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Review

Cholesterol-5,6-epoxides: Chemistry, biochemistry, metabolic fate and cancer

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ABSTRACT

In the nineteen sixties it was proposed that cholesterol might be involved in the etiology of cancers and cholesterol oxidation products were suspected of being causative agents. Researchers had focused their attention on cholesterol-5,6-epoxides (5,6-ECs) based on several lines of evidence: 1) 5,6-ECs contained an oxirane group that was supposed to confer alkylating properties such as those observed for aliphatic and aromatic epoxides. 2) cholesterol-5,6-epoxide hydrolase (ChEH) was induced in pre-neoplastic lesions of skin from rats exposed to ultraviolet irradiations and ChEH was proposed to be involved in detoxification processes like other epoxide hydrolases. However, 5,6-ECs failed to induce carcinogenicity in rodents which ruled out a potent carcinogenic potential for 5,6-ECs. Meanwhile, clinical studies revealed an anomalous increase in the concentrations of 5,6-EC in the nipple fluids of patients with pre-neoplastic breast lesions and in the blood of patients with endometriosis cancers, suggesting that 5,6-ECs metabolism could be linked with cancer. Paradoxically, ChEH has been recently shown to be totally inhibited by therapeutic concentrations of tamoxifen (Tam), which is one of the main drugs used in the hormonotherapy and the chemoprevention of breast cancers. These data would suggest that the accumulation of 5,6-ECs could represent a risk factor, but we found that 5,6-ECs were involved in the induction of breast cancer cell differentiation and death induced by Tam suggesting a positive role of 5,6-ECs. These observations meant that the biochemistry and the metabolism of 5,6-ECs needed to be extensively studied. We will review the current knowledge and the future direction of 5,6-ECs chemistry, biochemistry, metabolism, and relationship with cancer.

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1. Introduction

Cholesterol is a tetracyclic lipid of growing biological importance since its discovery by François Poulletier de la Salle in 1758 and was first named cholesterine by Christian Chevreul in 1815 [1]. Since the last century cholesterol is known to be subject to oxidation leading to the formation of mono- or poly-oxygenation products called oxysterols. The main functional groups containing

oxygen atoms are epoxides, ketones, hydroxyl and peracids [2]. Oxysterols are produced through enzymatic reactions reflecting the existence of a metabolic pathway and are also produced by autoxidation through non-enzymatic mechanisms, which are associated with inflammatory-linked pathologies [2]. It is however interesting to note that most oxysterols can be produced through chemical reactions and were discovered by chemists and biochemists before the enzymes responsible for their biosynthesis were characterized [3–5]. Among these oxysterols, 5,6-ECs have stimulated the interest of researchers some years after the photo-oxidation products of cholesterol were suspected to be involved in photo-carcinogenesis [6]. Because of the presence of an oxirane group, it was supposed that 5,6-ECs could be electrophilic and behave like alkylating agents with direct carcinogenic properties. Recent data from literature ruled out that 5,6-ECs could be direct alkylating substances [7] and provides evidence that 5,6-ECs may be involved in physiological processes that result in metabolites with tumor promoter properties as well as to the production of steroidal alkaloids which are anti-oncogenic.

Abbreviations: AEBS, antiestrogen binding site; ChEH, cholesterol epoxide hydrolase; Tam, tamoxifen, trans-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-N,N-dimethylethylamine; D8D7I, 3 β -hydroxysterol- Δ^8 - Δ^7 -isomerase; DHCR7, 3 β -hydroxysterol- Δ^7 -reductase; cholesterol, cholest-5-en-3 β -ol; 5,6-EC, 5,6-epoxy-cholesterol; CT, cholestane-3 β ,5 α ,6 β -triol; 5,6 α -EC, 5,6 α -epoxy-5 α -cholestestan-3 β -ol; 5,6 β -EC, 5,6 β -epoxy-5 β -cholestestan-3 β -ol; 5,6-ECS, 5,6 α -epoxy-5 α -cholestestan-3 β -sulfate; LXR α , Liver-X-Receptor alpha; LXR β , Liver-X-Receptor beta; SULT2B1, Steroid sulfotransferase 2B1.

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2. Nomenclature, structure and reactivity of Cholesterol-5,6-epoxides (5,6-ECs)

2.1. Nomenclature of 5,6-ECs

Cholesterol-5,6-epoxides (5,6-ECs) are products of the oxidation of cholesterol at the $\Delta 5$ double bond between C5 and C6 of the B ring of the steroid backbone (Fig. 1A, 1–4). Two diastereoisomers or epimers exist: cholesterol-5 α ,6 α -epoxide (5,6 α -EC) and cholesterol-5 β ,6 β -epoxide (5,6 β -EC) (Fig. 1B). Their common names given by the lipid maps organization (www.lipidmaps.org) are 5,6 α epoxy-cholesterol (lipid maps ID: LMST01010011) and 5,6 β epoxy-cholesterol (lipid maps ID: LMST01010010). Their systematic names are 5,6 α -epoxy-5 α -cholestan-3 β -ol and 5,6 β -epoxy-5 β -cholestan-3 β -ol. However a lot of publications used different names which renders difficult an exhaustive bibliography on 5,6-ECs. For example, trivial names used (that do not take into account their stereochemistry) are: epoxy-cholesterol, cholesterol oxide and cholesterol epoxide. Other names can be found in the PubChem database at pubchem.ncbi.nlm.nih.gov (5,6 α -EC: CID 1081109; 5,6 β -EC: CID 227037).

2.2. Structure of 5,6-ECs

5,6 α -EC and 5,6 β -EC are different by virtue of the oxygen of the epoxide ring being on the alpha side or on the beta side of the steroid core (Fig. 1B). The common structural representation of 5,6-EC diastereoisomers (Fig. 1B) suggests a dramatic conformational difference between 5,6 α -EC and 5,6 β -EC. However, our recent experimental data led us draw different conclusions. The A, C and D rings are identical in both 5,6-ECs and are in a chair conformation. The B ring of both 5,6-ECs is twisted with a different distortion at the junction of the A and B rings. In 5,6 α -EC the angle C1-C10-C9 is 110.91° and the angle C4-C5-C6 is 120.23° whereas in 5,6 β -EC these angles are 106.68° and 119.21° respectively. The conformational differences are highlighted through the superimposition of the 5,6-

ECs [7]. We measured a displacement of 0.68 Å of the oxygen from the hydroxyl in C3 and a displacement of 0.83 Å of the C19 methyl groups. The van der Waals volume of the steroid backbone of 5,6-ECs was 241.84 Å³ for 5,6 α -EC and 242.16 Å³ for 5,6 β -EC. The difference in the van der Waals volume after superimposition of the 5,6-ECs was 22.19 Å³, showing that 5,6 α -EC and 5,6 β -EC have 87% of their van der Waals volume in common but there was a 13% difference at the level of ring A, of the methyl C19 and of the epoxide ring from 5,6 α -EC. The calculation of the total energy of the 5,6-ECs showed that 5,6 β -EC is in a lower energy state and thus more stable than 5,6 α -EC ($\Delta E = 15.1$ kcal/mol). These data constitute the first experimental evidence of the existence of conformational differences between 5,6 α -EC and 5,6 β -EC. We observed less difference in the conformations of the 5,6-ECs in solution than that observed in the solid state [7], showing that the bending of the rings at the A, B junction was also less in solution than in the solid state.

2.3. Reactivity of 5,6-ECs

The reactivity of the 5,6-ECs has mainly been studied with 5,6 α -EC and is summarized in Fig. 2A, with 5,6 β -EC being marginally reactive or giving a mixture of products under forced conditions.

In acidic aqueous media 5,6-ECs can give cholestane-3 β ,5 α ,6 β -triol (CT) as a single product of hydration [8]. In the presence of 37% hypochloric and 48% hypobromic acid, 5,6 α -EC can produce 6 β -chloro- and 6 β -bromo-cholestan-3 β ,5 α -diol and 6 β -fluoro-cholestan-3 β ,5 α -diol is obtained by reaction of 5,6 α -EC with boron trifluoride etherate [9,10]. In gastric juice, which contains hydrochloric acid, 5,6 α -EC and 5,6 β -EC converted respectively to 6 β -chloro-cholestan-3 β ,5 α -diol and 5 α -chloro-cholestan-3 β ,6 β -diol and then both compounds gave CT [11]. 5,6 α -EC can be hydrogenated in acetic acid with a palladium catalyst to give cholestane-3 β ,5 α -diol, cholestane-3 β -ol and cholestane-3 β ,5,6-triol [12]. In nucleophilic conditions, 5,6 α -EC-3 β -acetate, in the presence of acetonitrile and boron trifluoride etherate gave the

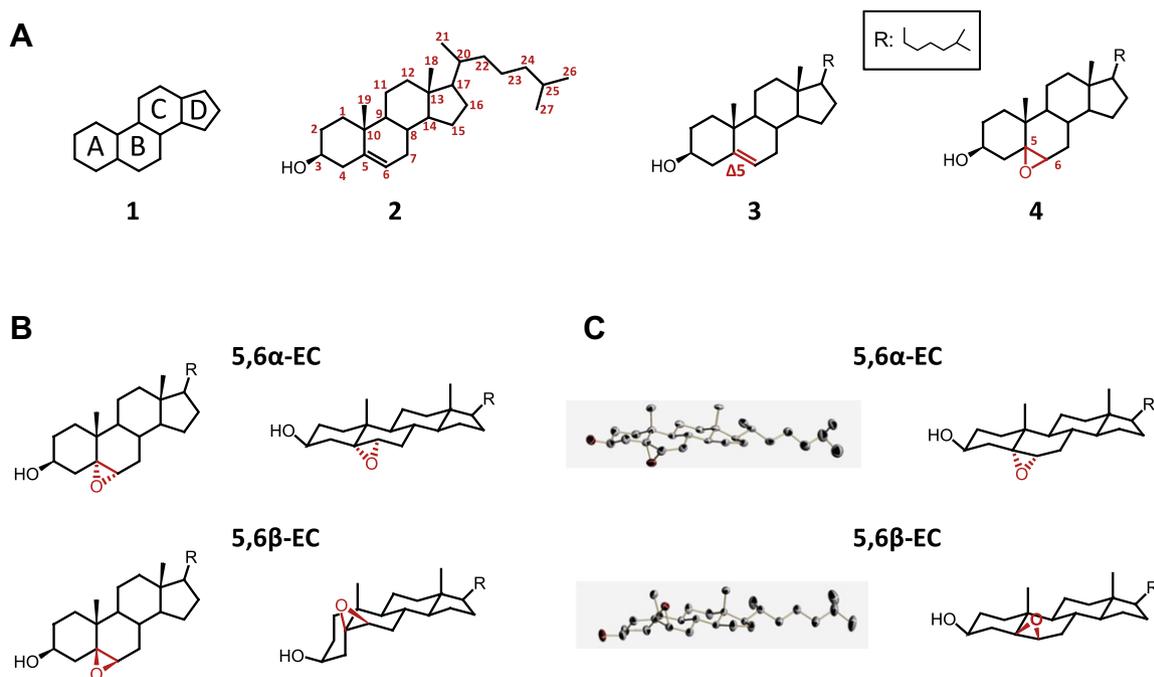


Fig. 1. Structure of 5,6-ECs. A) 1) Ring numbering of the tetracyclic steroid frame; 2) numbering of carbon atoms of the sterol nucleus and side chain; 3) positioning of the C5-C6 double bond; 4) representation of 5,6-ECs; B) Commonly used 2D representation of 5,6 α -EC and 5,6 β -EC epimers; C) Solid-state structure of 5,6 α -EC and 5,6 β -EC epimers as established by X-ray analysis and 2D representation of both diastereoisomers.

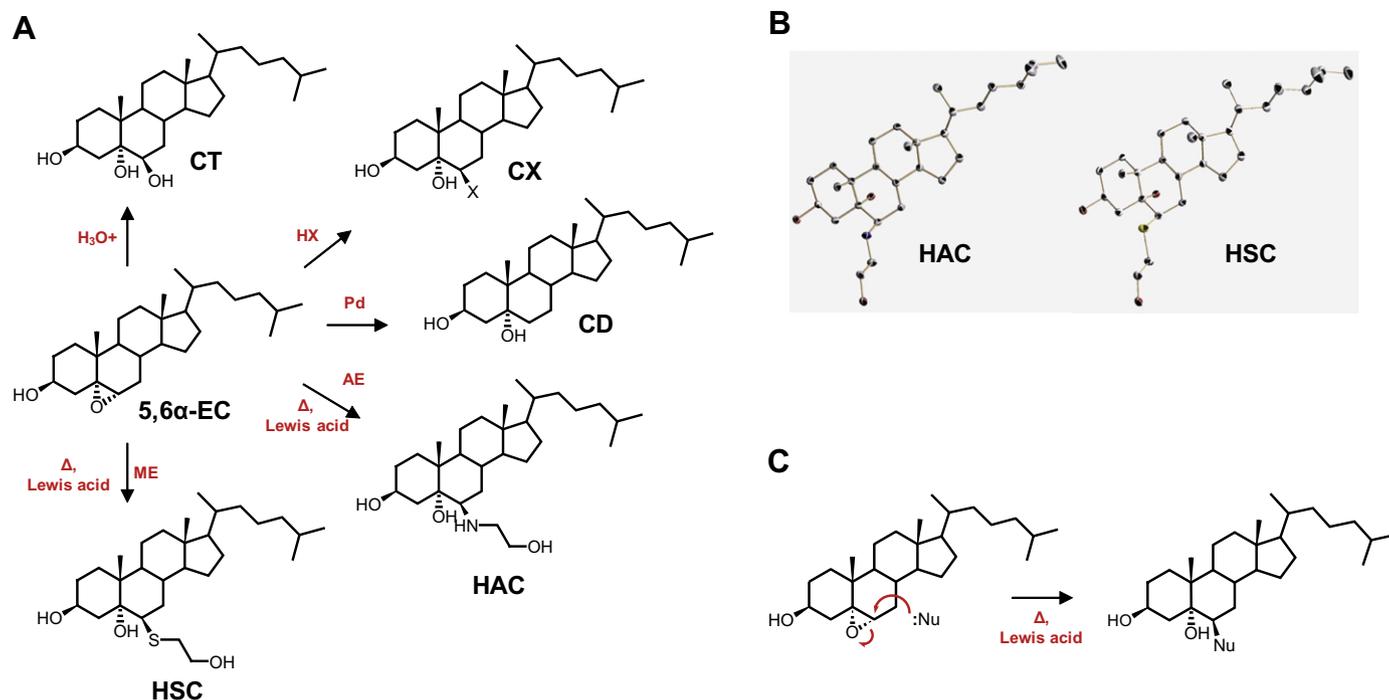


Fig. 2. Chemical reactivity of 5,6-EC. A) 5,6-EC in acidic aqueous medium (H_3O^+) gives cholestane-3 β ,5 α ,6 β -triol (CT); in the presence of concentrated hydrogen halogenide (HX) that can be hydrochloric acid or hydrobromic acid, it gives 6 β -chloro- and 6 β -bromo-cholestan-3 β ,5 α -diol respectively (CX); in the presence of palladium (Pd) it produces cholestane-3 β ,5 α -diol (CD); in the presence of 2-amino-ethanol (AE) or 2-mercapto-ethanol (ME) and Lewis acid and heat gives 5 α -hydroxy-6 β -[2-hydroxyethylamino]cholestan-3 β -ol (HAC) and 5 α -hydroxy-6 β -[2-hydroxyethylsulfanyl]cholestan-3 β -ol (HSC). B) Solid state structure of HAC and HSC as determined by X-ray analysis. Oxygen atoms are in red, carbon atoms are in gray, and sulfur atoms are in yellow. 5,6-EC gives a single product of addition with nucleophiles (Nu) under catalytic conditions.

hydroxyl-amide 6-acetylamino-5 α -cholestane 3 β ,5 α -diol 3-acetate. Under the same conditions or reaction, 5,6-EC gave a complex mixture [13]. We recently reported results from studies comparing the reactivity 5,6-EC diastereoisomers towards nucleophiles, and we found that they were both totally un-reactive at ambient and at physiological temperatures [7]. In each case we found that 5,6-ECs were stable up to several days establishing their absence of spontaneous reactivity even if nucleophiles were present at extremely high concentrations. Tested in the presence of a Lewis acid catalyst such as $LiClO_4$, we found that 5,6 α -EC but not 5,6 β -EC can give a reaction of addition. Structure analysis of the single products obtained by the reaction of 5,6 α -EC with 2-amino-ethanol or 2-mercapto-ethanol showed that this reaction resulted in the production of 5 α ,6 β -substituted derivatives (Fig. 2B) showing that a S_N2 occurred through a trans-diaxial ring opening (Fig. 2C) [7]. Under these conditions, we found that 5,6 α -EC can react with a number of biogenic amines such as histamine, putrescine, spermidine or spermine to give 5 α ,6 β -substituted derivatives [14]. Altogether these data established that 5,6-ECs are not spontaneously reactive towards nucleophilic compounds and that 5,6 α -EC is subject to chemical transformation at the level of the oxirane ring.

3. Control of 5,6-EC production

Cholesterol is known to be sensitive to oxidants, and the delta 5 double bond is the target of reactive oxygen species as well as acidic C7 allylic protons.

Cholesterol epoxidation were first reported by Westphalen in 1915 using perbenzoic acid and cholesterol [8,15]. A number of reactive oxygen species were tested against cholesterol oxidation in aqueous media or in organic solvents [3–5]. The ratio of α/β -epoxides is usually determined by chemists by integration at C6 proton signals in the 1H NMR spectra of crude residues

($\delta = 2.75$ – 2.95 for α -epoxides and $\delta = 3.00$ – 3.15 for β -epoxides) [16], whereas biochemists preferred gas chromatography or liquid chromatography [2,17–19]. We report on Table 1 the impact of oxygen reactive species on the production of 5,6-EC diastereoisomers, CT is the hydration product of 5,6-EC and 6-oxo-cholestan-3 β ,5 α -diol (OCDO) the oxidation product of CT. The triatomic oxygen species ozone (O_3) induced the production of 5,6-ECs and 5,6-secosteroids in water [20,21] and of 5,6-ECs, CT and OCDO in bronchial epithelial cells [22] and in lung [23]. 5,6 β -EC was the predominant 5,6-EC produced. In the latter case it is probable that CT and OCDO are products of the cellular metabolism of 5,6-ECs through ChEH. Reaction with ground state dioxygen (3O_2) is best exemplified by the natural air aging of cholesterol and gave 3 β -hydroxycholest-5-ene-7-hydroperoxydes and products of degradations and produced in low yield 5,6-ECs [21]. Dioxygen reactive species such as the electronically excited (singlet) dioxygen (1O_2) and the superoxide radical anion (O_2^-) were found inefficient to produce 5,6-ECs, CT and OCDO [20]. Dioxygen cation (O_2^+) were reported to give 5,6-EC in gas phase [20]. Peroxide anion

Table 1

Oxidation of cholesterol by various oxygen species. Cholesterol is prone to oxidation by different reactive oxygen species. We report here their impact on 5,6-EC production as well as their product of hydration and oxidation CT and OCDO.

Oxysterol	Defined oxygen species						
	O_3	$^3O_2^c$	$^1O_2^d$	$O_2^-^d$	$O_2^+^d$	O_2^{2-d}	HO^d
5,6-EC	$\beta > \alpha^{a,b}$	$\beta > \alpha$	–	–	+	$\beta > \alpha$	$\alpha > \beta$
CT	$+^b$	+	–	–	–	+	+
OCDO	$+^b$	–	–	–	–	–	–

^a [20,21].

^b [22,23].

^c [21].

^d [20].

(O₂⁻) was reported to give a mixture of 5,6-EC and the hydroxyl radical HO· gave 5,6-ECs along with C-7 oxides of cholesterol [20]. 5,6-ECs were obtained as a mixture of epimers in water and in lipid dispersions [24,25], vesicles [26–28] or lipoproteins [18,29–31].

The formation of 5,6β-EC was favored to the detriment of 5,6α-EC when oxidation occurred in low density lipoprotein (LDL) in the presence of 0.5 mM peroxynitrite (ONOO⁻) whereas increasing the concentration in peroxynitrite increased the production of 5,6α-EC [29].

Table 2 presents the proportion of 5,6-EC diastereoisomers obtained in given chemical and biochemical conditions. Air [5], hydroperoxides in aqueous alkaline dispersion [20], and hydrogen peroxide (H₂O₂) [32,33] gave a mixture of 5,6-ECs, 5,6β-EC being the predominant diastereoisomer over 5,6α-EC (α/β: 1/3–11) [32]. ADP with iron II in the presence of aliphatic hydroperoxides [34], xanthine oxidase [34], soybean lipoxygenase [35,36] and rat liver microsomes [36,37] gave a mixture of 5,6-ECs, in which 5,6β-EC was predominant (α/β: 1/2–4). Stereo-selective production of 5,6β-EC was demonstrated using porphyrin [38], ruthenium (II) bisoxazoline complex [39], permanganate ion [40], and a bulky alpha substituted ketone in the presence of oxone [41]. H₂O₂ in the presence of FeSO₄ [34], meta-chloroperbenzoic acid (mCPBA) [14], hydroxyl radical (HO·) (produced in the presence of N₂O, water or gamma radiations) [34] gave a mixture of 5,6-ECs, in which 5,6α-EC was predominant (α/β: 2–3.5/1). *In vivo* NO₂-induced peroxidation gave a mixture of 5,6-ECs, in which 5,6α-EC was predominant (α/β: 8/1) and bovine adrenal microsomes gave stereoselectively 5,6α-EC [42]. The stereoselective chemical synthesis of 5,6α-EC was achieved using sodium carbonate [43] and organosulfonic peracids [44].

Ionizing radiations of cholesterol in organic solvent [45] and liposomes [26] give a mixture of 5,6-ECs. Long time light illumination of cholesterol led to the production of 5,6-ECs [46,47], although UV illumination of cholesterol in solution does not give 5,6-ECs [48]. UV irradiation of the skin was shown to induce the production of 5,6α-EC [49,50]. Heating an aqueous dispersion of cholesterol produced 5,6-ECs [24,51,52].

The chemical reactivity of 5,6-ECs described above established that 5,6-ECs can be produced through the intermediate production of lipid peroxides in membranes and by peroxidases and a monoxygenase in biological systems. 5,6 ECs were found to be produced in rat liver homogenates as a consequence of lipid peroxidation [53]

Table 2

Chemical and biochemical synthesis of 5,6-ECs. 5,6-EC epimers can be produced chemically or biochemically as a mixture of diastereoisomers or as a pure diastereoisomer.

Conditions	α:β ratio	Reference
Air, aqueous dispersion pH 8	1:3	[5]
Hydroperoxide (aqueous alkaline dispersion)	1:11	[20]
H ₂ O ₂	1:9	[32,33]
Fe(II)-ADP, ROOH or LOOH	1:2–5	[34]
Xanthine oxidase	1:2	[34]
Soybean lipoxygenase	1:4	[35,36]
Liver microsomes	1:4	[36,37]
Porphyrin	<1:9	[38]
Ruthenium (II) bisoxazoline	<1:9	[39]
Permanganate ion	<1:9	[40]
Substitute ketone + oxone	<1:9	[41]
FeSO ₄ , H ₂ O ₂	2:1	[34]
mCPBA	2.5:1	[14]
OH radical (N ₂ O, H ₂ O, γ-radiations)	3.5:1	[34]
NO ₂ -induced <i>in vivo</i> peroxidation	8:1	[34]
Cytochrome P450 (Bovine Adrenal)	>9:1	[42]
Sodium carbonate	>9:1	[43]
Organosulfonic peracids	>9:1	[44]

Abbreviations: ROOH for tert-butyl, tert-amyl, or cumene hydroperoxide; LOOH for fatty acyl hydroperoxide or phospholipid hydroperoxides.

with the 5,6β-EC being the major isomer formed [54]. It has been reported by several authors that lipoxygenase [35,36,54], myeloperoxidase [55] and heme oxygenase 1 [56] were involved in the endogenous production of 5,6-ECs. A stereospecific transformation of cholesterol into 5,6α-EC was reported in the microsomal fraction of the bovine adrenal cortex by Watabe and Sawata [42]. The authors gave evidence that 5,6α-EC formation involved a yet unidentified cytochrome P450 [42].

Production of 5,6-ECs was shown to be blocked by the lipid peroxidation inhibitor EDTA [36] and potassium cyanide [54] in rat liver microsomes. Nitric oxide [57,58], phenolic antioxidants such as vitamin E [59,60], green tea catechin, quercetin [61], resveratrol [62] were reported to inhibit 5,6-EC biosynthesis through the blockage of the lipid peroxidation process. In that case they may inhibit the production of 5,6-EC epimers, but since 5,6β-EC is the major product obtained through peroxidation these inhibitors may control mainly 5,6β-EC production. Several proteins involved in lipid peroxidation inhibition such as paraoxonase 1 (PON1) [63], and lecithin-cholesterol acyltransferase were shown to inhibit the production of 5,6-ECs [64,65]. In the case of PON-1, the recent discovery of a promoter specific aryl hydrocarbon receptor (Ahr) ligand (Z)-2,3-bis-(4-nitrophenyl)-acrylonitrile, which activated PON-1 transcription opens new possibilities in the control of lipid and sterol oxidation [66]. Carbon monoxide and general inhibitors of cytochrome P450 such as proadifen (SKF-525A) can inhibit the stereoselective synthesis of 5,6α-EC [42].

Altogether these data established that the production of 5,6-ECs in a α/β ratio of 1:3 might be of peroxidative origin, assuming that a stereoselective differential metabolism does not occur. In that case, 5,6-ECs formation can be blocked by inhibitors of peroxidation or lipid peroxidation such as vitamin E. On the other hand an α/β ratio different from 1:3 may reflect a different mode of production and/or metabolism of 5,6-ECs.

4. Metabolism of 5,6-ECs

A scheme describing what is known about the metabolism of 5,6-EC epimers is given in Fig. 3.

4.1. Metabolism at the epoxide ring

5,6-EC diastereoisomers can be hydrated by cholesterol-epoxide hydrolase (ChEH) (EC 3.3.2.11) to give cholestane-3β,5α,6β-triol (CT) [67]. ChEH was first described and most studied in the nineteen sixties [36,37,42,53,68–75]. Pharmacogenomic studies enabled us to solve the molecular structure of ChEH and established that it was pharmacologically and molecularly similar to the microsomal anti-estrogen binding site (AEBS), which is a secondary target of the anticancer drug Tamoxifen (Tam). We established that the catalytic subunit of ChEH is 3β-hydroxysteroid-Δ⁸-Δ⁷-isomerase (D8D71 or EBP) and 3β-hydroxysteroid-Δ⁷-reductase (DHCR7) was found to be a regulatory subunit of ChEH. D8D71 and DHCR7 are membranous enzymes that catalyze two different steps in the post-lanosterol cholesterol biosynthesis pathway and are subunits of the AEBS involved in the binding of Tam [76,77]. ChEH can be inhibited by AEBS ligands that include different families of pharmacologically important drugs including 1) selective estrogen receptor modulators (SERM) such as Tam, 2) tricyclic antidepressors such as trifluoroperazine, 3) antiarrhythmic compounds such as amiodarone, and 4) natural substances including polyunsaturated fatty acids such as docosahexaenoic acid and ring B oxysterols including 7-ketocholestanol [67] and 7-dehydrocholesterol-5,6-epoxide [78]. These two oxysterols are autoxidation products of zymosterol, the substrate of D8D71, and of 7-dehydrocholesterol, the substrate of DHCR7, respectively, establishing a regulatory loop between the

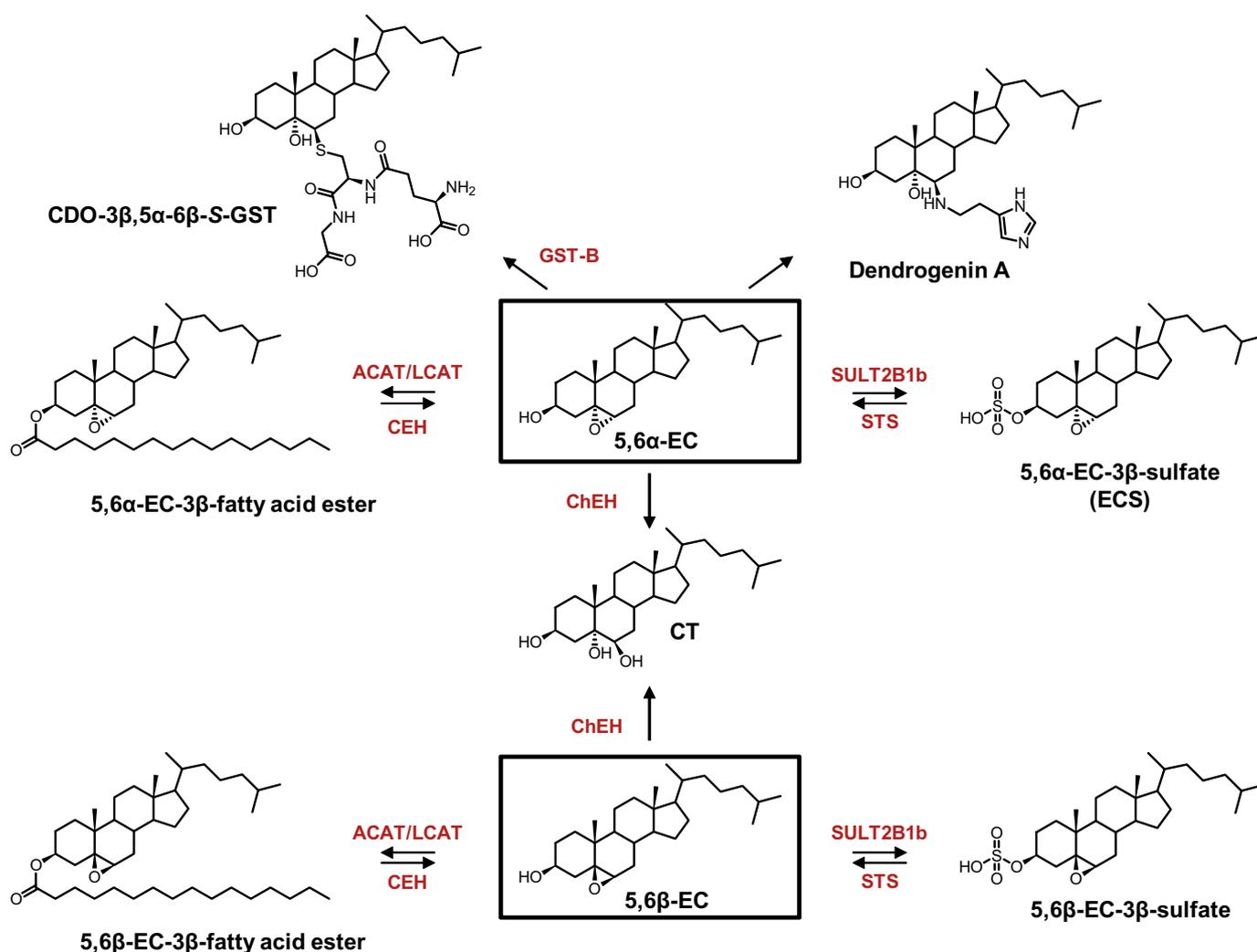


Fig. 3. 5,6 α -EC and 5,6 β -EC diastereoisomers are differently metabolized. 5,6 α -EC and 5,6 β -EC are hydrated by ChEH to give CT; they are sulfated by a sulfotransferase (SULT2B1b) to give 5,6 α - and 5,6 β -EC-3 β -sulfate; they are esterified with fatty acid by Acyl-coA: Cholesterol AcylTransferases (ACAT) and Lecithin-Cholesterol Acyl Transferase (LCAT). Additionally, 5,6 α -EC is specifically subject to an addition reaction with glutathione by rat liver glutathione transferase B (GST-B) to give 3 β -5 α -dihydroxycholestan-6 β -yl-S-glutathione (CDO-3 β ,5 α -6 β -S-GST), and with histamine, by a yet unknown enzyme, to give 5 α -hydroxy-6 β -[2-(1H-imidazol-4-yl)ethylamino]cholestan-3 β -ol (dendrogenin A).

cholesterol biosynthesis function of ChEH subunits and ChEH activity. ChEH has been reported to preferentially hydrate 5,6 β -EC over 5,6 α -EC [68,70] to give CT in rat liver microsomes but these parameters were not determined on intact cells (Fig. 4). CT can be metabolized in rodents to bile acids [79].

5,6 α -EC has been reported to be transformed into a -S-glutathione conjugate to give 3 β -5 α -dihydroxycholestan-6 β -yl-S-glutathione. This reaction of addition is catalyzed by the rat liver cytosolic glutathione B [75,80]. We have shown that 5,6-ECs can give different products of addition through trans-diaxial ring opening of the oxirane ring of 5,6 α -EC and the addition of amines [14,81,82].

4.2. 3 β -Esterification of 5,6-ECs and hydrolysis of 5,6-EC-3 β -esters

5,6-ECs can be metabolized at the level of the 3 β -hydroxyl group on ring A by esterification as observed with cholesterol. Plasma Lecithin-Cholesterol AcylTransferase (LCAT) was reported to esterify both 5,6-EC isomers with fatty acyl coenzyme A to give 5,6-EC-3 β -fatty acid esters in human sera [83]. In cells this reaction is catalyzed by the Acyl-coA: Cholesterol AcylTransferases (ACAT-1 and ACAT-2). In skin cells, macrophages, adrenal cells and CHO cells, ACAT-1 is the major iso-enzyme constituting more than 90%

of the total intact cell activity whereas in the liver and the intestine ACAT-2 seems to be the predominant isoform [84]. ACAT-1 and ACAT-2 do not discriminate between ring B and side chain oxysterols as substrates [85]. Although, it was not reported if these enzymes used 5,6-ECs as substrate, ACAT-1 displayed a selectivity towards 7 α -hydroxycholesterol over 7 β -hydroxycholesterol [85] suggesting that it could discriminate between 5,6-EC diastereoisomers. Sterol released from sterol esters is catalyzed by different enzymes such as hormone-sensitive lipase (HSL) [86], cholesteryl ester hydrolase (CEH/CES1) [87], and neutral cholesteryl ester hydrolase (KIAA1363) [88] but their implication in oxysterol esters hydrolyses has not been reported.

Sterols are known to be substrates of the sulfatase SULT2B1b [89] and this enzyme was shown to produce 3 β -sulfated derivatives of oxysterols and in particular 5,6-EC-3 β -sulfates with a substrate selectivity for 5,6 α -EC over 5,6 β -EC [90]. Since cholesterol-3 β -sulfate is desulfated by the sulfotransferase (STS) [91], it remains to be determined if STS can desulfate 5,6-ECs.

5. Biological properties of 5,6-ECs

5,6-ECs were initially found to inhibit 7- α hydroxylation of cholesterol [36]. The authors established that 5,6 β -EC was the

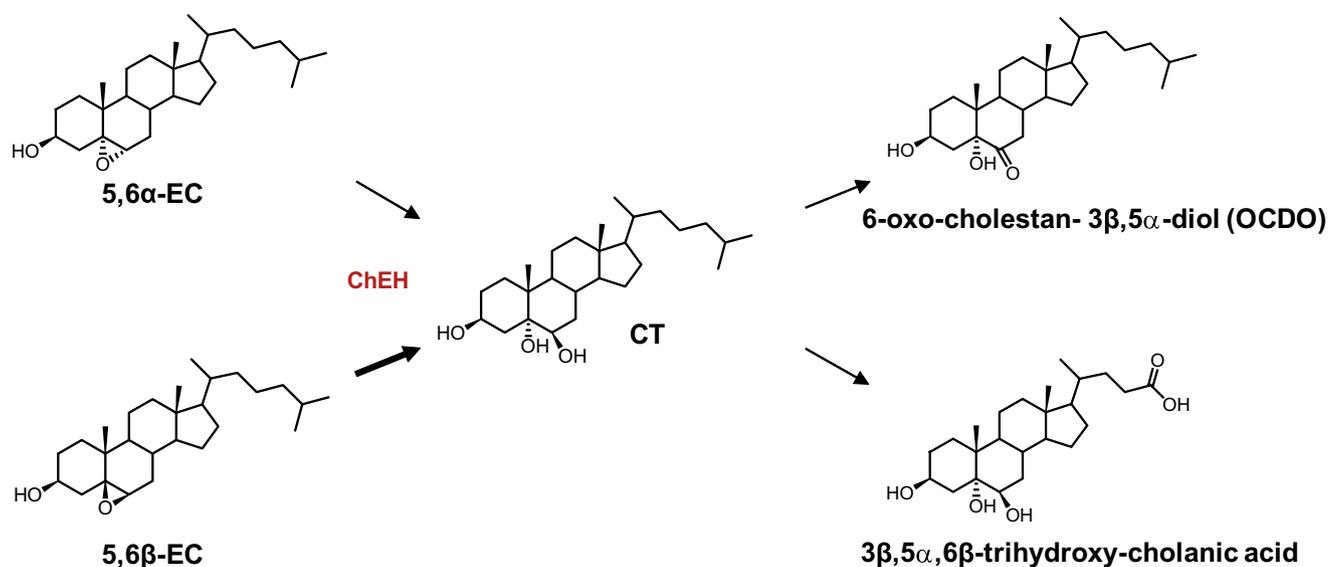


Fig. 4. Metabolism of 5,6-ECs through ChEH. 5,6 α -EC and 5,6 β -EC are hydrated by ChEH to give CT. 5,6 β -EC is the preferred substrate of ChEH. CT can be metabolized into 6-oxo-cholestan-3 β ,5 α -diol (OCDO) by a yet unknown enzyme and into 3 β ,5 α ,6 β -trihydroxy-cholanic acid.

active isomer to inhibit *cyp7 α* which is involved in the biosynthesis of bile acids. 5,6-ECs were shown to inhibit ChEH [68]. Ring B oxysterols were reported to stimulate cholesterol ester formation in cultured fibroblasts [92] and 5,6 α -EC was shown to be the most potent allosteric activator for ACAT-1 whereas 5,6 β -EC was found to be inefficient [93]. 5,6-ECs were found to modulate the biophysical properties of membranes but not as effectively as cholesterol [94]. It was found that 5,6 α -EC has tighter interactions with phospholipids than 5,6 β -EC and would be considered a better raft-stabilizing sterol [94]. 5,6 α -EC was reported to interact with DNA [95,96] but this was contradicted by Ishimaru et al. [97]. In the latter case, they reported that 5,6 α -EC did not interact with DNA but inhibited Topoisomerase II [97]. In these studies 5,6 β -EC was not investigated. 5,6 β -EC, used at 10 μ M, was found to be a modulator of estrogen receptors alpha in a transcriptional activation assay using HELA cells [98]. 5,6 α -24(S), 25-diepoxycholesterol was reported to be a dual ligand of LXR α and LXR β but a selective modulator of LXR α on the LXR-responsive element in the *cyp7 α* gene [99]. More recently, Berrodin et al. reported that 5,6 α -EC was a modulator of LXR α and LXR β with cell and gene context-dependent antagonist, agonist and inverse agonist activities [100]. 5,6 α -EC-3 β -sulfate (ECS), but not 5,6 β -EC-3 β -sulfate is an antagonist of LXR α and LXR β [101,102] and contributed to the induction by Tam of breast cancer cells differentiation and death [103]. The targeting of LXR α and LXR β by 5,6 α -EC and ECS suggest they might be involved in several pathologies including atherosclerosis, diabetes, Alzheimer's diseases, skin disorders, immune diseases, reproductive disorders and cancers [104,105].

6. 5,6-EC metabolites and cancer

The idea that cholesterol oxidation products can be linked with cancer came from observations that UV exposure can produce skin cancers and that cholesterol was sensitive to photo oxidations leading to cholesterol oxides [6]. Several years later, it was reported that subcutaneous injections of an aqueous suspension of 5,6 α -EC induced local sarcomas in Marsh-Buffalo strains of mice [106]. However these results were found to be dependent on the murine strain and on the administration route of the 5,6 α -EC. 5,6-ECs were found to be inactive in the induction of tumorigenicity in rats when injected into mammary glands [107] and in rat colon carcinogenesis

[108]. Homer Black et al. proposed that ultraviolet light induced the formation of 5,6 α -EC in the skin of hairless mice [49,50,109], although proof of the structure of the 5,6 α -EC isomers was lacking. They proposed that 5,6 α -EC was involved in the etiology of ultraviolet-induced skin carcinogenesis and established that ultraviolet light stimulated ChEH activity in the skin of mice [110]. These data suggested that CT, the product of ChEH, or a CT metabolite could contribute to carcinogenesis [111]. 5,6-ECs were found to accumulate in several cancer situations: 5,6-ECs were found in increased concentrations in nipple aspirates of human breast fluids [112] and interestingly 5,6 β -EC was found in higher amounts in breast fluids from woman with breast epithelial hyperplasia which is associated with an increased breast cancer risk [113]. 5,6 β -EC plasma concentration was found to be increased in an endometrial cancer patient [114]. Smith's group studied the mutagenicity of 5,6-ECs using the Ames test and found they were not mutagenic [115]. This observation was confirmed by Cheng et al. who found instead that CT, the product of ChEH, was weakly mutagenic through reactive oxygen species production suggesting that one or more oxidation products of CT was involved in this effect [116]. It was reported that 5,6-ECs were mutagenic at high concentrations on V79 Chinese hamster lung fibroblasts [117–119] and induced the transformation of murine embryo cells [120,121], with 5,6 β -EC having a greater activity than 5,6 α -EC. Mutagenesis on eukaryotic cells was done by counting the number of 8-azaguanine [119] and ouabain or 6-thioguanine resistant colonies [117,118]. No genotoxicity was observed with exposure of two fibroblastic cell lines (CHO and Indian Muntjac) to 5,6-ECs measuring DNA strand breaks frequency and sister chromatid exchanges [122]. The cytotoxicity of 5,6-ECs was studied in fibroblast [119,121], murine embryo cells [121], breast cancer cells [103] and it was found that 5,6 β -EC was more toxic than 5,6 α -EC, and less toxic than CT. From these studies it can be concluded that 5,6-ECs are unlikely to be direct carcinogenic substances. This is consistent with the recent molecular identification of ChEH, which led us establish that ChEH was a target of drugs of broad use [67]. Long-term use of these drugs may induce the accumulation of 5,6-ECs in patients with no appearance of cancers. On the contrary, Tam, which is the main drug used for the treatment of estrogen receptor positive breast cancer was shown to prevent against the appearance of breast cancers and was approved by the Food and Drug Administration for the prevention of breast cancer

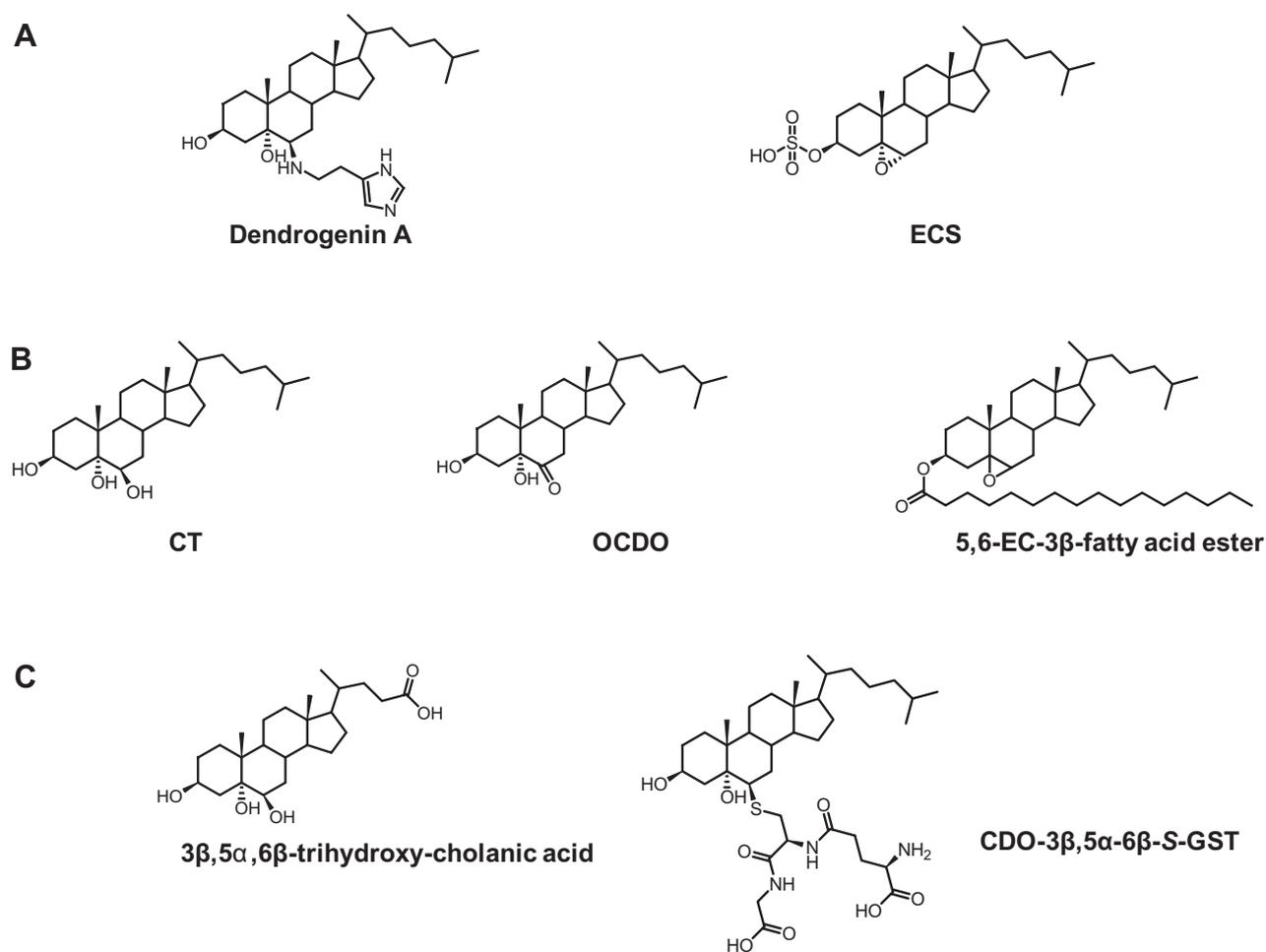


Fig. 5. Metabolism of 5,6-ECs and cancer. A) Metabolites 5,6-EC: dendrogenin A and ECS displayed redifferentiation of tumor cells and activation of tumor cell death. Both possessed anti-oncogenic properties. B) Preferred metabolites of 5,6-EC: CT and OCDO (coming from the ChEH pathway); and 5,6-EC-3β-fatty acid ester are potentially associated with cancer development. C) Metabolites of 5,6-EC and 5,6β-EC with yet unknown relationship with cancer: 3β,5α,6β-trihydroxy-cholanic acid and CDO-3β,5α-6β-S-GST.

[123]. This ruled out that 5,6-ECs are directly involved in carcinogenic processes but may support the view that 5,6-ECs can lead to metabolites involved in tumor promotion. For example cholesteryl esters were shown to possess tumor promoting activities [124,125], and we found that 5,6-EC esters were also tumor promoters (Silvente-Poirot S et al, unpublished results). CT, the product of ChEH, was reported to be involved in carcinogenesis indirectly through the stimulation of oxidation processes [116] and thus inhibition of ChEH and of CT production may contribute to the chemopreventive action of Tam and other ChEH inhibitors. CT has been reported to give OCDO [36] and 3β,5α,6β-trihydroxy-cholanic acid [79] (Fig. 4). However further exploration of the implications and the metabolic fate of CT during oncogenesis is warranted.

Our group found that dendrogenin A, a derivative of 5,6α-EC displayed redifferentiation properties on tumors of various origins [14]. Interestingly, DDA is a metabolite of 5,6α-EC found in normal tissues but absent from tumor cells and is a potent inhibitor of ChEH establishing a deregulation in DDA biosynthesis in cancer situations and underlying the importance of ChEH inhibition in DDA action. DDA is currently being developed by the company Affichem for several clinical anticancer applications.

7. Conclusions

5,6-ECs were discovered almost a hundred years ago and were suspected to be involved in the etiology of cancers. 5,6-EC

diastereoisomers are different compounds with different chemical and biological properties. Among epoxide bearing substances, 5,6-ECs are different from others since they are exceptionally stable with no spontaneous reactivity towards nucleophiles ruling out that 5,6-ECs are spontaneous alkylating substances and making it unlikely that they are directly carcinogenic or tumor initiators. We found evidence that 5,6-EC diastereoisomers have different biological properties, different susceptibility to metabolic fates, and different selectivity to modulate ligand-dependent transcription factors. This data delineate the existence of a possible metabolic fork controlling different pathways confirming the importance of the delta-5 double bond of cholesterol. We found that dendrogenin A and 5,6α-EC-3β-sulfate have antitumor activity establishing the potential anti-oncogenic effect of these 5,6-EC metabolites (Fig. 5A). We found evidence that fatty acid esters of 5,6-ECs and primary and secondary products of ChEH activity had tumor promoting and carcinogenic activity (Fig. 5B). Further research is required to determine if 3β,5α,6β-trihydroxy-cholanic acid and CDO-3β,5α-6β-S-GST have any oncogenic or anti-oncogenic properties.

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