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A comparative study of the sulfation of bile acids and a bile alcohol by the Zebra danio (*Danio rerio*) and human cytosolic sulfotransferases (SULTs)

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ABSTRACT

The current study was designed to examine the sulfation of bile acids and bile alcohols by the Zebra danio (*Danio rerio*) SULTs in comparison with human SULTs. A systematic analysis using the fifteen Zebra danio SULTs revealed that SULT3 ST2 and SULT3 ST3 were the major bile acid/alcohol-sulfating SULTs. Among the eleven human SULTs, only SULT2A1 was found to be capable of sulfating bile acids and bile alcohols. To further investigate the sulfation of bile acids and bile alcohols by the two Zebra danio SULT3 STs and the human SULT2A1, pH-dependence and kinetics of the sulfation of bile acids/alcohols were analyzed. pH-dependence experiments showed that the mechanisms underlying substrate recognition for the sulfation of lithocholic acid (a bile acid) and 5α-petromyzonol (a bile alcohol) differed between the human SULT2A1 and the Zebra danio SULT3 ST2 and ST3. Kinetic analysis indicated that both the two Zebra danio SULT3 STs preferred petromyzonol as substrate compared to bile acids. In contrast, the human SULT2A1 was more catalytically efficient toward lithocholic acid than petromyzonol. Collectively, the results imply that the Zebra danio and human SULTs have evolved to serve for the sulfation of, respectively, bile alcohols and bile acids, matching the cholanoid profile in these two vertebrate species.

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1. Introduction

Bile acids and bile alcohols, collectively called the cholanoids, are end-metabolites of cholesterol that show striking cross-species diversity in chemical structure, ranging from C_{27}-bile alcohols in phylogenetically basal vertebrates (e.g., jawless fish, cartilaginous fish) to C_{24}-bile acids in most birds, reptiles, and mammals [1,2]. Based on a detailed survey of cholanoids in 1153 phylogenetically diverse vertebrate species [3–5], C_{27}-bile alcohols appear to be the ancestral (plesiomorphic) character state for cholanoids. Bile alcohols are the main cholanoids of all jawless and cartilaginous fish analyzed to date and are also found in some teleost fish (e.g., cypriniform fish such as the Zebra danio, L. *Danio rerio*) and amphibians, as well as a small number of species of birds, reptiles, and mammals. The biosynthesis of C_{27}-bile alcohols has been proposed to be via side chain hydroxylation of C_{27}-bile alcohol intermediates such as 3α,7α,12α-trihydroxy-5α/β-cholestane (cf. Fig. 1) [6]. Bile alcohols are typically secreted from the liver as sulfate conjugates. The stereochemistry of the juncture between the A and B rings of cholanoids is variable and influences the overall shape of the bile salt, with 5β-cholanoids having a "bent" orientation, and 5α('allo')-cholanoids having a flat (planar) structure (cf. Fig. 2). 5α-Bile alcohols are the main cholanoids of jawless fish and may represent the ancestral phenotype for stereochemistry of cholanoids in vertebrates. While bile acids and alcohols have not been detected to date in invertebrates, chemically similar compounds have been detected in some invertebrate species. Example includes a sperm chemoattractant in sea squirts (e.g., *Ciona intestinalis*) and the lifespan-regulating nuclear hormone receptor ligand dafacronic acid in the nematode *Caenorhabditis elegans* [7,8].

In vertebrates, the biosynthetic pathway for bile acids is more complicated and lengthy than that for bile alcohols. The formation of a C_{24} bile acid requires side-chain oxidation by cytochrome P450 (CYP) 27A1 and the subsequent catalytic action of four peroxisomal enzymes that reduce the side chain by 3 carbon atoms (from 27 to

Abbreviations: SULT, cytosolic sulfotransferase; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; SDS-PAGE, sodium dodecyl sulfate-polyacryl gel electrophoresis; TLC, thin-layer chromatography.

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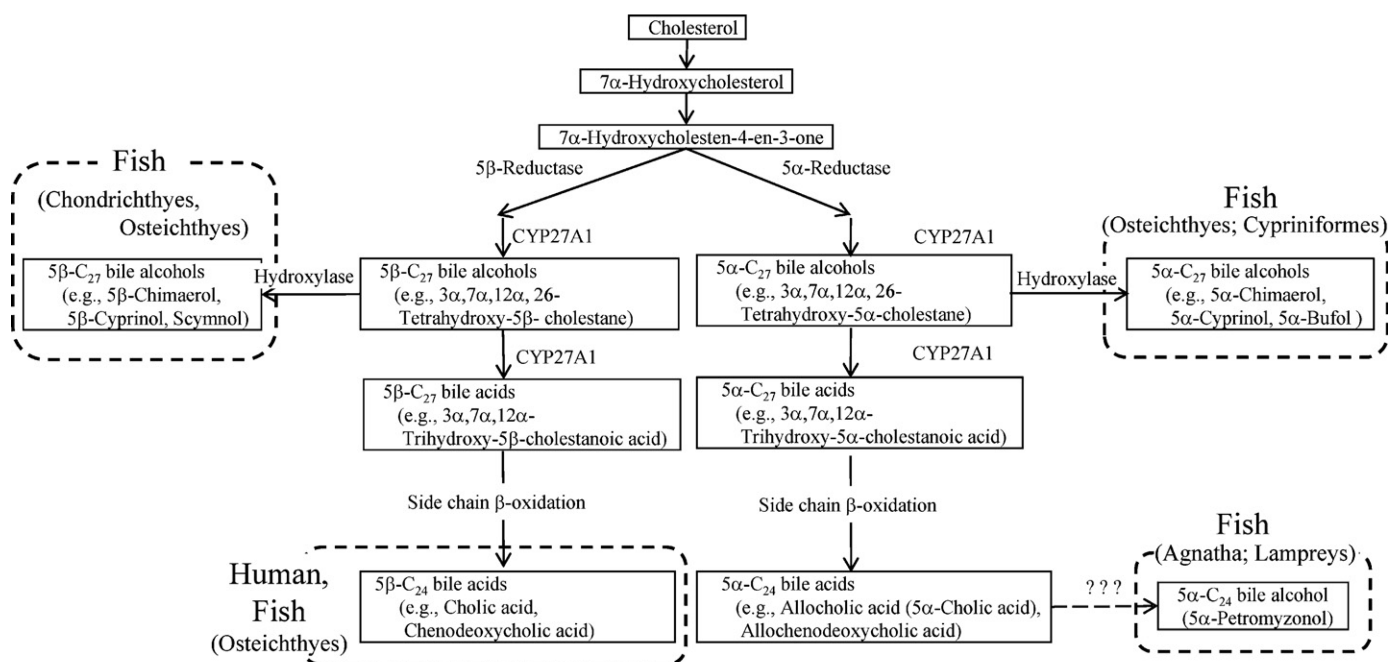

Fig. 1. Proposed biosynthetic pathway for bile acids and bile alcohols in the human and fish. All cholanoids including bile acids and bile alcohols are derived from cholesterol. C_{27}-bile alcohols such as 5α/β-cyprinol are produced from 3α,7α,12α-trihydroxy-5α/β-cholestane which are intermediates for biosynthesis of C_{24}-bile acids. In osteichthyes, the cholanoids of cypriniformes including the Zebra danio are unusual in being 5α-configured C_{27}-bile alcohols [2].

24) and oxidize the shortened side-chain to form a bile acid [9]. Primary bile acids are generally conjugated (*N*-acylamidated) with the amino acids taurine or glycine.

Humans have two main primary bile acids known as chenodeoxycholic acid (CDCA; 3α,7α-dihydroxy-5β-cholan-24-oic acid) and cholic acid (CA; 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) along with other minor bile acids. Primary bile acids are synthesized in the liver and secreted via the bile duct into the duodenum where they facilitate digestion and absorption of lipids [10]. Under pathological conditions, bile acids may cause, among other adverse effects, bile duct infarction, cholestasis, and liver fibrosis [11,12]. From studies in mammals, it is known that anaerobic bacteria in the distal intestine may alter ("damage") primary bile acids, principally by deconjugation (e.g., removal of the

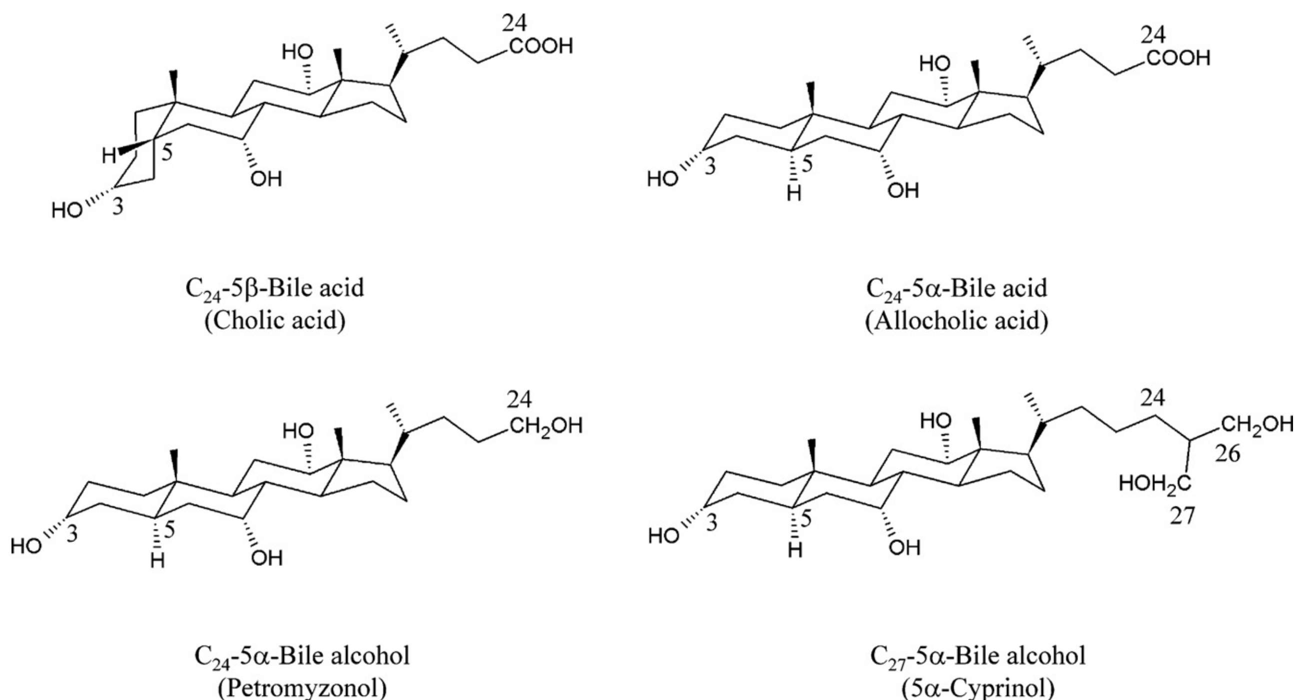

Fig. 2. Stereochemical structures of bile acids and bile alcohols. Cholanoids have an stereochemically variable juncture at C_5 position (A/B ring juncture), which affects the overall shape of the bile salt, with 5β-cholanoids having a "bent" orientation, and 5α-cholanoids having a flat (planar) structure. Another structural variation is related to the presence of hydroxyl or carboxyl group in the side chain, as well as the side chain length. The variation in the side chain generates the bile alcohols with hydroxyl group and the bile acids with carboxyl group, as well as C_{24}/C_{27} cholanoids.

taurine or glycine) and removal of hydroxyl groups. Such bile acids are called "secondary" to distinguish them from the primary bile acids formed in the liver from cholesterol. The most common bacterial modification of bile acids is the removal of the hydroxyl group at C-7 (7-dehydroxylation) to form 7-deoxy bile salts that can be toxic. The most toxic secondary bile acid in mammals is lithocholic acid (LCA; 3α-hydroxy-5β-cholan-24-oic acid), which is formed by deconjugation and 7-dehydroxylation of the common primary bile acid CDCA. LCA has poor water-solubility, having only a single hydrophilic hydroxyl group, and can cause a variety of problems, including damage to the intestinal mucosa and formation of gallstones [13]. It is an interesting question to determine the detoxification mechanisms that guard against the potential adverse effects of bile acids.

In vertebrates, sulfate conjugation is a key process that serves for the biotransformation of endogenous steroid/thyroid hormones and catecholamines, as well as for the detoxification of xenobiotics [14,15]. The responsible enzymes, the cytosolic sulfotransferases (SULTs), are a group of enzymes that catalyze the transfer of a sulfonate group from the active sulfate, 3′-phosphoadenosine 5′-phosphosulfate (PAPS), to a variety of substrate compounds containing hydroxyl or amino groups [16]. All SULTs from vertebrates constitute a gene superfamily within which distinct gene families may be classified [17]. In humans, eleven SULTs that fall into three SULT gene families have been identified and characterized [17]. A variety of bile acid sulfates, including CA sulfate, CDCA sulfate, deoxycholic acid (DCA; 3α,12α-dihydroxy-5β-cholan-24-oic acid) sulfate, and LCA sulfate, have been identified in human urine [18,19]. These sulfate-conjugated bile acids are believed to be derived from the sulfation of the corresponding bile acids. Indeed, previous studies have demonstrated the sulfation of a number of bile acids by the human SULT2A1 [20,21].

Zebra danio is an example of a teleost fish that utilize bile alcohols and not bile acids. Most teleost fish surveyed to date use C_{24} bile acids similar to humans [4]. In Zebra danio and other cypriniform fish (e.g., Asiatic carp), C_{27} bile alcohol sulfates are the dominant primary cholanoids [22], with the main bile alcohol being termed 5α-cyprinol (3α,7α,12α,26,27-pentahydroxy-5α-cholestane) 27-sulfate [23,24]. Whether these bile alcohol sulfates are derived from specific SULT-catalyzed sulfation reactions, however, remains unknown. We have previously cloned and expressed fifteen distinct Zebra danio SULTs, including eight SULT1 STs, three SULT2 STs, three SULT3 STs, and one SULT6 [25–36]. We were interested in finding out whether these zebrafish SULTs are involved in the sulfation of bile acids and/or bile alcohols, and, if so, how they may differ from the human SULT2A1 with regard to substrate specificity for bile acids and bile alcohols that are produced differentially in Zebra danio and humans (cf. Fig. 1). If the SULTs catalyze sulfation of cholanoids, one logical evolutionary hypothesis would be that the substrate specificity of cholanoid-sulfating SULT would match the cholanoid profile of a given species. In the case of Zebra danio, the SULTs would be predicted to show selectivity for 5α-bile alcohols (the Zebra danio cholanoids) compared to 5β-bile acids (that are not produced by zebrafish). In humans, cholanoid-sulfating SULTs would be predicted to efficiently catalyze sulfation of 5β-bile acids and less efficiently catalyze sulfation of bile alcohols.

In this paper, we report a comparative study of the sulfation of cholanoids by the human and Zebra danio SULTs. Eleven known human SULTs and fifteen zebrafish SULTs were tested using a panel of representative bile acids (5α and 5β) and sole commercially available bile alcohol, 5α-petromyzonol (PZ; 3α,7α,12α,24-tetrahydroxy-5α-cholestane), as substrates. The pH-dependence and kinetic parameters of the sulfation of representative bile acids and bile alcohol by the responsible human and Zebra danio SULTs were determined.

2. Materials and methods

2.1. Materials

CA, DCA, LCA, sodium CDCA, ursodeoxycholic acid (UDCA; 3α,7β-dihydroxy-5β-cholan-24-oic acid), hyodeoxycholic acid (HDCA; 3α,6α-dihydroxy-5β-cholan-24-oic acid), glycocholic acid (GCA), sodium glycochenodeoxycholate (GCDCA), sodium taurochenodeoxycholate (TCDCA), taurocholic acid sodium salt hydrate (TCA), adenosine 5′-triphosphate (ATP), sodium dodecyl sulfate (SDS), dithiothreitol (DTT), dimethyl sulfoxide (DMSO), sodium acetate, 2-morpholinoethanesulfonic acid (Mes), 3-(*N*-morpholino)propanesulfonic acid (Mops), 3-[*N*-tris-(hydroxymethyl)methylamino]-propanesulfonic acid (Taps), 2-(cyclohexylamino)ethanesulfonic acid (Ches), 3-(cyclohexylamino)-1-propanesulfonic acid (Caps) were from Sigma Chemical Company. 5α-Cholic acid (also known as allocholic acid; ACA) and PZ were products of Toronto Research Chemical, Inc. Carrier-free sodium [^{35}S]sulfate was from MP Biomedicals. Cellulose thin-layer chromatography (TLC) plates were from EMD Chemicals. All other chemicals were of the highest grade commercially available.

2.2. Preparation of purified human and zebrafish SULTs

Recombinant human P-form (SULT1A1 and SULT1A2) and M-form (SULT1A3) phenol SULTs, thyroid hormone SULT (SULT1B2), two SULT1Cs (SULT1C2 and SULT1C4), estrogen SULT (SULT1E1), dehydroepiandrosterone (DHEA) SULT (SULT2A1), two SULT2B1s (designated a and b), and a neuronal SULT (SULT4A1) were prepared as previously described [37–41]. Fifteen recombinant Zebra danio SULT enzymes, including eight SULT1s (SULT1 ST1 through SULT1 ST8), three SULT2s (SULT2 ST1, ST2, and ST3), three SULT3s (SULT3 ST1, ST2, and ST3), and one SULT6 expressed using pGEX-2Tk or pET23c prokaryotic expression system and purified by affinity chromatography, were prepared as previously described [25–35].

2.3. Enzymatic assay

The sulfating activity of purified recombinant human or Zebra danio SULTs was assayed using PAP[^{35}S] as the sulfonate group donor. The standard assay mixture, in a final volume of 12.5 μl, contained 50 mM Mops buffer at pH 7.0, 14 μM PAP[^{35}S] (15 Ci/mmol), 1 mM DTT, and 50 μM substrate. The substrates tested were prepared in DMSO. Control with DMSO, in place of substrate, was also prepared. The reaction was started by the addition of 1.0 μg enzyme, allowed to proceed for 20 min at 28 °C (for Zebra danio SULTs) or 37 °C (for human SULTs), and terminated by heating at 100 °C for 3 min. The precipitates formed were cleared by centrifugation at 16,000 × *g* for 3 min, and the supernatant was subjected to the analysis of [^{35}S]sulfated product using a previously developed TLC procedure [42], with *n*-butanol/isopropanol/formic acid/water (3:1:1:1; by volume) as the solvent system. Upon completion of the TLC, the plate was air-dried and scanned using a Bioscan AR-2000 Imaging Scanner. The results obtained were calculated and expressed in nanomoles of sulfated product formed/min/mg protein for the specific activities. To examine the pH-dependence of sulfation of LCA and PZ by the human SULT2A1, and Zebra danio SULT3 ST2 and ST3, different buffers (50 mM sodium acetate at 4.5, 5.0, or 5.5; Mes at 5.5, 6.0, or 6.5; Mops at 6.5, 7.0, or 7.5; Hepes at 7.0, 7.5, 8.0; Taps at 7.5, 8.0, or 8.5; Ches at 9.0, 9.5, or 10.0; and Caps at 10.0, 10.5, 11.0, 11.5), instead of 50 mM Mops (pH 7.0), were used in the reaction. For the kinetic studies on the sulfation of CDCA, glycochenodeoxycholic acid (GCDCA), LCA and PZ, varying concentrations of each of these substrate compounds and designated buffers (50 mM Mops at pH 7.0 for the sulfation of all four

Table 1
Specific activities of the responsible human SULT, SULT2A1, toward bile acids and bile alcohol as substrates.[a]

Substrate	Specific activity (nmol/min/mg)
Primary bile acids	
Cholic acid (CA)	ND[b]
Allo cholic acid (ACA)	2.48 ± 0.07
Chenodeoxycholic acid (CDCA)	3.54 ± 0.19
Ursodeoxycholic acid (UDCA)	4.92 ± 0.06
Conjugated bile acids	
Glycocholic acid(GCA)	ND
Glycochenodeoxycholic acid (GCDCA)	2.80 ± 0.05
Taurocholic acid (TCA)	ND
Taurochenodeoxycholic acid (TCDCA)	2.48 ± 0.15
Secondary bile acids	
Deoxycholic acid (DCA)	8.19 ± 0.55
Lithocholic acid (LCA)	23.61 ± 0.69
Hyodeoxycholic acid (HDCA)	0.79 ± 0.04
Bile alcohol	
Petromyzonol (PZ)	3.59 ± 0.15

[a] Specific activity refers to nmol substrate sulfated/min/mg purified enzyme. Data represent mean ± SD derived from three determinations. The concentration of the substrate used in the assay mixture was 50 μM.
[b] ND refers to activity not detected. Specific activity determined was lower than the detection limit (estimated to be ~0.01 nmol/min/mg protein).

substrates by human SULT2A1, 50 mM Taps at pH 8.5 for the sulfation of CDCA, GCDCA and LCA by Zebra danio SULT3 ST2 or SULT3 ST3, and 50 mM Mes at pH 6.0 for the sulfation of PZ by Zebra danio SULT3 ST2 or SULT3 ST3) were used. Kinetic parameters were calculated from the Michaelis–Menten equation using GraphPad Prism5 software and non-linear regression.

2.4. Miscellaneous methods

PAP[^{35}S] was synthesized from ATP and carrier-free [^{35}S]sulfate using the recombinant human bifunctional PAPS synthase and its purity was determined as previously described [43]. The PAP[^{35}S] synthesized was adjusted to the required concentration and a specific activity of 15 Ci/mmol at 1.4 mM by the addition of unlabelled PAPS. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% polyacrylamide gels using the method of Laemmli [44]. Protein determination was based on the method of Bradford with bovine serum albumin as the standard [45].

3. Results

3.1. Sulfation of bile acids and a bile alcohol by the human SULTs

Previous studies had demonstrated the sulfating activities of human SULT2A1 toward a number of bile acids [20,21]. To investigate in greater detail the sulfating activities of SULT2A1 and other known human SULTs (SULT1A1, SULT1A2, SULT1A3, SULT1B2, SULT1C2, SULT1C4, SULT1E1, SULT2B1a, SULT2B1b, and SULT4A1), a systematic analysis was carried out. All eleven known human SULTs, prepared in our previous studies [37–41], were assayed for sulfating activities toward a panel of eleven bile acids (CA, GCA, TCA, ACA, CDCA, GCDCA, TCDCA, DCA, HDCA, UDCA, and LCA) and a bile alcohol (PZ). Results showed that, of the eleven human SULTs, SULT2A1 was the only enzyme that exhibited detectable sulfating activities toward the bile acids/alcohol we tested (Table 1). Among these substrates, SULT2A1 displayed the strongest activity toward LCA (at 23.61 ± 0.69 nmol/min/mg enzyme), low to moderate activities (ranging from 0.79 to 8.19 nmol/min/mg enzyme) toward ACA, CDCA, UDCA, GCDCA, TCDCA, DCA, and HDCA, and no detectable activities toward CA, GCA, and TCA. Interestingly, SULT2A1 also showed a significant sulfating activity (at 3.59 ± 0.15 nmol/min/mg enzyme) toward the bile alcohol PZ.

3.2. Sulfation of bile acids and a bile alcohol by the Zebra danio SULTs

Attempts were made to identify the Zebra danio SULT enzyme(s) that are capable of sulfating bile acids and bile alcohol. We have recently embarked on the molecular cloning of the Zebra danio SULTs and have cloned, expressed, and characterized fifteen distinct Zebra danio SULTs [25–37]. These fifteen SULTs were examined for sulfating activities toward the panel of eleven bile acids and the bile alcohol mentioned above. All SULT1 STs and the SULT6 ST showed no detectable activities toward the bile acids and bile alcohol we tested. The three SULT2 STs showed weak sulfating activities toward the bile alcohol, PZ, but not the bile acids with the exception of the 5α-bile acid ACA (Table 2). The three SULT3 STs, on the other hand, displayed differential activities toward the bile acids and bile alcohol tested (Table 2). Of the three, SULT3 ST1 showed strong activity (at 1.3 ± 0.02 nmol/min/mg enzymes) toward a bile acid (UDCA) and a weak activity toward the bile alcohol PZ. In contrast, SULT3 ST3 showed strong activity (at 3.2 ± 0.03 nmol/min/mg enzymes) toward the bile alcohol, PZ, and weak activities toward the panel of bile acids tested as substrates. SULT3 ST2 exhibited in general weak activities toward the bile acids and bile alcohol.

3.3. pH-Dependence and kinetic properties of the sulfation of bile acids and bile alcohol by human vs. Zebra danio SULTs

To further characterize the sulfation of bile acids and bile alcohol by human vs. Zebra danio SULTs, pH-dependence and the kinetic parameters of the SULT-mediated sulfation reactions were analyzed.

3.4. pH-Dependence

Human SULT2A1 and Zebra danio SULT3 ST2 and ST3 were tested with regard to the pH-dependence of the sulfation of LCA and PZ. As shown in Fig. 3A, human SULT2A1 exhibited a broad pH optimum spanning pH 6.5–9.0 toward both LCA and PZ. With LCA, the enzyme appeared to exhibit two peak activities at pH 6.5 and 9.0, respectively. Zebra danio SULT3 ST2 showed a broad pH optimum spanning 8.5–10.5 toward LCA and, similar to human SULT2A1, with two peaks at pH 8.5 and 10.5, respectively. With PZ as substrate, however, a distinct pH optimum of 6.0 was observed (Fig. 3B). In contrast to human SULT2A1 and Zebra danio SULT3 ST2, Zebra danio SULT3 ST3 displayed a distinct optimum at pH 8.5 or pH 6.0 with LCA or PZ as substrate (Fig. 3C).

3.5. Kinetic properties

The kinetic parameters of the three enzymes in catalyzing the sulfation of CDCA, GCDCA, LCA, and PZ were determined. Based on the pH-dependence profiles (Fig. 3), pH 7.0 (for human SULT2A1) and 8.5 (for Zebra danio SULT3 ST2 and ST3) were chosen for studying the sulfation of bile acids (CDCA, GCDCA, and LCA); and pH 7.0 (for human SULT2A1) and 6.0 (for Zebra danio SULT3 ST2 and ST3) were chosen for studying the sulfation of PZ. Table 3 shows the kinetic constants determined for the sulfation of CDCA, GCDCA, LCA and PZ by the three enzymes. Based on the calculated V_{max}/K_m values, the catalytic efficiency of human SULT2A1 with LCA as substrate appeared to be nearly forty times that with CDCA, GCDCA, or PZ as substrate. For Zebra danio SULT3 ST2 and ST3, comparable K_m values were found with the three bile acids as substrate, while the V_{max} values were much lower than those detected for human SULT2A1. With PZ as substrate, the catalytic efficiency, as reflected by V_{max}/K_m, was nearly 1.7 times higher for Zebra danio SULT3 ST3 than for human SULT2A1.

Table 2
Specific activities of the responsible zebrafish SULTs, SULT2 STs or SULT3 STs, toward bile acids and bile alcohol.[a]

Substrate	Specific activity (nmol/min/mg)					
	SULT2			SULT3		
	ST1	ST2	ST3	ST1	ST2	ST3
Primary bile acids						
Cholic acid (CA)	ND[b]	ND	ND	ND	0.08 ± 0.01	0.10 ± 0.01
Allo cholic acid (ACA)	0.04 ± 0.01	ND	ND	ND	0.12 ± 0.01	0.12 ± 0.01
Chenodeoxycholic acid (CDCA)	ND	ND	ND	ND	0.32 ± 0.01	0.66 ± 0.02
Ursodeoxycholic acid (UDCA)	ND	ND	ND	1.3 ± 0.02	0.12 ± 0.01	0.35 ± 0.02
Conjugated bile acids						
Glycocholic acid (GCA)	ND	ND	ND	ND	0.06 ± 0.01	0.14 ± 0.01
Glycochenodeoxycholic acid (GCDCA)	ND	ND	ND	ND	0.40 ± 0.04	0.95 ± 0.03
Taurocholic acid (TCA)	ND	ND	ND	ND	0.17 ± 0.01	0.29 ± 0.02
Taurochenodeoxycholic acid (TCDCA)	ND	ND	ND	ND	0.44 ± 0.03	0.71 ± 0.03
Secondary bile acids						
Deoxycholic acid (DCA)	ND	ND	ND	ND	0.11 ± 0.01	0.32 ± 0.02
Lithocholic acid (LCA)	ND	ND	ND	0.15 ± 0.01	0.22 ± 0.01	0.86 ± 0.04
Hyodeoxycholic acid (HDCA)	ND	ND	ND	ND	ND	0.27 ± 0.01
Bile alcohol						
Petromyzonol (PZ)	0.23 ± 0.02	0.19 ± 0.02	0.33 ± 0.01	0.04 ± 0.01	0.79 ± 0.05	3.2 ± 0.03

[a] Specific activity refers to nmol substrate sulfated/min/mg purified enzyme. Data represent mean ± SD derived from three determinations. The concentration of the substrate used in the assay mixture was 50 μM.
[b] ND refers to activity not detected. Specific activity determined was lower than the detection limit (estimated to be ~0.01 nmol/min/mg protein).

4. Discussion

It is generally accepted that cholanoids, including bile acids and bile alcohols, have undergone changes during the evolution of the vertebrates. A major transition is believed to be from C_{27}-bile alcohols (putative ancestral state) in phylogenetically basal vertebrates to the C_{24}-bile acids that predominate in higher vertebrates such as birds and mammals [1,2].

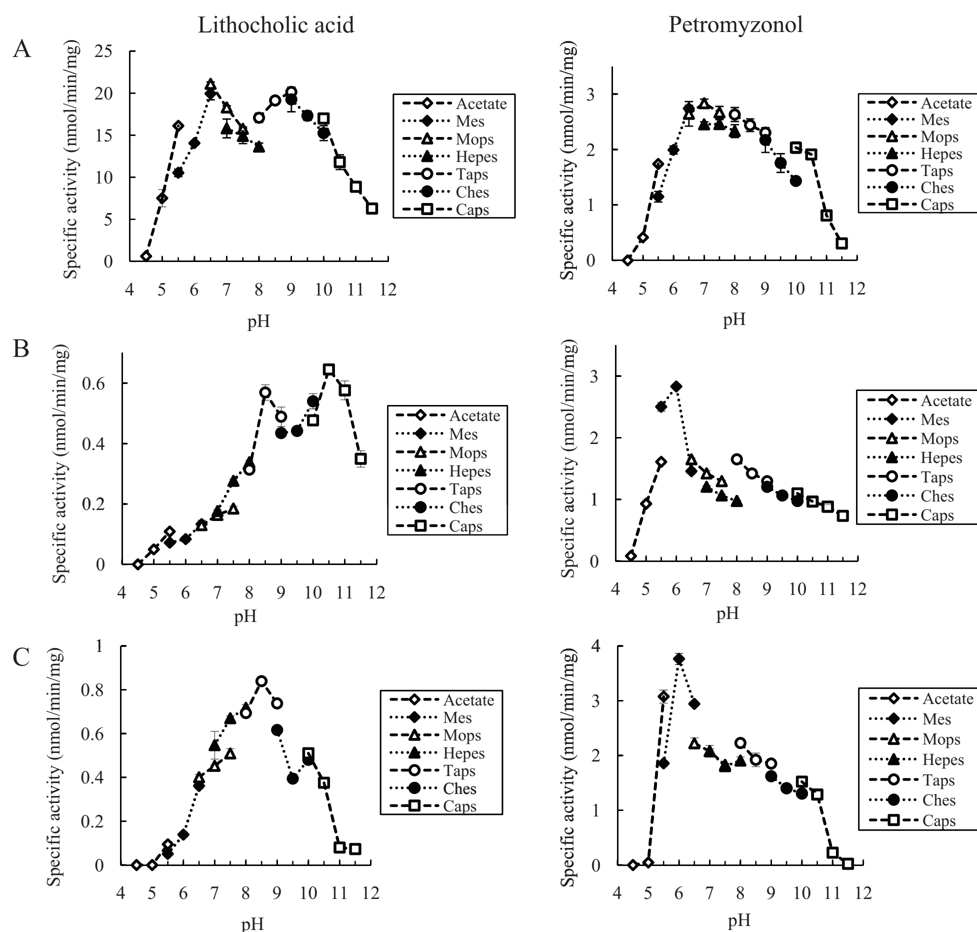

Fig. 3. pH-dependency of the sulfating activity toward lithocholic acid (LCA) and petromyzonol (PZ) by human SULT2A1 (A), Zebra danio SULT3 ST2 (B), and Zebra danio SULT3 ST3 (C). The enzymatic assays with 50 μM each substrate were carried out under standard assay conditions as described in Section 2, using different buffer systems as indicated. The data represent calculated mean ± SD derived from three experiments.

Table 3
Kinetic constant of the sulfation of cholanoids by human SULT2A1, zebrafish SULT3 ST2, and zebrafish SULT3 ST3.[a]

Substrate	hSULT2A1			ZF SULT3 ST2			ZF SULT3 ST3		
	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m
CDCA	42.30 ± 2.97	3.95 ± 0.17	0.09	21.75 ± 2.74	0.75 ± 0.04	0.03	74.04 ± 7.90	1.35 ± 0.05	0.02
GCDCA	36.73 ± 2.47	3.80 ± 0.15	0.10	19.89 ± 1.32	0.97 ± 0.02	0.05	64.31 ± 4.52	1.34 ± 0.05	0.02
LCA	8.36 ± 0.39	35.97 ± 1.16	4.30	6.00 ± 0.55	0.51 ± 0.02	0.09	7.87 ± 0.05	1.35 ± 0.05	0.17
PZ	49.38 ± 4.62	5.49 ± 0.25	0.11	22.36 ± 1.47	2.54 ± 0.06	0.11	31.12 ± 3.33	5.79 ± 0.42	0.19

[a] Results represent means ± SD derived from three determinations.

In mammals, previous studies have demonstrated that sulfation of the C_{24}-bile acids at the 3-OH position by human SULT2A1 decreases their intestinal absorption rate and enhances their fecal excretion [46,47]. Sulfation has also been shown to play a role in decreasing or abolishing cholestatic activity of CA and CDCA [48], and the cytotoxicity and carcinogenicity of LCA [49,50]. Bile alcohol sulfates are known to be reproductive pheromones in the sea lamprey (*Petromyzon marinus*) [51], and the olfactory systems of a number of teleost fish, including Zebra danio, have been shown to be highly sensitive to the detection of cholanoids in the water, possibly as chemoattractants or proximity markers [52,53].

These latter results imply that the sulfate group of chemoattractive cholanoids may confer a crucial characteristic, i.e., a negative charge, for the olfactory receptor binding. An interesting issue is how the sulfated bile alcohols and bile acids are generated in Zebra danio and other teleost fish. The current study was designed to examine the sulfation of bile acids and bile alcohols by the Zebra danio SULTs, in comparison with the human SULTs.

A systematic analysis initially performed revealed that, of the eleven known human SULTs, SULT2A1 was the only enzyme capable of sulfating the bile acids and bile alcohol tested as substrates (Table 1). The enzyme displayed the strongest activity toward LCA and lower, but significant, activities toward UDCA, GCDCA, TCDCA, DCA, and HDCA. No activities, however, were detected with CA, GCA, and TCA as substrates. These results were in line with those previously reported, indicating that, in terms of substrate-binding, SULT2A1 may recognize the steroid nucleus structure of bile acids [20,54]. It is worthwhile mentioning that a recent kinetic study using human SULT2A1 expressed in stably transfected HEK293 cells showed that the sulfation affinity of SULT2A1 was inversely proportional to the number of hydroxyl groups of bile acids. Among the bile acids tested as substrates, LCA, a mono hydroxyl bile acids, and two conjugated LCA (glycol-LCA and tauro-LCA), displayed the highest affinity as substrates [54]. It may be of physiological importance that SULT2A1 exhibited the highest catalytic activity toward LCA, which is the most toxic and hydrophobic bile acid in humans. In contrast, SULT2A1 showed low activity against the more hydrophilic bile acid CA.

It is interesting to note that SULT2A1 was not only capable of sulfating the 5β-bile acids common in human but also a 5α-configured bile acid (ACA) and bile alcohol (PZ). The main structural difference between 5α- and 5β-cholanoids is the overall structure of the steroid nucleus, with an overall planar structure in 5α-cholanoids and a bent structure in 5β-cholanoids (cf. Fig. 2). 5α-Bile acids are known to be produced by human fetus and infant, and may reappear in adults during hepatocarcinogenesis or liver regeneration, but generally constitute only a minor fraction of circulating bile acids [55–57]. Bile alcohols have been shown to be present in patients with rare inborn errors of bile acid synthesis including a disorder called cerebrotendinous xanthomatosis, which is caused by the mutations of CYP27A1, an essential enzyme for bile acid synthesis [58] (cf. Fig. 1). It is therefore an important question whether the human SULT2A1 is also involved in the detoxification and excretion of allo-bile acids and bile alcohols, as the cholanoids may be found in humans, either early in development or pathologically in hepatobiliary disorders.

A systematic study performed revealed that, of the fifteen known Zebra danio SULTs we tested, SULT3 ST2 and SULT3 ST3 were the major enzymes capable of sulfating bile acids (Table 2). It was somewhat surprising to note that both enzymes displayed the sulfating activities toward not only 5α-cholanoids but also 5β-bile acids. Since zebrafish is known to generate exclusively 5α-configured bile alcohols [23,24], these results may indicate that Zebra danio is capable of sulfating its own endogenous bile alcohols as well as exogenous bile alcohols and 5β-bile acids absorbed from the diet. It is noted also that both the two SULT3 STs, as well as the three SULT2 STs, displayed sulfating activity toward the 5α-bile alcohol PZ. The strong activity of SULT3 ST3 toward the 5α-bile alcohol PZ suggests that this may be the main SULT in Zebra danio for the sulfation of bile alcohols. It had been reported that the predominant 5α-bile alcohol in Zebra danio is 5α-cyprinol. The sulfation position of 5α-cyprinol is likely the C_{27} hydroxyl group [59], whereas the sulfation position of PZ is the C_{24} hydroxyl group [51]. Further study is warranted in order to clarify the responsible SULT enzyme(s) for the sulfation of 5α-cyprinol and the hydroxyl group(s) therein that may be sulfated.

To investigate further the characteristics of the human and Zebra danio SULT enzymes (human SULT2A1, Zebra danio SULT3 ST2, and Zebra danio SULT3 ST3) in sulfating cholanoids, the pH-dependences and kinetic parameters were examined (Fig. 3 and Table 3). The results showed distinct pH optima observed with the sulfation of LCA and PZ by human SULT2A1, Zebra danio SULT3 ST2, and Zebra danio SULT3 ST3, indicating possible differences in the mechanism of substrate recognition between the human SULT2A1 and the Zebra danio SULT3 ST2 and SULT3 ST3 (Fig. 3). Significantly, the sulfation of PZ by human SULT2A1 took place with a somewhat broad pH optimum; whereas both Zebra danio SULT3 ST2 and SULT3 ST3 displayed a narrow pH optimum at pH 6. In the subsequent comparative characterization of the cholanoids sulfating activity among the human and Zebra danio responsible SULTs, the kinetic parameters of the human SULT2A1 and Zebra danio SULT3 ST2 and ST3 were determined with CDCA, GCDCA, LCA, and PZ as substrates (Table 3). The V_{max}/K_m values, calculated based on the V_{max} and K_m values determined, indicated a much higher catalytic efficiency of the human SULT2A1 with LCA as substrate than with the other three cholanoids tested. In contrast, the V_{max}/K_m values of the Zebra danio SULT3 ST2 and ST3 with LCA were much lower than that of the human SULT2A1, even though the K_m values of three responsible SULTs with LCA were comparable (8.36 μM for human SULT2A1, 6.00 μM for Zebra danio SULT3 ST2, and 7.87 μM for Zebra danio SULT3 ST3). It is noted that the V_{max}/K_m values of both Zebra danio SULT3 ST2 and ST3 for the sulfation of PZ (0.11 and 0.19, respectively) were equivalent to those for the sulfation of LCA (0.09 and 0.17, respectively), and were also similar to that of human SULT2A1 for the sulfation of PZ (0.11). Previous studies showed that both Zebra danio SULT3 ST2 and ST3 displayed high V_{max}/K_m values with the hydroxysteroid DHEA (3.42 and 11.2, respectively) [33,34]. It appears that the catalytic efficiency of these

two Zebra danio SULT3 STs with bile salts was much lower than that with DHEA and the sulfating activity of the two SULT3 STs toward bile salts may be fortuitous. Whether 5α-cyprinol, the predominant 5α-bile alcohol in Zebra danio [59], may in fact be the true physiological substrate and be sulfated much more efficiently than the bile salts by the two SULT3 STs therefore is an important issue that needs to be resolved.

The evolutionary relationship between vertebrate SULTs and enzymes that catalyze the sulfation in invertebrate species is currently unclear. Putative SULT orthologs have been cloned and characterized from *Drosophila melanogaster*, *Bombyx mori*, and *C. elegans* [60–62], although none has yet been shown to have catalytic activity toward steroidal compounds. However, sulfation of steroidal compounds is common in invertebrate [63], including a bile acid-like compound that functions as a sperm chemoattractant in *Ciona intestinalis* [7], suggesting that the sulfotransferases that esterify bile alcohols or steroids with sulfate in vertebrates may have an evolutionary history that pre-dates the evolution of vertebrates, with considerable diversification of sulfotransferases amino acid sequence and function across hundreds of millions of years of evolution.

In conclusion, the present study demonstrated for the first time the sulfation of cholanoids by the Zebra danio SULTs, particularly SULT3 ST2 and SULT3 ST3. A systematic analysis revealed that these two Zebra danio SULTs were capable of sulfating a diverse group of cholanoids including primary bile acids, secondary bile acids, conjugated bile acids, and bile alcohol, although the activities were in general lower than those of the human SULT2A1. Kinetic analysis clearly showed that the human SULT2A1 to be more catalytically efficient with LCA, compared with the two Zebra danio SULT3 STs. It is at present unknown whether SULTs catalyze the sulfation of 5α-bile acids and 5α/β-bile alcohols *in vivo* in human and Zebra danio. Additional work will also be needed in order to fully elucidate the biochemical and physiological relevance of the sulfation of chemoattractive cholanoids by the SULTs in Zebra danio, as well as other teleost fish.

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