, the biliary 3α-sulfated TLCA excretion rate was clearly increased (7.2-fold) in wild-type mice treated with PCN, compared with vehicle-treated wild-type mice. The biliary 3α-sulfated TLCA excretion rate, however, was only 4.2% of the total bile acids (total CDCA, total MDCA, total HDCA, total LCA, total UDCA and 3α-sulfated TLCA) excretion rate, and TCDCA excretion was estimated at 75% of total bile acid excretion in wild-type mice treated with PCN. 3α-sulfated LCA was not detected in biliary bile acids.

Faecal bile acid excretion amount

In wild-type mice treated with PCN, faecal total 3α-sulfated LCA (3α-sulfated LCA and 3α-sulfated TLCA) excretion was higher (1.6-fold) than faecal total CDCA (CDCA and TCDCA) excretion (Figure 4). Total 3α-sulfated LCA was the most abundant bile acid component in the faeces. In FXR-null mice, faecal amounts of total 3α-sulfated LCA were more than 20-fold higher than those of faecal total CDCA. The ratios of faecal excretion to biliary excretion of total CDCA and total 3α-sulfated LCA were 0.02 and 0.49, respectively, in wild-type mice treated with PCN (Table II). These results suggest extremely high excretion of 3α-sulfated LCA excretion from bile to faeces.

Faecal and urinary 3α-sulfated LCA excretion

To compare 3α-sulfated bile acid excretion from the body to urine and faeces, urine and faeces were collected for 24 h after diet change from LCA supplemented to the normal. Total 3α-sulfated bile acid levels in faeces were significantly increased by treatment of wild-type mice with PCN, but the levels were still lower than those in FXR-null mice treated with vehicle (Figure 5). No significant difference in urinary amounts of 3α-sulfated bile acid was found between FXR-null and wild-type mice treated with or without PCN pretreatment. Furthermore, urinary levels of 3α-sulfated bile acids were much lower compared with the faecal excretion. Urinary and serum levels of 3α-sulfated bile acids were not increased in PCN-treated wild-type mice, although hepatic Mrp3 mRNA levels were significantly increased (data not shown).

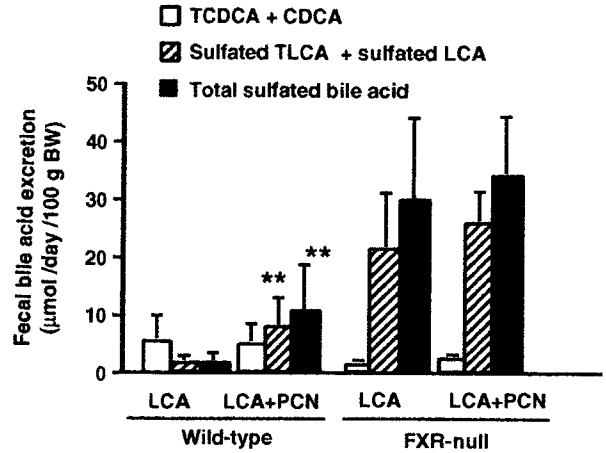

Figure 4. Influence of PCN treatment in faecal excretion of sulfated LCA and CDCA. Faeces were isolated for 24 h from wild-type and FXR-null mice fed a 1% LCA diet for 9 days and injected with PCN (100 mg kg^{-1}, intraperitoneally) or vehicle (corn oil) the last 4 days. Total CDCA (TCDCA and CDCA), total sulfated LCA (sulfated TLCA and sulfated LCA) and total sulfated bile acids were measured by HPLC, LC/MS and enzyme-colorimetric method, respectively. Data are shown as the mean ± standard deviation ($n = 5$). Significantly different from the corresponding control group ($^{**}p < 0.01$).

Table II. Relative faecal/biliary ratios of total sulfated LCA and CDCA excretion levels.

	Wild-type		FXR- null	
Metabolite	LCA	LCA + PCN	LCA	LCA + PCN
Total sulfated LCA	>1.0	0.49	0.28	0.68
Total CDCA	0.047	0.023	0.004	0.010

Total sulfated LCA, 3α-sulfated LCA and 3α-sulfated TLCA; Total CDCA, CDCA and TCDCA. The ratio (R) was calculated from the following equation. R = faecal bile acid (nmol/day/100 g BW)/biliary bile acid output rate (nmol/30 min/100 g BW) × 48.

Influence of DHEA feeding on LCA-induced toxicity and faecal 3α-sulfated LCA excretion

To assess the influence of Sult2a on LCA-induced hepatotoxicity, FXR-null female mice expressing high level of Sult2a protein were fed a diet supplemented with 1% LCA and 0.4% DHEA as DHEA is known to competitively inhibit sulfation catalysed by Sult2a in vitro (Nagata and Yamazoe 2000). Serum AST activity was significantly increased in FXR-null mice fed both DHEA and LCA (Figure 6). No significant increases in AST activity were observed in FXR-null mice fed a DHEA or an LCA diet compared with control FXR-null mice. Furthermore, when wild-type mice were fed 0.5% LCA and 0.4% DHEA diets, no significant increases in the activity were observed, compared with wild-type mice fed either control or 0.5% LCA diets (data not shown). The influence of DHEA on LCA-induced toxicity was observed in FXR-null mice expressing high Sult2a protein, but not in wild-type mice thus indicating that the DHEA effect in FXR-null mice is likely to depend on LCA sulfation. No significant changes in hepatic Sult2a protein level were observed in FXR-null mice fed a DHEA diet.

Consistent with the increase in the serum AST activity, the hepatic TLCA concentration was significantly increased in FXR-null mice fed the DHEA and LCA diet compared with

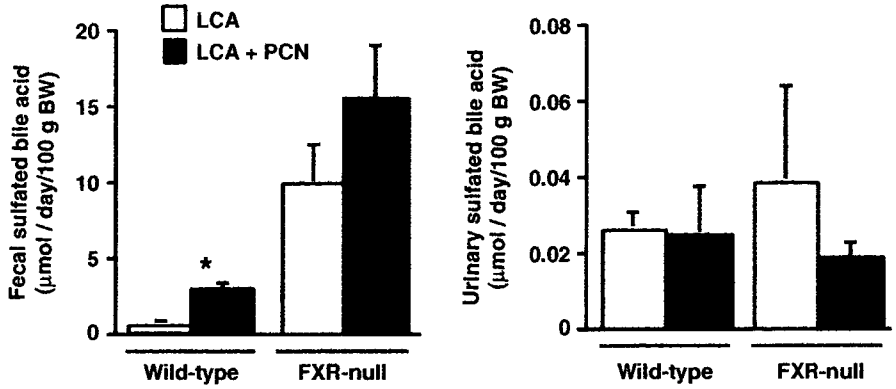

Figure 5. Faecal and urinary excretion of sulfated bile acids. Faeces and urine were collected for 24 h from wild-type mice and FXR-null mice fed a control diet after the mice were fed a 1% LCA diet for 9 days and injected with PCN (100 mg kg^{-1}, intraperitoneally) or vehicle (corn oil) the last 4 days. Faecal and urinary sulfated bile acids were measured by enzyme-colorimetric method. Data are shown as the mean ± standard deviation ($n = 5$). Significantly different from the corresponding control group ($^*p < 0.05$).

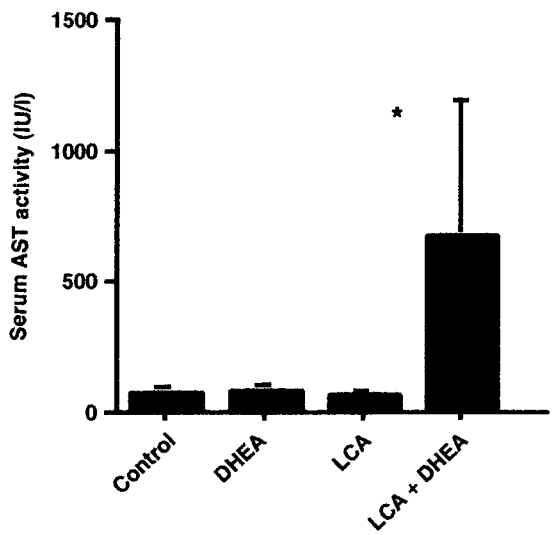

Figure 6. Influence of DHEA feeding in serum AST activity of LCA fed FXR-null mice. FXR-null mice were fed 1% LCA, 0.4% DHEA or 1% LCA and 0.4% DHEA diet for 9 days. Data are shown as the mean ± standard deviation ($n = 3 - 5$). Significantly different from control group ($^*p < 0.05$).

FXR-null mice fed the LCA diet (Figure 7A). As expected, faecal excretion of total 3α-sulfated LCA in FXR-null mice fed the LCA and DHEA diet was reduced to 62% of FXR-null mice fed a diet containing only LCA (Figure 7B).

Discussion

The present study provides evidence that hepatic LCA sulfation is critical for protection against LCA-induced hepatotoxicity. LCA-induced toxicity was attenuated in PCN-treated

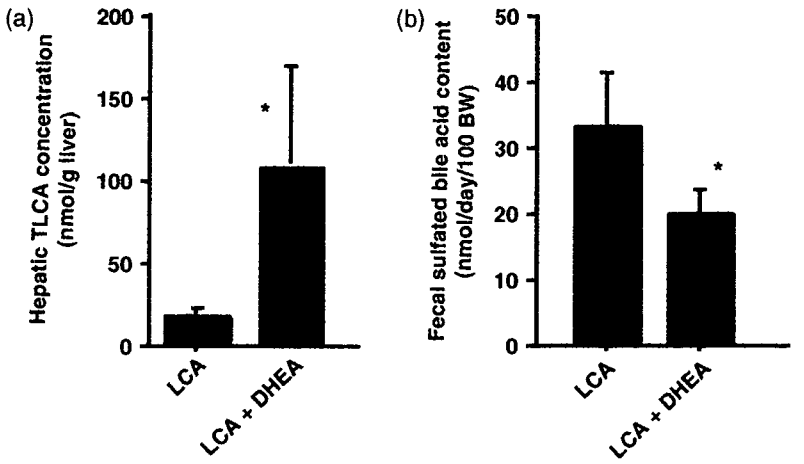

Figure 7. Influence of DHEA feeding in hepatic TLCA concentration and faecal sulfated bile acid contents. FXR-null mice were fed 1% LCA, 0.4% DHEA or 1% LCA and 0.4% DHEA diet for 9 days. (A) Hepatic TLCA concentration. Hepatic TLCA concentrations were measured by HPLC. (B) Faecal sulfated bile acid contents. Faecal sulfated bile acid contents were measured by enzyme-colorimetric method. Data are shown as the mean ± standard deviation ($n = 3 - 5$). Significantly different from the corresponding control group ($^*p < 0.05$).

wild-type mice in which hepatic Sult2a protein, biliary total 3α-sulfated LCA and faecal total 3α-sulfated LCA were markedly increased. Furthermore, LCA-induced toxicity was enhanced after feeding DHEA to FXR-null mice, in which faecal total 3α-sulfated LCA were significantly decreased. These results suggest that hepatic formation of 3α-sulfated TLCA is a crucial factor for protection against LCA-induced hepatotoxicity. Furthermore, our data demonstrate that 3α-sulfated TLCA produced in liver was efficiently excreted into faeces, which is direct in contrast to the main metabolite TCDCA. Thus, 3α-sulfated TLCA might be poorly reabsorbed by the ileal bile acid transporter or other systems, whereas biliary 3α-OH bile acids are efficiently reabsorbed. The excretion rate of sulfated TLCA from bile to faeces is more than tenfold higher than that of TCDCA (Table II). Even though the biliary total 3α-sulfated LCA level is less than 5% of total bile acids, it may be sufficient to reduce hepatic LCA contents in mice fed 1% LCA diet.

Biliary and faecal total 3α-sulfated LCA (TLCA and LCA) levels were positively correlated with hepatic Sult2a protein levels in FXR-null and wild-type mice treated with PCN or vehicle. Thus, hepatic formation but not transport of 3α-sulfated TLCA, is likely the rate limiting step for faecal LCA excretion. Biliary and faecal total 3α-sulfated LCA levels in FXR-null mice fed a 1% LCA diet were higher than those in PCN-treated wild-type mice fed a 1% LCA diet. These results also support the idea that animal species possessing a potent capacity to produce 3α-sulfated LCA like humans, are resistant to LCA-induced hepatotoxicity. 3α-sulfated TLCA, but not 3α-sulfated LCA was detected in biliary bile acid (Table I). This raises the possibility that tauro-conjugation of LCA is a preferential metabolic step and/or the biliary excretion system of 3α-sulfated LCA is absent in the canalicular membrane. The appearance of 3α-sulfated LCA together with 3α-sulfated TLCA in faeces, suggest the occurrence of deconjugation by intestinal bacteria.

In humans and rodents, hepatic LCA is mainly transformed to LCA amino acid conjugates in liver. A portion of LCA amino acid conjugates further undergo 3α-sulfation by SULT2A. Hepatic 3α-sulfated LCA amino acid conjugates are transported into bile

by the canalicular transporter, Mrp2 (Kuipers et al. 1988; Takikawa et al. 1991; Stieger et al. 2000) or also transported into blood by the basolateral transporters, Mrp3 and Mrp4 (Hirohashi et al. 2000; Zelcer et al. 2003). It has been suggested that Sult2a1 and Mrp4 participate in an integrated pathway mediating elimination of sulfated bile acids from mouse liver (Assem et al. 2004). The present study suggests that the contribution of urinary excretion of 3α-sulfated bile acids might be of limited significance in protection against LCA-induced toxicity, because of no clear increase in contents even after PCN treatment. Furthermore, the rate of urinary excretion was more than 100-fold lower than faecal excretion in PCN-treated wild-type mice. Thus, LCA sulfate seems to be selectively excreted into the faeces in mice. These results are consistent with the observation in humans intravenously injected with radiolabelled LCA where LCA was rapidly and predominantly excreted as sulfated conjugates in bile (Cowen et al. 1975a, b). In FXR-null mice, 43% of biliary excreted total LCA was 3α-sulfated derivatives. High hepatic production of LCA sulfate is likely to be responsible for the resistance to LCA-induced toxicity in humans and FXR-null mice.

Faecal 3α-sulfated bile acid excretion was decreased in DHEA-fed mice in the current study. Sulfated DHEA is not excreted by Mrp2, which transports 3α-sulfated TLCA (Sasaki et al. 2002; Zelcer et al. 2003). Thus, the observed enhancement of the LCA-induced toxicity in FXR-null mice fed a DHEA diet was likely due to DHEA-mediated competitive inhibition of hepatic TLCA sulfation but not biliary 3α-sulfated TLCA excretion. It has been reported that DHEA activates PXR and induces Cyp3a (Ripp et al. 2002). However, hepatic LCA clearance was decreased in the DHEA co-treated mice. Thus, DHEA-mediated competitive inhibition of hepatic TLCA sulfation is likely to be primary determinant of increase in hepatic LCA concentration.

Cyp3a-mediated hydroxylation of LCA has also been considered important for protection against LCA-induced toxicity (Hofmann 2004). Under conditions used in the present study, CYP3A-mediated metabolism was not important for protection against LCA-induced toxicity. Mouse Cyp3a isoforms catalyse LCA 6α-hydroxylation and thus, PCN treatment was expected to increase biliary total HDCA (6α-OH LCA). However, a decrease in biliary total HDCA was observed in PCN-treated wild-type mice. Although the possibility that THDCA is further hydroxylated or glucuronidated remains to be clarified, our data support a minor contribution of Cyp3a to PCN-mediated protection against LCA-induced hepatotoxicity, as compared with Sult2a.

Monohydroxy bile acids such as LCA are preferentially sulfated, compared with di- and trihydroxy bile acids (Kitada et al. 2003). Thus, small amounts of monohydroxy, secondary bile acids produced by bacterial dehydroxylation of primary bile acids during enterohepatic circulation are preferably sulfated and efficiently excreted into the faeces. This latter system, which is active under normal conditions, is likely a critical defence mechanism to prevent the accumulation of toxic hydrophobic bile acids. It has been reported that human SULT2A1 mRNA is induced in human primary hepatocytes treated with PXR agonists such as dexamethasone or rifampicin (Duanmu et al. 2002; Fang et al. 2005). Thus, PXR agonists that induce SULT2A may be useful for the improvement of hepatic function under conditions of cholestasis where hydrophobic bile acids such as LCA accumulate.

In the present study, we have provided direct evidence that hepatic production of 3α-sulfated LCA contributes to decrease hepatic TLCA concentrations in LCA-fed mice. The data raise the possibility that the sulfated bile acid excretion pathway is a potential therapeutic target for treatment of cholestatic liver disease.

Acknowledgements

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Epigenetic Control Using Natural Products and Synthetic Molecules

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Abstract: The term "epigenetics" is defined as "heritable changes in gene expression that occur without changes in DNA sequence". Recently, it has been revealed that DNA methylation and histone modifications such as acetylation, methylation and phosphorylation are epigenetic mechanisms according to this definition. In other words, these posttranslational modifications are important factors in determining when and where a gene will be expressed. To date, several enzymes that catalyze DNA or histone modifications have been identified, such as DNA methyltransferases and histone deacetylases. Inhibitors and activators of enzymes controlling epigenetic modifications are considered useful not only as tools for the elucidation of cellular and biological phenomena, but also as therapeutic agents, since disruption of the balance of epigenetic networks is known to cause some disease states such as cancer. In this review, we present natural products and synthetic molecules that inhibit or activate enzymes catalyzing DNA methylation or histone modifications, and discuss the potential of epigenetic therapy.

Keywords: Epigenetics, DNA methylation, histone modification, inhibitor, activator, epigenetic therapy.

INTRODUCTION

According to the "Central Dogma of Molecular Biology" [1], DNA is the only source of genetic information, which flows linearly from DNA to RNA to protein. However, a number of phenomena cannot be explained by this Central Dogma. For example, the function and morphology of brain cells are completely different from those of liver cells although these cells contain the same set of genes. It is because the kinds of genes expressed in brain cells are different from those expressed in liver cells. Therefore, it is important to understand when and where a gene will be expressed.

The term "epigenetics" was defined as "heritable changes in gene expression that occur without changes in DNA sequence" by Dr Alan Wolffe in 1999 [2]. Recent studies have revealed that DNA methylation and histone modifications such as acetylation and methylation are epigenetic mechanisms according to this definition [3, 4]. Thus far, several enzymes that methylate DNA or posttranslationally modify histones have been identified. For instance, DNA methyltransferases add a methyl group at the 5-position of cytosine residues in DNA and histone acetyltransferases transfer an acetyl group to lysine residues of histones [5, 6]. Inhibitors and activators of these enzymes are considered useful as tools for the understanding of the role of these modifications in genome function and regulation. In addition, since disruption of the balance of epigenetic networks is known to cause some disease states such as cancer [7, 8], these enzyme inhibitors or activators could be therapeutic agents. In this review, we present natural products and synthetic molecules that inhibit or activate enzymes catalyzing DNA methylation or histone modifications, and discuss the potential of epigenetic therapy.

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DNA METHYLATION INHIBITORS

DNA Methylation

DNA methylation at the 5-position of cytosine in CpG dinucleotides is an important mechanism for the epigenetic regulation of gene expression. Hypermethylation occurs in the CpG-rich sequence, so called CpG islands, where core promoters and transcription initiation sites are located. The hypermethylation in the CpG islands leads to the silencing of genes [9-11]. CpG island-specific hypermethylation is a common characteristic of cancer cells. This causes the silencing of tumor suppressor genes such as $p16^{INK4a}$ and *human mutL homologue 1* (*hMLH1*) which are involved in the tumorigenic process including DNA repair, cell cycle regulation and apoptosis [12-15]. Therefore, DNA methylation inhibitors may work against cancer.

DNA Methyltransferases

DNA methylation is catalyzed by DNA methyltransferases (DNMTs) using *S*-adenosyl-L-methionine (SAM) as the methyl donor (Fig. **1**). At present, four mammalian DNMTs, namely, DNMT1 [16], DNMT2 [17], DNMT3A and DNMT3B [18], have been identified. DNMT1 is regarded as a maintenance methyltransferase because it has a preference for hemi-methylated DNA substances that are methylated in one strand and unmethylated in the other. The primary function of DNMT1 might be the copying of the methylation patterns from the parental DNA strand to the newly replicated daughter strand during the DNA replication process [19]. The function of DNMT2 is still unknown. Direct evidence of catalytic activity has not been provided yet, thus it has been suggested that DNMT2 might not function as a DNA methyltransferase [20, 21]. However, according to a more recent report by Liu *et al*, weak DNA methyltransferase activity of DNMT2 was observed in mouse and human cells [22]. In contrast to

DNMT1, DNMT3A and DNMT3B add a methyl group to unmethylated CpG base pairs. Such *de novo* methylation by these enzymes is responsible for the establishment of DNA methylation patterns during embryogenesis [14, 18, 23, 24].

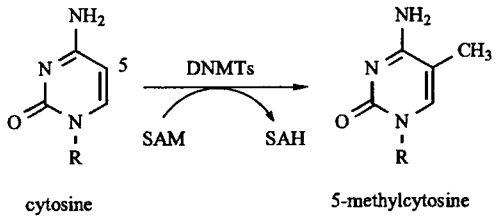

Fig. (1). DNA methylation at the 5-position of cytosine by DNMTs. SAH: *S*-adenosyl-L-homocysteine.

The catalytic mechanism for the methylation of cytosine-5 has been studied extensively and is well understood [25-27]. As depicted in Fig. **2**, a thiol of the cysteine residue in the active site of DNMTs serves as a nucleophile that attacks the 6-position of cytosine to generate a covalent DNA-protein intermediate which possesses nucleophilic properties at the 5-position. This reactive intermediate accepts a methyl group from SAM to form the 5-methyl covalent adduct and *S*-adenosyl-L-homocysteine (SAH). Following the methyl transfer, the proton at the 5-position is abstracted by a basic residue in the active site of the enzyme which is removed from the 6-position by β-elimination to generate the methylated cytosine and free enzyme.

DNA Methylation Inhibitors

To date, several DNA methylation inhibitors have been developed. Cytidine analogues such as 5-azacytidine (5-aza-CR) **1** and 5-aza-2'-deoxycytidine (5-aza-CdR) **2** (Fig. **3**) have long been known to have activity to inhibit DNA methylation [28]. It has also been reported that inhibition of methylation induced by 5-aza-CR **1** and 5-aza-CdR **2** reactivates the expression of genes that have been repressed by DNA methylation [29-31]. 5-Aza-CR **1** and 5-aza-CdR **2** have antiproliferative activity against cancer cells and are used for the clinical treatment of acute myeloid leukemia and myelodysplastic syndrome [32-34]. However, 5-aza-CR **1** and 5-aza-CdR **2** have problems such as instability in aqueous media, toxicity and poor bioavailability [35]. To overcome these problems, the novel cytidine analogues 5-fluoro-2'-deoxycytidine (FCDR) **3** and zebularine **4** were developed [28]. In particular, zebularine **4** is a promising compound [36]. Zebularine **4** is stable and minimally toxic both *in vitro* and *in vivo* [37]. Furthermore, zebularine **4** was orally administered to achieve the reactivation and demethylation of a silenced and hypermethylated *p16* gene in human bladder tumor cells grown in nude mice [37]. In addition, it has also been shown that the continuous treatment of cultured cancer cells with zebularine **4** effectively sustains demethylation of the *p16* 5' region and prevents gene resilencing [38].

Cytidine analogues **1**–**4** are mechanism-based inhibitors of DNMTs. Their inhibitory mechanisms are well investigated. As mentioned above, the catalytic mechanism of DNMTs involves the addition of a thiol to the 6-position of the target cytosine, which activates the carbon at the 5-position allowing a nucleophilic reaction with SAM (Fig. **4a**). When 5-aza-CR **1** or 5-aza-CdR **2**, in which the carbon at the 5-position of the cytosine is replaced with a nitrogen, is incorporated into a DNA sequence, nucleophilic attack is facilitated at the 6-position and a slow methyl transfer takes place, but there is no hydrogen at the 5-position to abstract and the covalent complex persists [39-41] (Fig. **4b**). FCDR **3** inhibits DNMTs in a similar manner. Following the formation of a covalent DNA-enzyme complex at the 6-position and methylation at the 5-position, the analogue remains bound to the active site, because the abstraction of fluorine can not be achieved [26, 42] (Fig. **4c**). The X-ray crystal structure of a bacterial DNMT from *Haemophilus haemolyticus* with an oligodeoxynucleotide duplex containing zebularine **4** made clear the inhibitory mechanism of zebularine **4** [43]. As with 5-aza-CR **1**, 5-aza-CdR **2** and

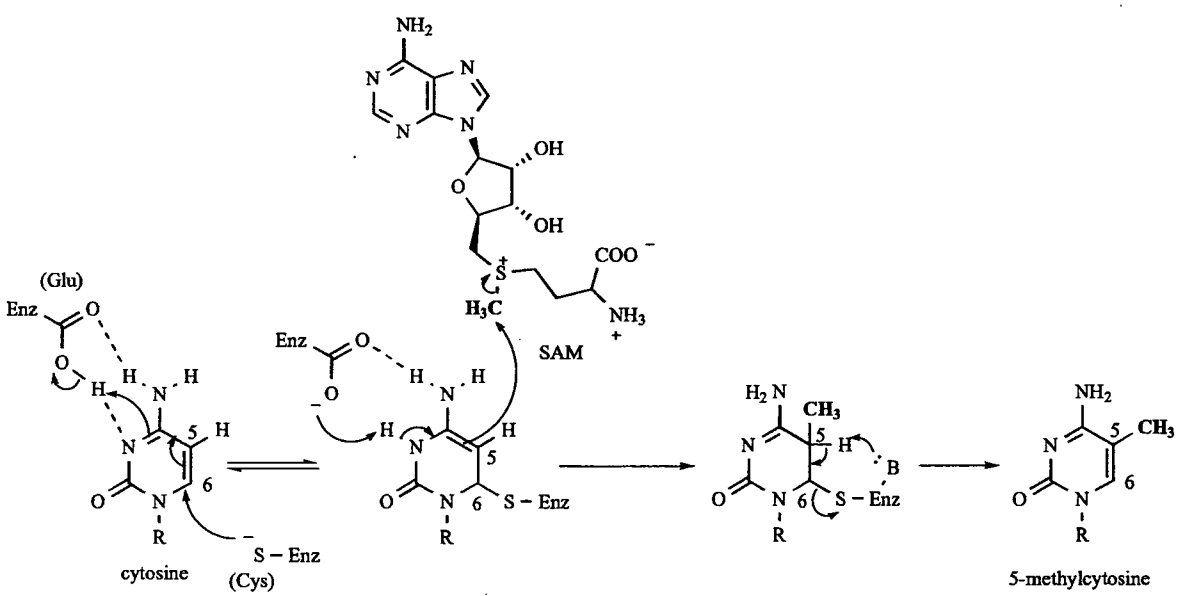

Fig. (2). Proposed catalytic mechanism for the methylation of cytosine by DNMTs [25-27].

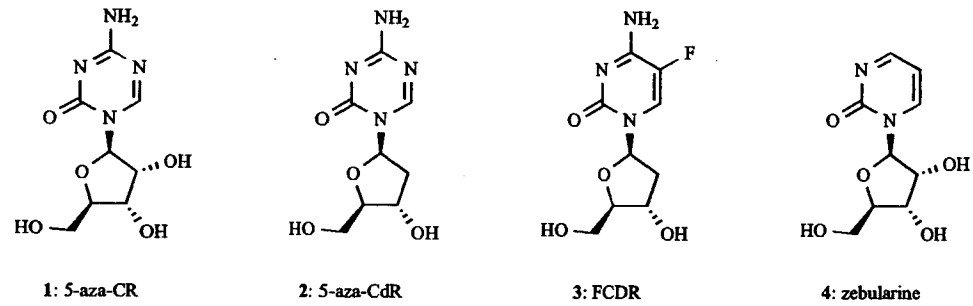

Fig. (3). Cytidine analogues as DNA methylation inhibitors.

FCDR **3**, following nucleophilic attack at the 6-position, proton transfer occurs at the 5-position instead of methyl transfer to form a stable covalent complex (Fig. **4d**).

Non-nucleoside DNA methylation inhibitors have also been reported (Fig. **5**). Procainamide **5**, approved by the FDA for the treatment of cardiac arrhythmias, has been

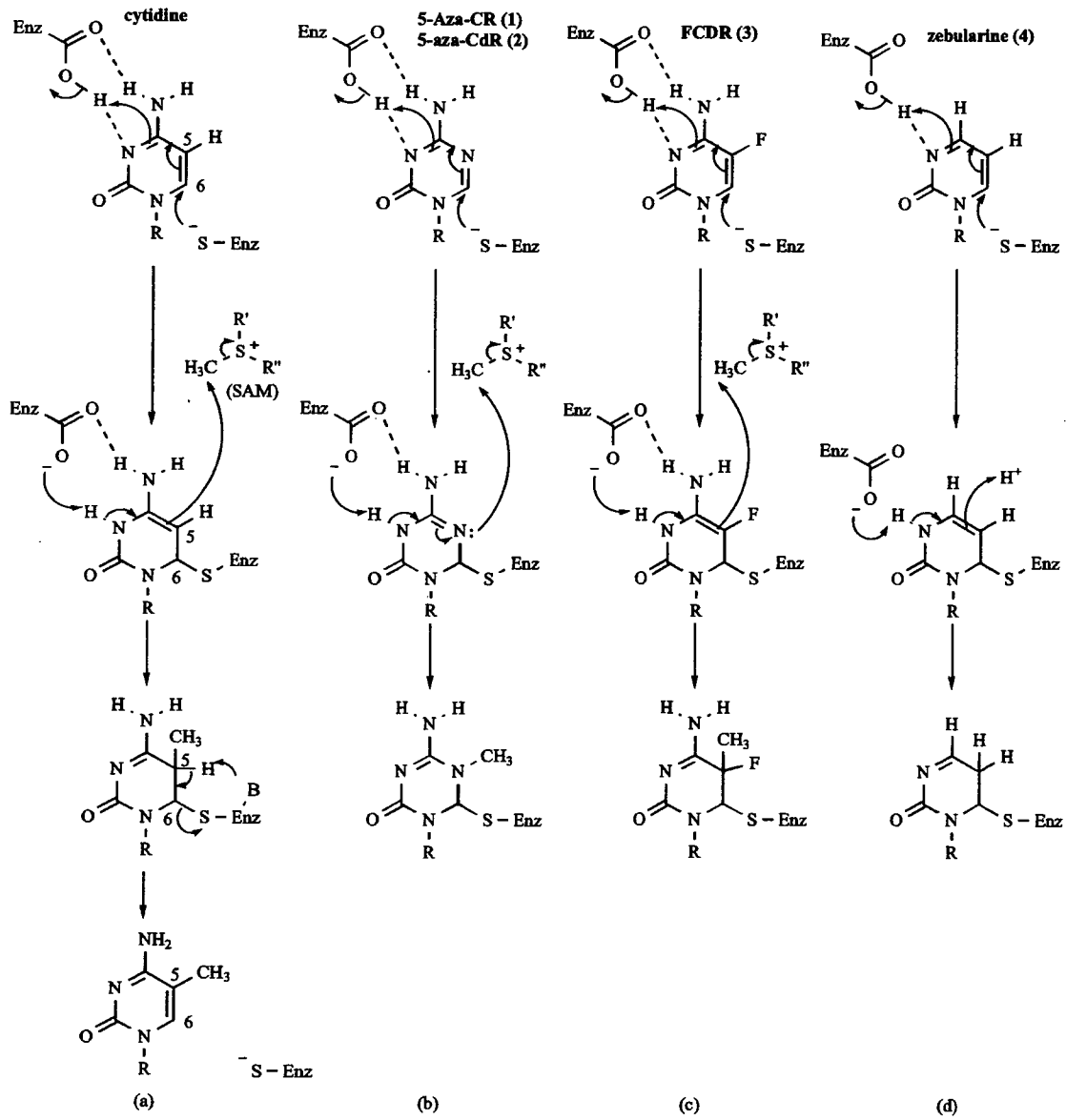

Fig. (4). Catalytic mechanism for the methylation of cytosine by DNMTs (a) and inhibitory mechanism of 5-Aza-CR **1** (b), 5-aza-CdR **2** (b), ECDR **3** (c) and zebularine **4** (d) [26, 39-43].