Novel Role of Soluble Epoxide Hydrolase in Regulating Cholesterol in Mammalian Cells

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Abstract: Soluble epoxide hydrolase (sEH) is becoming an attractive therapeutic target in cardiovascular disease. Recently, known human sEH polymorphisms were associated with elevated plasma cholesterol and atherosclerosis. In this study we evaluated the potential role of sEH in regulating cholesterol metabolism through modulating the levels of fatty acid epoxide substrates and/or their corresponding diol products known to activate peroxisome proliferator activated receptors (PPARs). We measured changes in cholesterol levels induced by expressing sEH proteins in mammalian cell lines and in response to treatment with various sEH-related compounds. Our results indicate that sEH has a cholesterol lowering effect that is mediated at least in part through its C-terminal hydrolase activity. In addition, several fatty acid epoxides and their corresponding diols showed cholesterol lowering effects in the current study. In conclusion, this study provides evidence that fatty acid epoxides and diols are endogenous cholesterol lowering molecules and that sEH may be involved in cholesterol regulation by modulating their levels.

Keywords: Soluble epoxide hydrolase, PPAR, cholesterol, fatty acid epoxides.

INTRODUCTION

Cholesterol plays an indispensable role in various aspects of mammalian cell biology. In addition to being a vital component of eukaryotic cell membranes and the myelin sheath in the nervous system, cholesterol is also the precursor of bile acids, vitamin D as well as several steroid hormones such as sex hormones, mineralocorticoids and glucocorticoids. However, high levels of cholesterol have been found to be toxic to cells and have been associated with human diseases such as cardiovascular disease and atherosclerosis; hence its level is precisely regulated. Cholesterol levels in the cell are regulated partly by de novo synthesis as well as by mechanisms which control efflux and influx across the cell membrane. Several regulatory proteins and transcription factors have been identified to contribute to cholesterol regulation, such as sterol regulatory element binding protein (SREBP) reviewed in [1] and peroxisome proliferator-activated receptors (PPAR) reviewed in [2].

PPAR alpha and gamma are members of the nuclear receptor family of ligand-activated transcription factors. Several synthetic ligands of PPAR alpha and gamma such as fibric acid derivatives and glitazone drugs respectively, have been found to lower cellular cholesterol levels [3,4] and alter plasma lipid profile both in rodents [5] and in humans [6-8]. A diversity of naturally occurring fatty acids and fatty acid derivatives, has been identified to bind PPARs with varying degrees of selectivity to the different PPAR subtypes [9-11]. Due to the diversity of fatty acids that are capable of binding PPARs at low micromolar concentrations, it has been suggested that their physiological functions are perhaps determined through the interaction with several molecules rather than a single high-affinity ligand [2].

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Soluble epoxide hydrolase (sEH) is a member of the epoxide hydrolase family (EH: EC3.3.2.3) with broad distribution in human tissues [12]. sEH has been shown to possess a C-terminal epoxide hydrolase (C-term) domain [13,14] and an N-terminal (N-term) phosphatase domain [15,16]. Endogenous substrates of the C-term domain include fatty acid epoxides such as arachidonic acid epoxides and linoleic acid epoxides (EETs, EpOMEs, respectively) [17,18]. The vasoactive and anti-inflammatory properties of EETs and their corresponding diols (DHETs) implicate sEH as a potential attractive therapeutic target in the management of cardiovascular disease such as hypertension and atherosclerosis (reviewed in [19,20]). In addition to the association of known human variants of sEH with increased risk of coronary heart disease and atherosclerosis [21-23], the R287Q variant has been associated with increased plasma cholesterol and triglycerides levels in familial hypercholesterolemia patients [24]. Several sEH C-term inhibitors, substrates and products (such as AUDA, EETs, DHETs, EpOMEs and their corresponding diols (DiHOMEs)) have been shown to activate PPAR alpha and gamma [25-29]. Interestingly, the R287Q variant of sEH was previously shown to be significantly associated with insulin resistance in type 2 diabetics [30], which could also be related to PPAR gamma activation by sEH epoxide substrates. Furthermore, several isoprenoid phosphate precursors of cholesterol such as GPP (geranyl pyrophosphate) and FPP (farnesyl pyrophosphate) have been shown to be substrates of the sEH N-term domain [31,32]. Taken together this data suggests that sEH may be involved in modulating lipid metabolism and that this effect may be a contributing factor to the association of sEH variants with coronary heart disease and atherosclerosis. In this study we test the hypothesis that sEH is involved in regulating cholesterol levels in mammalian cells and that such effect is mediated by sEH-related compounds perhaps through their PPAR activity.

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MATERIALS AND METHODS

Purified hsEH Proteins

His-tagged human sEH (EPHX2) wild type (WT) whole protein and wild type C-term protein (amino acids 218-555) were expressed in SF21 cells using a baculovirus expression system as previously described [33]. His-tagged proteins were purified using His-select Nickel columns (Sigma, St. Louis MO) as recommended by the manufacturer. Protein purity was evaluated by SDS-PAGE and was found to be more than 95%. The activity of the purified proteins was evaluated by t-DPPO hydrolase assay [34].

Cell Culture

The Human Hepatoma (HepG2) cell line was obtained from ATCC (Manassas, VA). Cells were grown in Eagle's Minimum essential medium (MEM) with 2 mM L-glutamine, 1% penicillin-streptomycin, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 10% (v/v) fetal-bovine serum, at 37ºC and 5% CO_2. Human cervical carcinoma cells (Hela) and Chinese hamster ovary cells (CHO) were obtained from ATCC (Manassas, VA). Hela and CHO cells were grown in Ham's F12K medium with 2 mM L-glutamine, 1% penicillin-streptomycin, 1.5 g/L sodium bicarbonate and 10% (v/v) fetal-bovine serum, at 37ºC and 5% CO_2. Treatment compounds used in this study; including fatty acid epoxides and their corresponding diols; were obtained from Cayman chemicals (Ann Arbor, MI) and Biomol (Plymouth Meeting, PA). All treatments for measuring cholesterol in the media or the cells were carried in 1% fetal-bovine serum. The concentrations for the different treatments were selected based on relevant previous studies [25,27,29,35,36]. For each experiment, vehicle treated cells were used as control.

Protein Transport into Mammalian Cells

Transport protein delivery reagent was obtained from Cambrex (Rockland, ME). The protocol for protein delivery was essentially that provided by the manufacturer. β-galactosidase was provided by the manufacturer and was used as a positive control for protein delivery in the cell lines used. The cells were then fixed and stained for β-galactosidase using the standard β-gal staining protocol provided by the manufacturer. sEH protein delivery was evaluated by measuring t-DPPO C-terminal hydrolase enzymatic activity [34], western blotting [12] and rhodamine labeled sEH proteins (see below). Cells treated with protein delivery reagent only without sEH proteins were used as a control for the cholesterol experiments.

Rhodamine Labeling of Purified hsEH Proteins

To determine the efficiency of the purified sEH proteins, rhodamine labeled sEH proteins were delivered into cells cultured in glass bottom dishes (Biosciences Tools, San Diego, CA). Purified sEH proteins were labeled using the EZ-label Rhodamine labeling kit as recommended by the manufacturer (Pierce Biotechnology, Rockford, IL). The cells were then examined using a Leica confocal microscope. The fluorescent images were overlayed with simultaneous light transmitted images to determine efficiency of the protein delivery as the percentage of cells showing positive red fluorescence.

Amplex Red Cholesterol Assay

Total cholesterol was measured using amplex red cholesterol assay obtained from Molecular Probes (Eugene, OR). The cholesterol assay was carried out as described by the manufacturer. In this assay total cholesterol is determined by including cholesterol esterase in the assay buffer. For determination of cellular cholesterol, the cells were lysed in lysis buffer containing 50 mM Tris-HCL, 150 mM NaCl, 5 mM EDTA and 1% TritonX 100. Protein concentration was determined with bicinchoninic acid assay (Pierce, Rockford, IL) using BSA as a standard and cellular cholesterol was reported as ug total cholesterol/mg total protein. Total cholesterol in the media was directly measured from aliquots obtained at the indicated time points.

Statistical Analysis

All experiments were repeated at least 3 times with 2-5 replicates for each treatment. Statistical comparison of cholesterol levels from multiple replicates of both the control and treatment was evaluated using t-test or ANOVA with $P<0.05$. Error bars on the figures represent standard deviation (SD).

RESULTS

Protein Delivery in HepG2 Cells

The incorporation of purified hsEH into HepG2 cells was verified by using rhodamine labeled purified hsEH proteins. The efficiency of the protein delivery was more than 95% (Fig. **1A**). In addition, western blot analysis before and after protein delivery demonstrated non-detectable levels of sEH expression in these cells before introducing purified sEH proteins (Fig. **1B**). Similarly, standard sEH (H^3) t-DPPO hydrolase assay showed no detectable activity before introduction of purified sEH proteins and also verifies intact enzymatic activity following protein delivery (Fig. **1C**).

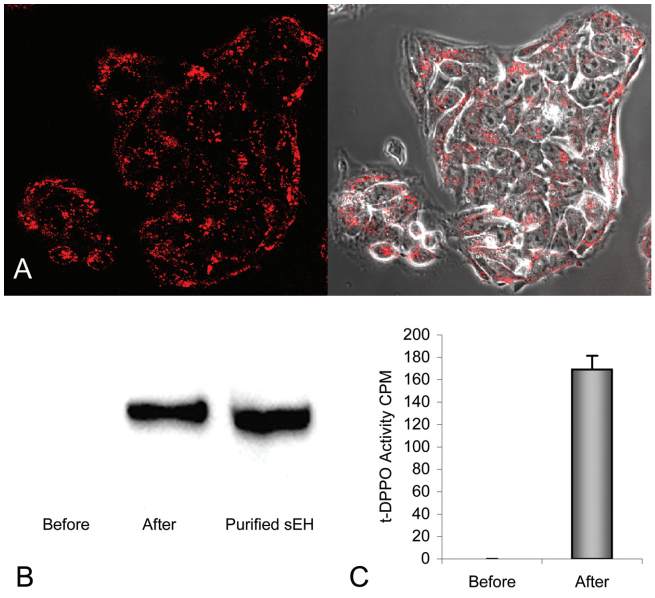

Fig. (1). A) Rhodamine labeled purified human sEH (red) delivered into HepG2 cells demonstrating an incorporation efficiency ≥95%. (**B**) Western blot showing sEH expression and (**C**) t-DPPO activity in HepG2 cells before and after protein transport.

Effect of Soluble Epoxide Hydrolase on Cholesterol in HepG2 Cells

At 24 hours both the wild type (WT) hsEH whole protein as well as the C-term hydrolase domain protein construct (CT) showed a similar cholesterol lowering effect in the media (Fig. **2A**). This indicates that the cholesterol lowering effect of sEH is mediated at least in part by the C-term hydrolase activity. Further lowering of cholesterol was detected when a mixture of EET regioisomers (10 μM) was added to either WT or CT treated cells (Fig. **2A**). Interestingly, treatment with a mixture of EET regioisomers (5 μM 11,12 EET + 5 μM 14,15 EET) in absence of sEH showed a cholesterol lowering effect which was greater than that of simvastatin (5 μM) at 12 hours (Fig. **2B**). However, simvastatin treatment for 24 hours resulted in a significantly lower total cholesterol level than with the EETs (Fig. **2B**).

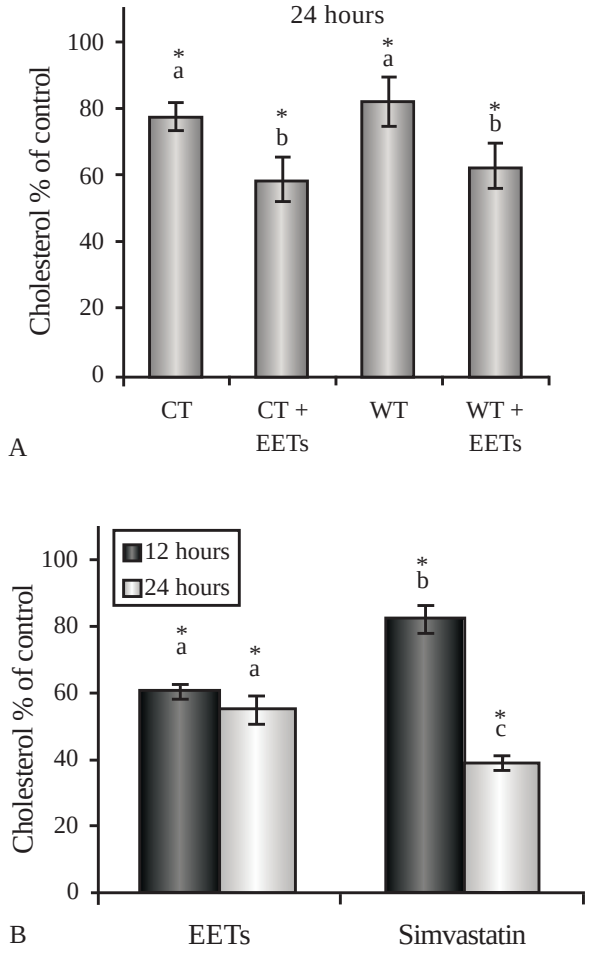

Fig. (2). A) Total cholesterol in the media from HepG2 cells expressing sEH wild-type (WT) and C-terminal hydrolase construct (CT) with and without 11,12 (5μM) and 14,15 (5μM) EET regioisomers mixture. **B**) EET 11,12 (5μM) and 14,15 (5μM) regioisomers mixture in absence of sEH as compared to simvastatin (5μM) at 12 and 24 hours. * indicates statistically significant difference as compared to control (P<0.05). Within each chart, different letters indicate statistically significant difference as compared to each other (P<0.05).

Effect of sEH-Related Compounds on Cholesterol in HepG2 Cells

Several sEH-related compounds; fatty acid epoxide substrates, diols and inhibitors; were found to lower total cholesterol in the media in HepG2 cells (Fig. **3**). Similar regioisomer specificity was found for the EETs and DHETs (Figs. **3A,B**) where only the 11,12 and 14,15 regioisomers but neither the 5,6 nor the 8,9 regioisomers had an effect on total cholesterol. Interestingly, EETs and DHETs produced similar effects (Figs. **3A,B**). Similarly, both linoleic acid epoxide (EpOME) and the corresponding diol (DiHOME) had comparable cholesterol lowering effect (Fig. **3C**). In addition to sEH epoxide substrates and diol products, hydrolase inhibitors such as AUDA and CUDA; also known to activate PPAR [29]; were found to lower total cholesterol levels in the media as well (Fig. **3D**). However, the effect of AUDA was more pronounced than that of CUDA (P<0.05).

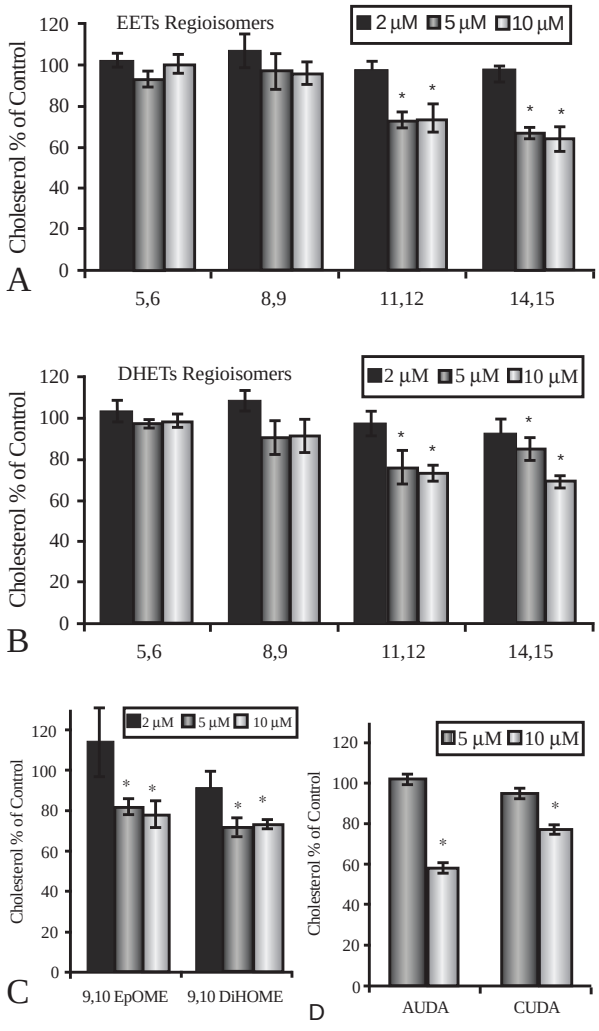

Fig. (3). Total cholesterol in the media at 24 hours from HepG2 cells treated with different sEH-related compounds. **A**) Arachidonic acid epoxide (EET) regioisomers, **B**) Arachidonic acid diol (DHET) regioisomers, **C**) 9,10-linoleic acid epoxide (EpOME) and diol (DiHOME), and **D**) sEH hydrolase inhibitors AUDA and CUDA. (* = different from control with P<0.05).

Effect on Cellular Cholesterol

In addition to the effects seen on total cholesterol levels in the media; here we evaluated total cellular cholesterol in response to treatment with sEH-related compounds (Fig. **4**). Generally, the cholesterol lowering response was comparable to that seen with simvastatin except for CUDA which did not elicit a statistically significant response as compared to control. For each compound we used the highest concentration that produced a maximal effect on cellular cholesterol without significant cytotoxicity or cell death as evaluated by cell morphology and number of floating cells (not shown).

Effect of sEH-CT Domain in CHO and Hela Cells

Introducing sEH-CT domain protein construct into cell lines other than HepG2; such as CHO and Hela cells; produced similar effects (Fig. **5**). sEH expression and activity before and after protein delivery were evaluated (not shown) and results obtained were similar to those obtained with HepG2 cells (Fig. **1**).

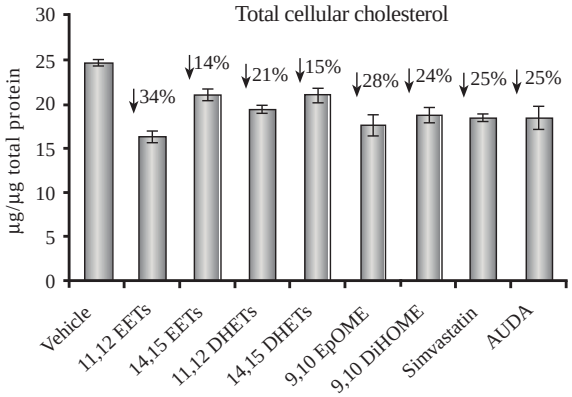

Fig. (4). Total cellular cholesterol at 48 hours from HepG2 cells treated with EETs (30µM), DHETs (30µM), 9,10-EpOME (30µM), 9,10-DiHOME (30µM), Simvastatin (5µM), AUDA (10µM) and CUDA (10µM). (* = different from control with P<0.05).

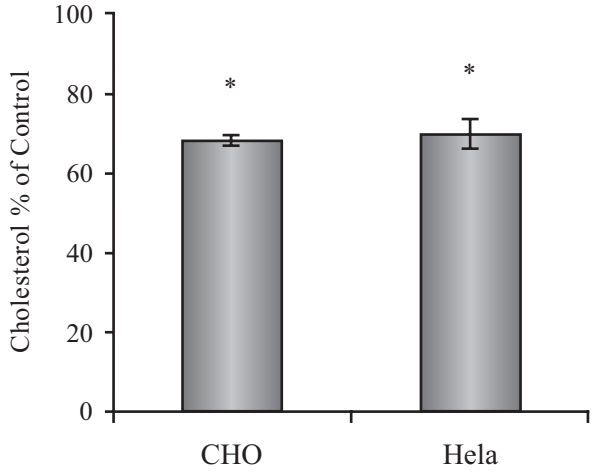

Fig. (5). Total cholesterol in the media from CHO and Hela cells expressing sEH-CT protein construct as compared to control. (* = different from control with P<0.05).

DISCUSSION

Several sEH C-term epoxide hydrolase substrates, diol products, and inhibitors have been shown to activate PPARs [25-27,29]. Ligands for both PPAR alpha and gamma are known to modify plasma lipid profile in humans and many such compounds have been in clinical use for years [6-8]. In this study we evaluated the potential role of sEH in regulating cholesterol metabolism in HepG2 cells perhaps through regulating the levels of various fatty acid epoxides and their corresponding diols.

Indeed, our results demonstrate that introducing sEH or the C-term hydrolase domain in mammalian cells results in significant lowering of cholesterol levels. This cholesterol lowering effect of the C-term hydrolase domain is suggestive of a diol effect mediated through its PPAR activity [25,27]. Even with the epoxide treatments, it is possible that the effect is mostly mediated through the diols as supported by previous findings showing that intracellular DHET levels in HepG2 cells were much higher following exogenous EET treatment as compared to exogenous DHET treatment which remains mostly extracellular [25]. However, we cannot exclude that the effect of introducing sEH into the cells may be possibly mediated by various endogenous epoxide substrates and diol products other than those of arachidonic and linoleic acids evaluated in this study. In addition, both EETs and DHETs were previously found to exhibit a positive feedback on RNA expression of sEH and P450 epoxygenases in HepG2 cells, an effect which is also mediated by PPAR activation [25].

A number of endogenous fatty acids and fatty acid derivatives are known to bind PPARs [9,10] which led to the suggestion that the endogenous role of these receptors is not limited to a single selective ligand interaction but rather an interaction with various endogenous ligands [2]. The same concept may apply to our findings where the cholesterol lowering effect is not limited to a single fatty acid derivative. This suggests that the role of sEH in regulating cholesterol is an additive effect of various endogenous sEH-related ligands rather than accumulation of a single selective ligand. In this context, several PPAR fatty acid ligands are known to exist at micromolar concentrations in serum and tissues within the concentration range used in this study [2,37], which further supports the biological relevance of our findings *in vivo*.

Several recent genetic studies demonstrated the association of known human sEH polymorphisms such as Arg287gln [38] with atherosclerosis [21,23] and elevated plasma cholesterol in familial hypercholesterolemia patients [24]. Interestingly, Arg287Gln has been previously shown to possess lower hydrolase activity and lower stability [33,38]. Taken together, these findings support our hypothesis that the sEH hydrolase activity has a cholesterol lowering effect and that polymorphisms with lower hydrolase activity and/or stability may be associated with elevated cholesterol and related pathologies.

Currently, sEH is becoming an attractive potential therapeutic target in the management of cardiovascular disease due to the various beneficial biological effects of its epoxide substrates including vasodilatation and anti-inflammatory properties, (reviewed in [20]). However, here we suggest that inhibition of the sEH epoxide hydrolase domain could abol-

ish its cholesterol lowering effect thus resulting in an undesired side effect especially in cardiovascular disease.

Interestingly, however, substituted urea-derived sEH hydrolase inhibitors such as AUDA and CUDA have been also shown to activate PPARs similar to DHETs and at concentrations relevant to that used in this study [29]. Consistent with our findings those inhibitors had a cholesterol lowering effect in HepG2 cells in absence of sEH expression. This data suggests that such an effect on cholesterol is perhaps independent of sEH inhibition but rather mediated by PPAR activity. The maximal effect of simvastatin as a cholesterol lowering drug in our model was achieved at a concentration of 5 μM which is consistent with data from previous studies in HepG2 cells [36]. Increasing the concentration of simvastatin up to 10 μM did not produce a significantly higher effect on cholesterol but was associated with significant cytotoxicity and cell death as evaluated by cell morphology and number of floating cells (not shown). An interesting finding is that contrary to simvastatin, sEH related compounds did not show cytotoxicity even at concentrations up to 30 μM (not shown) yet produced a cholesterol lowering effect comparable to that of simvastatin (Fig. **4**).

In conclusion, this study suggests a biological role for the sEH epoxide hydrolase domain in regulating cholesterol metabolism in mammalian cells. The cholesterol lowering effect of the hydrolase domain suggests that hypercholesterolemia might be an unwanted effect of its potential therapeutic inhibition in cardiovascular disease, which warrants further investigation in this regard. On the other hand, sEH-related compounds shown here to lower cholesterol might be investigated as a prototype for developing hypolipidemic agents; however, further mechanistic understanding of their mode of action requires further investigation.

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ABBREVIATIONS

FPP	=	Farnesyl Pyrophosphate
GPP	=	Geranyl Pyrophosphate
AUDA	=	12-(3-Adamantan-1-yl-Ureido) Dodecanoic Acid
CUDA	=	*N*-cyclohexyl-*N*'-dodecyl urea
EET	=	Epoxyeicosatrienoic Acid
DHET	=	Dihydroxyeicosatrienoic Acid
EpOME	=	Epoxyoctadecenoic Acid
DiHOME	=	Dihydroxyoctadecenoic Acid

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