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Identification and characterization of 5α-cyprinol-sulfating cytosolic sulfotransferases (Sults) in the Zebrafish (Danio rerio)

Katsuhisa Kurogi[a,b], **Maki Yoshihama**[a,c], **Austin Horton**[a], **Isaac T. Schiefer**[a], **Matthew D. Krasowski**[d], **Lee R. Hagey**[e], **Frederick E. Williams**[a], **Yoichi Sakakibara**[b], **Naoya Kenmochi**[c], **Masahito Suiko**[b], and **Ming-Cheh Liu**[a,*]

[a]Department of Pharmacology, College of Pharmacy, University of Toledo Health Science Campus, Toledo, OH 43614 USA

[b]Biochemistry and Applied Biosciences, University of Miyazaki, Miyazaki 889-2192 Japan

[c]Frontier Research Center, University of Miyazaki, Miyazaki 889-2192 Japan

[d]Department of Pathology, University of Iowa Hospitals and Clinics, RCP 6233, 200 Hawkins Drive, Iowa City, Iowa 52242 USA

[e]Department of Medicine, University of California at San Diego, La Jolla, San Diego, CA 92093 USA

Abstract

5α-Cyprinol 27-sulfate is the major biliary bile salt present in cypriniform fish including the zebrafish (Danio rerio). The current study was designed to identify the zebrafish cytosolic sulfotransferase (Sult) enzyme(s) capable of sulfating 5α-cyprinol and to characterize the zebrafish 5α-cyprinol-sulfating Sults in comparison with human SULT2A1. Enzymatic assays using zebrafish homogenates showed 5α-cyprinol-sulfating activity. A systematic analysis, using a panel of recombinant zebrafish Sults, revealed two Sult2 subfamily members, Sult2st2 and Sult2st3, as major 5α-cyprinol-sulfating Sults. Both enzymes showed higher activities using 5α-cyprinol as the substrate, compared to their activity with DHEA, a representative substrate for mammalian SULT2 family members, particularly SULT2A1. pH-Dependence and kinetics experiments indicated that the catalytic properties of zebrafish Sult2 family members in mediating the sulfation of 5α-cyprinol were different from those of either zebrafish Sult3st4 or human SULT2A1. Collectively, these results imply that both Sult2st2 and Sult2st3 have evolved to sulfate specifically C_{27}-bile alcohol, 5α-cyprinol, in Cypriniform fish, whereas the enzymatic characteristics of zebrafish Sult3 members, particularly Sult3st4, correlated with those of human SULT2A1.

*Corresponding Author: Ming-Cheh Liu, Ph.D., Department of Pharmacology, College of Pharmacy, University of Toledo Health Science Campus, 3000 Arlington Avenue, Toledo, OH 43614 USA, Tel: (419) 383-1918, Fax: (419) 383-1909, ming.liu@utoledo.edu.

Keywords

Cytosolic sulfotransferase; SULT; sulfation; zebrafish; 5α-cyprinol

1. Introduction

Sulfation is a key conjugation reaction of bile salts in mammals and the reaction is mainly mediated by a hydroxysteroid sulfotransferase, SULT2A1, using 3′-phosphoadenosine 5′-phosphosulfate (PAPS) [1,2] as a sulfate donor. SULT2A1 is a member of the SULT2 family and is known to catalyze the sulfation of a variety of steroid hormones and bile salts [3,4]. Sulfation of bile acids plays an important role in the elimination and detoxification of bile acids, especially the cytotoxic bile acid lithocholic acid (LCA; 3α-hydroxy-5β-cholan-24-oic acid) [2,5,6]. While bile acids are the major end-metabolites in the biosynthesis of cholanoids (bile acids/alcohols) from cholesterol in mammals, birds, reptiles and some teleost fish, bile alcohols have been also known to be major end-metabolites in jawless, cartilaginous, and cypriniform fish, as well as in amphibians [7,8]. A detailed survey of cholanoids in over 1,000 different vertebrate species has revealed that the family contains a great diversity of chemical structures, ranging from C_{27}-bile alcohols in basal vertebrates to C_{24}-bile acids in higher vertebrates [9–11]. The synthesis of cholanoids requires a long series of enzymes, and is a pathway that has become progressively longer in parallel with the evolution of vertebrates. In most teleost fish, especially ray-finned fish, 5β-C_{24}-bile acids, such as cholic acid and chenodeoxycholic acid, are the main cholanoids, as they are in human bile [10]. However, cypriniform fish including the zebrafish commonly used in scientific research exclusively utilize a 5α-C_{27}-bile alcohol, 5α-cyprinol (3α,7α,12α,26,27-pentahydroxy-5α-cholestane) [10, 12]. Most bile salts are found with the A/B ring junction in the 5β-position. The unusual A/B ring junction in cypriniform fish appears to be the result of a special case where there is a double mutation in the bile acid synthesis pathway. The first mutation is a partial loss of function in the mitochondrial sterol 27-hydroxylase (CYP27A1), an enzyme that normally performs three sequential reactions. It first hydroxylates the sterol side-chain at carbon 27, and then progressively oxidizes this carbon, first to an aldehyde and then to the stereospecific 27R carboxylic acid isomer. Only the first step (hydroxylation at C27) has been observed in Cypriniform fish. The second mutation affects the enzyme Δ4-3-oxosteroid 5β-reductase (AKR1D1). This enzyme removes the double bond found in the 3-oxo-Δ4-structure of ring A in the steroid nucleus, and transforms the flat A/B ring junction into the bent 5β-ring configuration [10]. A previous study has shown that 5α-cyprinol (when sulfated at position C-27) retains physiological properties similar to the common taurine conjugated 5β-bile acids cholic acid and chenodeoxycholic acid [13].

In zebrafish, eighteen distinct Sults, including nine Sult1sts, three Sult2sts, five Sult3sts, and one Sult6, have been identified and characterized to date [14,15]. Previous work has shown that zebrafish Sult2 and Sult3 members are capable of catalyzing the sulfation of both 5β-bile acids and a bile alcohol, 5α-petromyzonol. In general, the catalytic activities of these zebrafish Sults are lower than that of human SUT2A1 [16]. Based on this study, we anticipated that members of the zebrafish Sult2 and Sult3 families would also be active for

5α-cyprinol. The zebrafish Sult2 family has been identified as a counterpart of the human SULT2 family (which includes SULT2A1 and SULT2B1) based on their similarity in amino acid sequence [17,18]. The zebrafish Sult3 family has been identified as an ortholog of mammalian arylamine SULT, a SULT3 family member [19–21]. For the 5β-bile acid previously studied, zebrafish Sult3 family members showed higher activities toward steroids and bile acids than did those of the Sult2 family.

The zebrafish is an excellent model for investigations into the physiological function of sulfation and the SULT enzymes [22,23]. In this paper, we investigated the identities and properties of the zebrafish SULT enzymes involved in the 27-sulfation of the major bile alcohol, 5α-cyprinol, and compare the activities of these enzymes with those of human SULT2A1, using structural isomers of 5α-cyprinol, including 5β-cyprinol and 3α,7α,12α-trihydroxy-5α-cholestanoic acid (5α-triOH CA) (Figure 1). The pH-dependence and kinetics of the sulfation of 5α and 5β-cyprinol by the relevante zebrafish and human SULTs were determined to clarify the effect of C5-configuration of cyprinol on their catalytic activity.

2. Materials and Methods

2.1. Materials

5α-Cyprinol and 5β-cyprinol were generated by deconjugating their sulfate salts purified from carp gallbladder as previously described [24]. 3α,7α,12α-trihydroxy-5α-cholestanoic acid (5α-triOH CA) were prepared as previously described [25]. Dehydroepiandrosterone (DHEA), 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), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 3-([1,1-dimethyl-2-hydroxyethyl]amino)-2-hydroxypropanesulfonic acid (Ampso), 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (Capso), 3-(cyclohexylamino)-1-propanesulfonic acid (Caps) were from Sigma Chemical Company. Carrier-free sodium [^{35}S]sulfate was from American Radiolabeled Chemicals. Silica gel thin-layer chromatography (TLC) plates were from Merck Millipore. All other chemicals were of the highest grade commercially available.

2.2. Preparation of zebrafish homogenate

Adult zebrafish (strain AB) were obtained from the Zebrafish International Resource Center at the University of Oregon (Eugene, OR, USA; P40 RR012546 from NIH-NCRR). The fish were kept in tanks containing buffered water (pH 7.2) at 28 °C, and were fed daily live brine shrimp naupli and Tetramin dried flake food (Tetra, Blacksburg, VA, USA). The day-night cycle was maintained at 14:10 h, and spawning and fertilization were stimulated by the onset of first light. Marbles were used to cover the bottom of the spawning tank to protect newly laid eggs and facilitate their retrieval for study. Fertilized zebrafish embryos were collected from the bottom of the tank by siphoning with a disposable pipette. The eggs were placed in Petri dishes and washed thoroughly with E3 water (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$). 50 zebrafish larvae at 7 dpf were chilled in ice-cold water for 20 min and then homogenized by glass tissue grinder in 50 μl homogenate buffer (20 mM

Hepes-NaOH, pH7.5, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, 1 mM PMSF, protease inhibitor cocktail). The homogenate was subjected to a centrifuge at 10,000 × g for 20 min and collected supernatant was stored at −80°C.

2.3. Enzymatic assay with zebrafish homogenate

The sulfating activity of zebrafish homogenate was assayed using [^{35}S]PAPS as the sulfonate group donor. [^{35}S]PAPS was synthesized from ATP and carrier-free [^{35}S]sulfate using the recombinant human bifunctional PAPS synthase as previously described [26]. The standard assay mixture, in a final volume of 20 μl, contained 50 mM Mops buffer at pH 7.0, 14 μM [^{35}S]PAPS (15 Ci/mmol), 1 mM DTT, 50 mM NaF, and 10 μM substrate, 5α-cyprinol. The substrates tested were prepared in DMSO. The reaction was started by the addition of 2.0 μg homogenate, allowed to proceed for 15 min at 28°C and terminated by heating at 100°C for 3 min. The precipitates formed were cleared by centrifugation at 16,000 × g for 5 min, and the supernatant was subjected to the analysis of [^{35}S]sulfated product by TLC with ethyl acetate/acetonitrile/*n*-butanol/isopropanol/formic acid (2:2:2:4:1; by volume) as the solvent system. Afterwards, the TLC plate was dried and the radioactive spots located by autoradiography, excised from the plate, and eluted in 0.5 ml H_2O in a glass vial. 4.5 ml of Ecolume scintillation liquid was then added to each vial and counted for the radioactivity of sulfur-35 using a liquid scintillation counter. The cpm count obtained was calculated into pmol of sulfated product formed based on the specific activity of the [^{35}S]PAPS prepared for the corresponding reactions and expressed in pmol of sulfated product/min/mg protein. Kinetic assay was performed using varying concentrations of 5α-cyprinol under the above-mentioned condition and the parameters were calculated from substrate inhibition kinetics using GraphPad Prism5 software and non-linear regression.

2.4. Enzymatic assay with purified recombinant SULTs

The sulfating activity of purified recombinant SULT enzymes was assayed using [^{35}S]PAPS as sulfonate group donor. Human SULT2A1 and eighteen zebrafish Sult enzymes were expressed using pGEX-2TK or pMALc5x prokaryotic expression and purified as previously described [17–21, 27]. The enzymatic assay was performed under the following conditions. The standard assay mixture, in a final volume of 20 μl, contained 50 mM Mops buffer at pH 7.0, 14 μM [^{35}S]PAPS (15 Ci/mmol), 1 mM DTT and 50 μM substrate. This substrate concentration (50 μM) was selected for screening the cyprinol-sulfating activity of the zebrafish Sults since it has been successfully used for the identification of the bile acid-sulfating zebrafish Sults in our previous study [16]. The reaction was started by the addition of 1.0 μg enzyme, allowed to proceed for 10 min at 37°C for human SULT and at 28°C for zebrafish Sults and terminated by heating at 100°C for 3 min. TLC analysis of sulfated product was carried out under the above mentioned procedure. The pH-dependent assays were performed with different pH buffers (50 mM sodium acetate at 4.5 or 5.5; Mes at 5.5, 6.0, or 6.5; Mops at 6.5 or 7.0; Hepes at 7.0, 7.5, or 8.0; Ampso at 8.0, 8.5 or 9.0; Capso 9.0, 9.5, or 10.0; and Caps at 10.0, 10.5, 11.0). For the kinetic studies on the sulfation of 5α-cyprinol and 5β-cyprinol by the relevant Sults, enzyme assays were performed with varying concentrations of the substrates and 50 mM Mops at pH 7.0. Kinetic parameters were calculated from the Michaelis-Menten and substrate inhibition equation using GraphPad Prism5 software and non-linear regression.

2.5. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) analyses of sulfated 5α-cyprinol

Enzymatic sulfation of 5α-cyprinol was performed with zebrafish Sult2st3 and nonradioactive PAPS as the sulfate donor based on the above-mentioned procedure. The final reaction mixture was applied to a Waters Sep-Pak C18 cartridge. The bound 5α-cyprinol and its sulfated metabolite were eluted using 80% methanol, and the eluate was dried using a SpeedVac concentrator. The eluate reconstituted into 50% methanol/1% formic acid was analyzed by direct-infusion electrospray ion-trap mass spectrometry in the negative ion mode using a Thermo-Finnigan LCQ DECA XP Plus mass spectrometer. Ionization conditions employed were: spray voltage, 5 kV; sheath gas flow rate, 40; capillary temperature, 315 °C, and the infusion flow rate, 5 μL/min. For NMR analysis, the eluate from Waters Sep-Pak C18 cartridge was further loaded into Oasis WAX column. The bound 5α-cyprino sulfate was eluted into 80% methanol/1% NH4OH and dried using a SpeedVac concentrator. The isolated sample was dissolved in CD_3OD, respectively, (Aldrich) and analyzed using a Bruker Advance 600 instrument (600 MHz) (Bruker) with ^{1}H-NMR, ^{1}H-^{1}H COSY, and HSQC modes. The data obtained are summarized in Table S1.

3. Results

3.1. Sulfation of 5α-cyprinol by zebrafish homogenate

Zebrafish homogenate at 7 dpf was used to detect the *in vivo* 5α-cyprinol-sulfating activity, performed with ^{35}S-labeled PAPS. As shown in Figure 2, zebrafish homogenate showed the sulfating activity toward 5α-cyprinol. The saturation curve analysis with 5α-cyprinol as a substrate exhibited the substrate inhibition kinetics for 5α-cyprinol sulfation by zebrafish homogenate (Figure 2 (B)), with K_m and V_{max}/K_m values of 0.60 μM and 172.3 pmol/min/mg/μM, respectively. These results indicated clearly the capacity of zebrafish homogenate in sulfating 5α-cyprinol.

3.2. Sulfation of 5α-cyprinol and its structural analogs by the zebrafish Sults and human SULT2A1

Systematic enzyme assays were carried out in order to identify the zebrafish Sult enzyme(s) responsible for the sulfation of 5α-cyprinol and investigate the structure-activity relationship of the sulfation reaction using 5β-cyprinol, 5α-triOH CA, and DHEA. Human SULT2A1 was also used in the enzymatic assay with those four substrates for comparison. All members of zebrafish Sult2 and Sult3 families showed activity toward 5α-cyprinol (Table 1). Among them, Sult2st2 and Sult2st3 were the most active, compared to the other zebrafish Sults. Although both Sult2st2 and Sult2st3 effectively sulfated both 5α-cyprinol and 5β-cyprinol (two isomers with opposite ring A/B junctions), neither were active in sulfating 5α-triOH CA. The activity of both enzymes using DHEA as substrate was much less than that shown for 5α- or 5β-cyprinol. In contrast to Sult2st2 and Sult2st3, zebrafish Sult3 enzymes showed only weak activities toward the 5α-cyprinol, 5β-cyprinol, and 5α-triOH CA. In contrast to the Sult2 enzymes, zebrafish Sult3 enzymes were quite active with DHEA as a substrate. Human SULT2A1, on the other hand, exhibited moderate activity with 5α-cyprinol and 5α-triOH as substrates, and a much higher activity with DHEA as substrate. Human SULT2A1 was more active with 5α-cyprinol than with 5β-cyprinol, indicating that

SULT2A1 is readily capable of catalyzing the sulfation of 5α-configured bile salts, with structures not normally encountered in human bile.

3.3 pH-Dependence and kinetic properties of the sulfation of 5α-cyprinol by zebrafish Sults and human SULT2A1

To further characterize the sulfation of 5α-cyprinol by the relevant zebrafish Sults and human SULT2A1, pH-dependence and kinetic properties were analyzed. Zebrafish Sult2st3 and Sult3st4 and human SULT2A1 were tested in the pH-dependent assay. As shown in Figure 3, zebrafish Sult2st3 was active over a pH range from 6.0 to 10.5, with a distinct optimal activity detected at pH 8.5. In contrast, zebrafish Sult3st4 exhibited a broad pH optimum spanning 6.0–10.5 with an optimal activity at pH 9.5. Similar to zebrafish Sult3st4, human SULT2A1 exhibited a broad pH optimum spanning 7.5–9.5. Zebrafish Sult2st1, Sult2st2, Sult2st3, and Sult3st4 and human SULT2A1 were analyzed in the kinetics study. To investigate the effect of cyprinol's A/B ring junction on the kinetic properties, 5β-cyprinol was also used as a substrate. Substrate saturation curves of zebrafish Sult2st3 and Sult3st4 and human SULT2A1 are shown in Figure 4. The saturation curve analysis demonstrated that the sulfation of both of 5α-cyprinol and 5β-cyprinol by zebrafish Sult2st2 and Sult2st3 could be fitted to substrate inhibition kinetics, which were further confirmed by a linear Eadie-Hofstee plot (Figure 4). In contrast to these two zebrafish Sults, zebrafish Sult2st1 showed Michaelis–Menten kinetics for the sulfation of both 5α-cyprinol and 5β-cyprinol (data not shown). Zebrafish Sult3st4 and human SULT2A1 showed substrate inhibition kinetics for 5α-cyprinol and Michaelis–Menten kinetics for 5β-cyprinol (Figure 4). Table 2 shows the kinetic constants determined for the sulfation of 5α-cyprinol and 5β-cyprinol by the five enzymes tested. The K_m values of zebrafish Sult2st1, Sult2st2, and Sult2st3 were much lower than those of human SULT2A1 and zebrafish Sult3st4. Furthermore, the catalytic efficiencies of all members of zebrafish Sult2 subfamily with 5α-cyprinol were much higher than those of human SULT2A1 and zebrafish Sult3st4 based on the calculated V_{max}/K_m values. The kinetic parameters of zebrafish Sult2 members with 5α-cyprinol as a substrate appeared to be comparable to those with 5β-cyprinol, except for the K_i values. These results suggest that zebrafish Sult2 members are the primary enzymes responsible for the sulfation of 5α-cyprinol and the human orthologous isoform, SULT2A1, is far less efficient in sulfating 5α-cyprinol.

3.4. Structure of 5α-cyprinol sulfated product

Following the sulfation of 5α-cyprinol with the zebrafish enzymes, the structure of the sulfated product was examined using mass spectrometry and NMR. Mass spectrometry analysis of the reaction product with Sult2st3 showed a prominent ion at m/z 531.33, equivalent to a deprotonated mono-sulfated pentahydroxy C27 bile alcohol ion (Figure 5A). Further MS/MS analysis was performed but showed no fragmentation (data not shown). The ^{1}H-NMR spectrum (600 MHz, CD_3OD) for the sulfated 5α-cyprinol clearly showed the five types of protons, at 3α-OH-bearing carbon (3.79 ppm), 7α-OH-bearing carbon (3.98 ppm), 12α-OH-bearing carbon (3.93 ppm), 26-OH-bearing carbon (3.59 ppm), and 27-*O*-sulfate-bearing carbon (4.02 ppm) (Figure 5B). All assigned protons were supported by H^1-H^1 COSY and H^1-C^{13} HSQC analyses (Figure S1 and Table S1). The spectrum obtained in

this study was similar to that of the NMR data previously reported for 5α–cyprinol 27-*O*-sulfate [28].

4. Discussion

Bile salts, including bile acids and bile alcohols, is known to vary in the structure among different vertebrates. While many species of teleost fish utilize taurine conjugated C24-5β-bile acids, zebrafish uniquely synthesize a C27-5α-bile alcohol, 5α-cyprinol, present in bile as the sulfate, 5α-cyprinol 27-*O*-sulfate [10,12]. The primitive nature of this bile alcohol is an unusual finding, given even the earliest teleost fish contain a bile acid synthetic pathway capable of converting cholesterol into an advanced C24 bile acid like cholic acid [29,30]. Previously, it was shown that some human SULTs, particularly SULT2A1, are capable of sulfating C24-5β-bile acids, while the relevant zebrafish Sults are less efficient at sulfating C24-5β-bile acids [16]. In this study, zebrafish larva homogenate actively sulfated 5α-cyprinol with a low K_m value (Figure 2). This suggests that zebrafish Sults are evolutionally better suited for sulfating bile salt structures with the A/B ring junctions in the flat *trans* position.

The current study also identified the zebrafish Sult enzyme(s) responsible for the sulfation of 5α-cyprinol and compared the catalytic properties of these enzymes with SULT2A1, the enzyme responsible for the sulfation of bile acids in humans. Of the eight 5α-cyprinol-sulfating zebrafish identified, Sult2st2 (K_m 7.73 μM) and Sult2st3 (K_m 9.47 μM) had the highest activity with 5α-cyprinol as substrate (Figure 4 and Table 2). The K_m value (0.60 μM) determined for the homogenate, however, was much lower than either Sult2st2 or Sult2st3 and closer in value to Sult2st1 (1.56 μM). Previous ontogenic expression analysis has shown that both Sult2st2 and Sult2st3 were fully expressed at 1-week post fertilization and that their expression continued into the adult stage [18]. These observations, therefore, suggested that either or both Sult2st2 and Sult2st3 (but not Sult2st1) are the major sulfating enzymes for 5α-cyprinol.

Interestingly, both Sult2st2 and Sult2st3 apparently did not distinguish between 5α-cyprinol and 5β-cyprinol, two bile salts with opposite A/B ring junctions (Table 1 and 2). Zebrafish Sult2 family members are expected to be involved in the sulfation of hydroxysteroids like DHEA and corticosterone [18]. However, the specific activities of zebrafish Sult2 members toward hydroxysteroids are much lower (and the K_m values much larger) than those with cyprinol. Significantly, none of the zebrafish Sult2 members examined were capable of sulfating C24-5β-bile acids, including chenodeoxycholic acid and lithocholic acid [16], two bile salts not encountered in zebrafish bile. The enzymatic properties of Sult2st2 and Sult2st3 match the profile of bile salts in the zebrafish, and suggest that their primary function in the fish is to sulfate 5α-cyprinol. Our observations of the effective DHEA-sulfating activities of zebrafish Sult3 members are consistent with previous studies showing high catalytic activity of zebrafish Sult3 members for 3β-hydroxysteroids but not for C24-5β-bile acids [16, 19–21]. Zebrafish Sult3 members are relatively comparable in their substrate preferences to human SULT2A1, being capable of sulfating C24-5β-bile acids [16]. However, it should be pointed out that a previous study showed that Sult3st1 exhibited specific sulfating activity toward ursodeoxycholic acid [16], while Sult3st1 displayed low

sulfating activities toward other C24-5β-bile acids, cyprinol, and DHEA. Amino acid sequence analysis indicated that zebrafish Sult3st1 shares relatively low amino acid sequence identities (50–55%) with other zebrafish Sult3 family members and can in fact be classified into a separate subfamily [19–21], which may underscore the unique enzymatic property of Sult3st1. Zebrafish Sult3 members are also expected to be similar to mammalian SULT3 family members (based on amino acid sequence homologies), which are capable of catalyzing arylamine compounds [19–21]. All mammalian SULT enzymes have in common three flexible loops which form the substrate-binding pocket and two of the loops are highly variable in amino acid sequence, implying the role of these loops in substrate selectivity and specificity [31]. Crystal structure analysis of human SULT2A1 have shown that these three flexible loops (short loop-1 in SULT2A1) and a loop between 2^{nd}-β-sheet and 1^{st}-α-helix (Pro14-Ser20) are involved in the recognition of a representative substrate, DHEA [32,33]. The three dimensional structures of zebrafish Sults are not yet available, but amino acid sequence alignments show significant differences within the four loops, for zebrafish Sult2 members, human SULT2A1, zebrafish Sult3 members, and mouse SULT3A1 (Figure 6).

A previous study has reported that Sult2st3 showed the strongest activity toward corticosterone among hydroxysteroids tested [18]. It appears that the catalytic pocket of Sult2st3 is wide enough to accept structurally dissimilar sterol side-chains, for example, C21 (in corticosterone) and C27 (as in α- and β-cyprinol), a flexibility that may have led to the subsequent evolutionary selection of this enzyme for the sulfation of 5α-cyprinol.

In our assay, a small amount of 5α-cyprinol was sulfated using human SULT2A1. This enzyme is known to be capable of sulfating both the 3α- and 3β-hydroxyl groups of C24 5β-bile acids [2,16], although the catalytic activity of human SULT2A1 toward 5β-bile acids, such as lithocholic acid, is much higher than what we observed with 5α-cyprinol. In addition, the enzyme has been implicated in the sulfation of 24-hydroxycholesterol at C24 [34], and the steroid drug tibolone (7α-methylnoretynodrel) at C21 [35]. We did not examine the position of the sulfate in α-cyprinol after the reaction with human SULT2A1, but the mixed ability of SULT2A1 to sulfate numerous positions on the steroid skeleton leaves enough uncertainty to believe that the sulfate could be found on either C3 or C27.

5. Conclusion

The present study has revealed Sult2st2 and Sult2st3 as the major 5α-cyprinol-sulfating Sults in zebrafish that sulfate 5α-cyprinol at C27 position. Whereas zebrafish Sult2 family members are active in the sulfation of bile alcohols, Sult3 family members sulfate 3β-hydroxysteroids such as pregnenolone and DHEA. Although the catalytic properties of zebrafish Sult3 family members are not the same as those of mammalian SULT3 enzymes, they are relatively comparable to that of human SULT2A1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

SULT	cytosolic sulfotransferase
PAPS	3′-phosphoadenosine-5′-phosphosulfate

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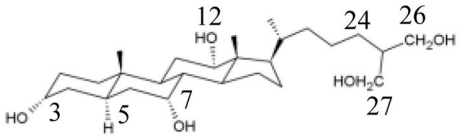
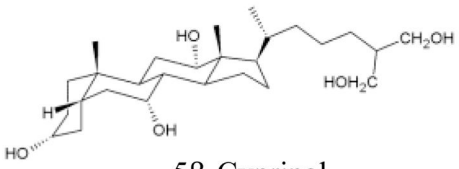
Highlights

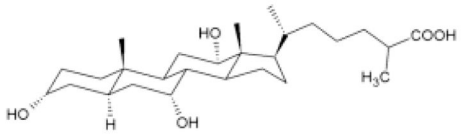
- Zebrafish homogenates are capable of sulfating 5α-cyprinol with a high affinity.
- Zebrafish Sult2 and Sult3 members exhibit strong 5α-cyprinol-sulfating activities.
- Kinetic studies indicate that zebrafish Sult2 members are more catalytically efficient toward cyprinol than zebrafish Sult3 members.
- The catalytic property of human SULT2A1 is comparable to that of zebrafish Sult3 members, but not zebrafish Sult2 members.



5α-Cyprinol
(C_{27}-5α-Bile alcohol)



5β-Cyprinol
(C_{27}-5β-Bile alcohol)



3α,7α,12α-Trihydroxy-5α-cholestanoic acid
(5α-TriOH CA, C_{27}-5α-Bile acid)

Figure 1.

Stereochemical structures of 5α- and 5β-cyprinol and 3α,7α,12α-Trihydroxy-5α-cholestanoic acid (5α-TriOH CA). 5α-cyprinol and 5α-TriOH CA possess a planar structure due to the 5α-configuration at A/B ring junction, in contrast to 5β-cyprinol which has a bent orientation at the C5 position.

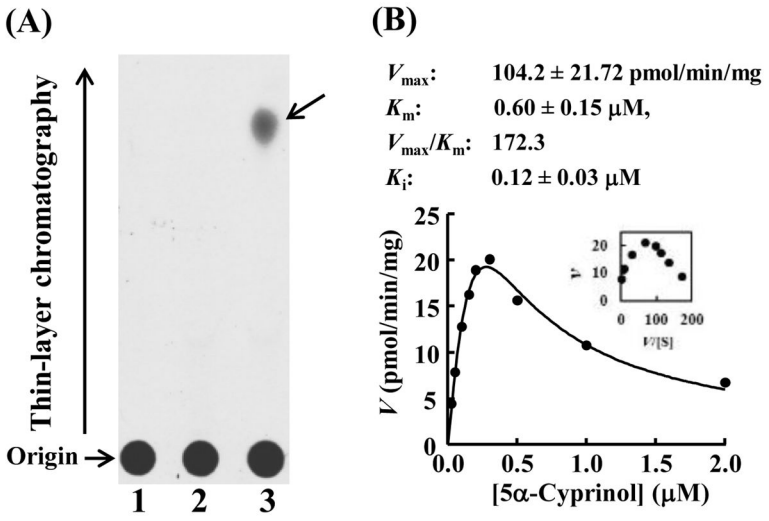


Figure 2.

Zebrafish homogenate exhibits a significant 5α-cyprinol-sulfating activity. (A) Enzymatic sulfation of cyprinol was performed with 7-dpf zebrafish homogenate under assay condition as described in **Materials and Methods**. The figure shows the autoradiograph of the TLC analysis for the reaction product without substrate (lane 1), without homogenate (lane 2), or with substrate and homogenate (lane 3). (B) Kinetic analysis for the sulfation of 5α-Cyprinol by zebrafish homogenate. The fitted curve was generated using substrate inhibition kinetics. An Eadie-Hofstee plot is inserted in the fitting curve. Kinetic parameters compiled in this figure were calculated based on the substrate inhibition kinetics. The data are calculated mean ± SD derived from three experiments.

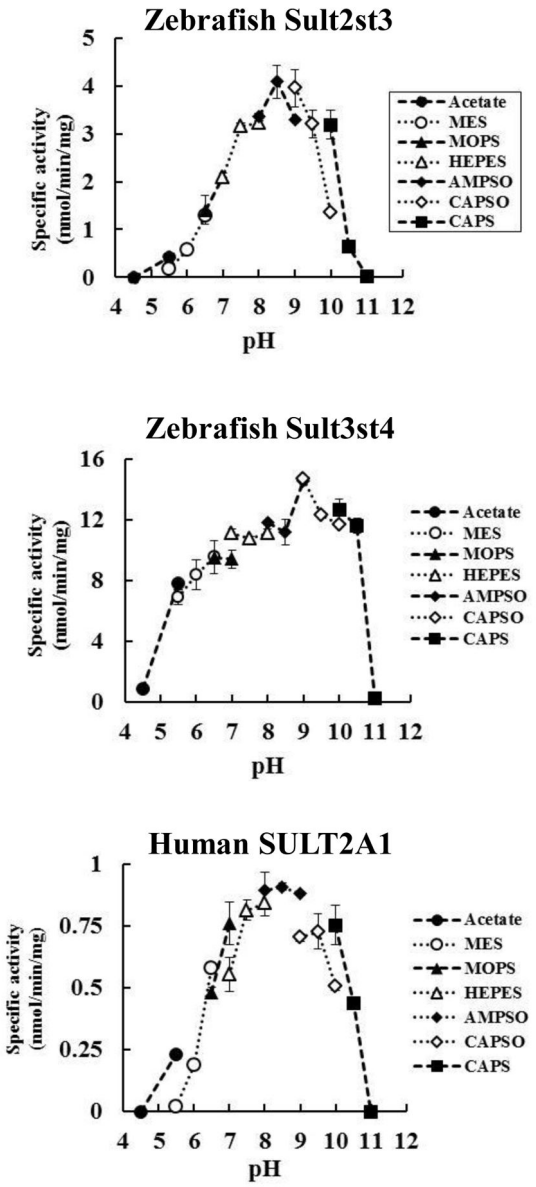


Figure 3.

Zebrafish Sult2st3, zebrafish Sult3st4, and human SULT2A1 exhibit differential pH profiles in the sulfation of 5α-cyprinol. The enzymatic assays were performed using different pH-buffer systems as indicated under standard assay conditions as described in Materials and Methods. The data are calculated mean ± SD derived from three experiments.

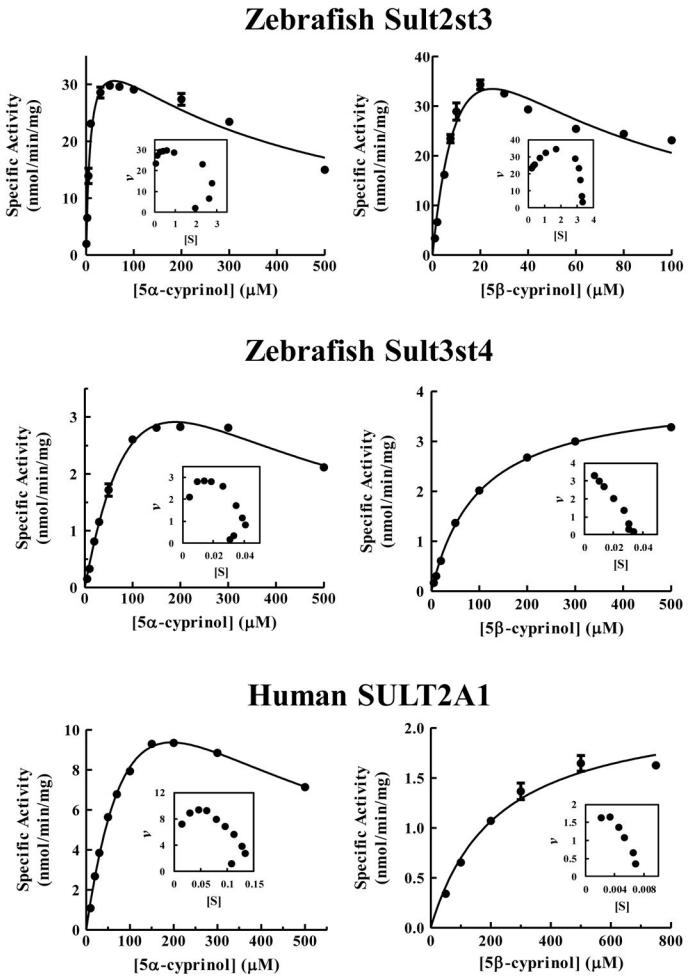


Figure 4.

Zebrafish Sult2st3, but not zebrafish Sult3st4 and human SULT2A1, displays similar kinetics in the sulfation of 5α- and 5β-cyprinol. Figures in the left panels show the kinetic assay with 5α-cyprinol and in the right panels the kinetics with 5β-cyprinol. The fitting curves were generated using Michaelis-Menten or substrate inhibition kinetics. Eadie-Hofstee plots are inserted under each fitting curve. The data are calculated mean ± SD derived from three experiments.

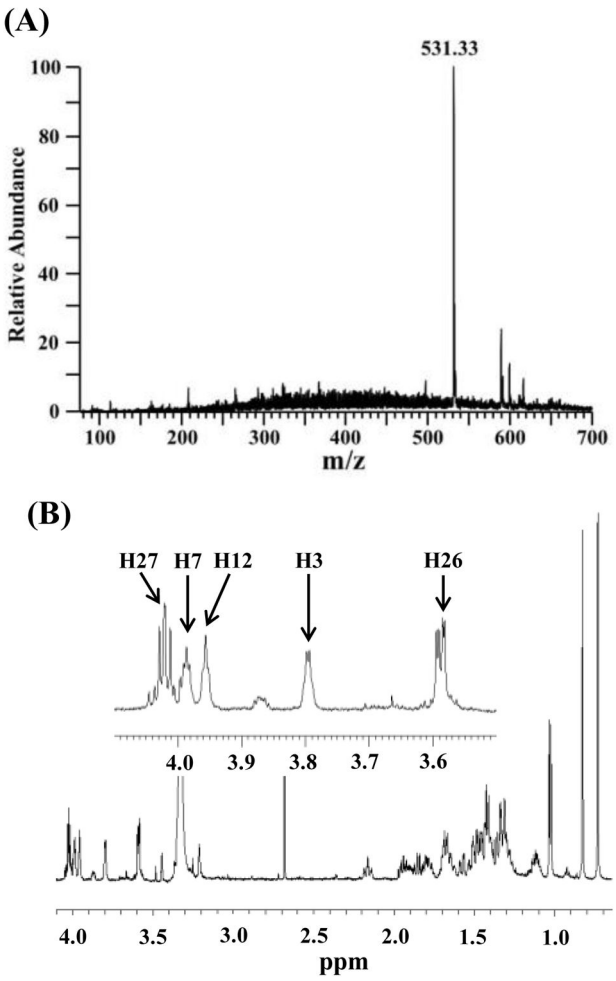


Figure 5.

Sulfation of 5α-cyprinol by zebrafish Sult2st3 occurs specifically at the 27-hydroxyl group. (A) Negative mode ESI-Mass spectrometry profile of sulfated 5α-cyprinol. (B) ^{1}H-NMR spectrum of the sulfated 5α-cyprinol. Assigned protons, H3, H7, H12, H26, H27 are referred to the 5α-cyprinol in Figure 1.

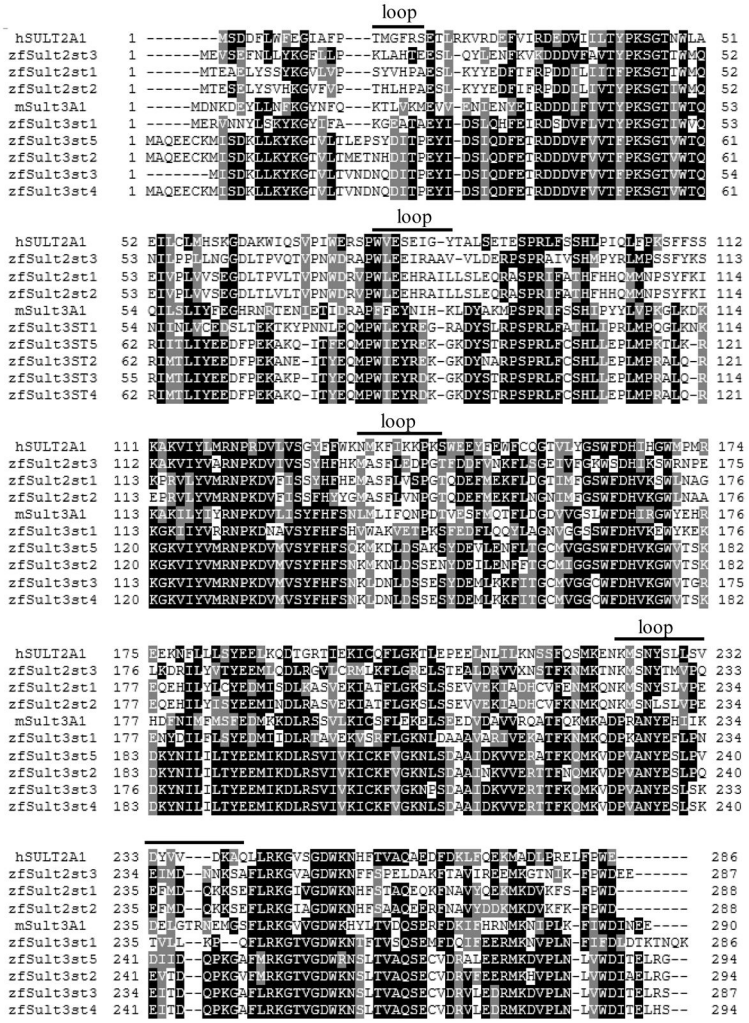


Figure 6.

Substrate recognition sites of zebrafish Sult2 and Sult3 family members differ significantly from those of human SULT2A1 and mouse Sult3A1. Alignment of amino acid sequences of members of SULT2 and SULT3 family were performed with the Clustal Omega tool. Identical and similar residues conserved among the enzymes analyzed are drawn in black and gray. Four loop structures involved in the substrate recognition are shown in the figure.

Table 1

Specific activities of the zebrafish Sults and human SULTs with 5α-cyprinol and its structural analogs as substrates[a]

	Specific activity (nmol/min/mg)			
Enzymes	**5α-cyprinol**	**5β-cyprinol**	**5α-triOH CA**	**DHEA**
zfSult2st1	1.60 ± 0.07	1.52 ± 0.10	N.D.[b]	1.45 ± 0.04
zfSult2st2	10.14 ± 0.68	10.08 ± 0.06	N.D.	0.90 ± 0.01
zfSult2st3	27.86 ± 0.20	26.42 ± 0.76	N.D.	0.11 ± 0.01
zfSult3st1	0.64 ± 0.03	0.04 ± 0.01	N.D.	0.34 ± 0.01
zfSult3st2	0.77 ± 0.06	0.35 ± 0.03	0.14 ± 0.01	16.72 ± 0.12
zfSult3st3	0.44 ± 0.03	0.22 ± 0.01	0.07 ± 0.01	12.53 ± 0.05
zfSult3st4	1.43 ± 0.03	1.15 ± 0.04	0.26 ± 0.01	26.88 ± 0.21
zfSult3st5	0.68 ± 0.07	0.60 ± 0.01	0.09 ± 0.01	5.35 ± 0.01
hSULT2A1	4.71 ± 0.20	0.34 ± 0.01	1.94 ± 0.04	13.94 ± 0.47

[a] Specific activity corresponds to nmol substrate sulfated/min/mg purified enzyme. Results shown represent mean ± standard deviation derived from three separate assays.

[b] ND refers to activity not detected. The detection limit of this assay was estimated to be ~0.01 nmol/min/mg protein.

Table 2

Kinetic constant of the sulfation of cyprinol by and the relevant zebrafish Sults and human SULT 2A1[a]

	V_{max} (nmol/min/mg)	K_m (μM)	V_{max}/Km	K_i (μM)
zfSult2st1				
5α	1.57 ± 0.05	1.56 ± 0.24	1.00	-
5β	1.78 ± 0.05	1.38 ± 0.19	1.29	-
zfSult2st2				
5α	24.73 ± 5.40	7.73 ± 2.67	3.20	27.43 ± 11.16
5β	22.84 ± 1.10	7.15 ± 0.67	3.19	58.15 ± 5.74
zfSult2st3				
5α	40.34 ± 1.31	9.47 ± 0.86	4.26	373.9 ± 38.0
5β	75.57 ± 7.71	15.77 ± 2.48	4.79	39.78 ± 6.70
zfSult3st4				
5α	9.59 ± 0.89	217.0 ± 26.0	0.04	164.9 ± 22.2
5β	3.97 ± 0.05	98.9 ± 2.89	0.04	-
hSULT2A1				
5α	26.73 ± 1.61	181.0 ± 14.6	0.15	210.8 ± 19.9
5β	2.24 ± 0.09	221.3 ± 23.1	0.01	-

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