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Electrophile-Modified Lipoic Derivatives of PDC-E2 Elicits Anti-mitochondrial Antibody Reactivity

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Abstract

Our laboratory has hypothesized that xenobiotic modification of the native lipoyl moiety of the major mitochondrial autoantigen, the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2), may lead to loss of self-tolerance in primary biliary cirrhosis (PBC). This thesis is based on the finding of readily detectable levels of immunoreactivity of PBC sera against extensive panels of protein microarrays containing mimics of the inner lipoyl domain of PDC-E2 and subsequent quantitative structure-activity relationships (QSARs). Importantly, we have demonstrated that murine immunization with one such mimic, 2-octynoic acid coupled to bovine serum albumin (BSA), induces antimitochondrial antibodies (AMAs) and cholangitis. Based upon these data, we have focused on covalent modifications of the lipoic acid disulfide ring and subsequent analysis of such xenobiotics coupled to a 15mer of PDC-E2 for immunoreactivity against a broad panel of sera from patients with PBC and controls. Our results demonstrate that AMA-positive PBC sera demonstrate marked reactivity against 6,8-bis(acetylthio)octanoic acid, implying that chemical modification of the lipoyl ring, i.e. disruption of the S-S disulfide, renders lipoic acid to its reduced form that will promote xenobiotic modification. This observation is particularly significant in light of the function of the lipoyl moiety in electron transport of which the catalytic disulfide constantly opens and closes and, thus, raises the intriguing thesis that common electrophilic agents, i.e. acetaminophen or non-steroidal anti-inflammatory drugs (NSAIDs), may lead to xenobiotic modification in genetically susceptible individuals that results in the generation of AMAs and ultimately clinical PBC.

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Introduction

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by chronic progressive destruction of small intrahepatic bile ducts and the presence of antimitochondrial antibodies (AMAs). The immunodominant epitopes recognized by AMA have been mapped to the inner lipoyl domain of the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2) within the inner mitochondrial matrix [1-3]. While the etiology of PBC remains unclear, there have been several lines of evidence suggesting that molecular mimicry may contribute to the breaking of self-tolerance in this disease [4-9]. Recently we have shown that replacement of the whole lipoyl residues of the native PDC-E2 molecule with select synthetic chemical compounds, particularly 2-octynoic acid and 2-nonynoic acid, found in cosmetics, lipstick, and chewing gums, demonstrates very high reactivity against PBC sera [10, 11]. Interestingly, immunization of experimental animals with these compounds when conjugated to bovine serum albumin (BSA) induces AMA and PBC-like liver lesions [12-14].

Based on the results from previous experiments, we carried out further studies aimed at determining the spectrum of xenobiotics that can serve as mimeotopes. We expanded those studies in efforts to determine the range of structural modifications that could show either a) reactivity with PBC sera and b) initiate the breakdown of self-tolerance. Herein, we focused on more detailed studies aimed at identifying the precise chemical structure of the xenobiotics that mimic lipoic acid by chemically modifying the lipoyl disulfide. To address this question, we synthesized a panel of lipoyl mimics which were subsequently conjugated to the 15-amino-acid-PDC-E2 peptide (the immunodominant PDC-E2 epitope) and analyzed them for their reactivity against sera from patients with PBC and controls using protein microarrays to establish quantitative structure-activity relationships (QSARs).

Methods

Sera

A panel of well-defined sera from our laboratory was used in the present study. These included samples from 46 AMA-positive PBC patients, 10 AMA-negative PBC patients, 14 primary sclerosing cholangitis (PSC) patients, 34 systemic lupus erythematosus (SLE) patients, and 28 healthy controls. The diagnosis of all patients was verified using published criteria [15-18]. The protocol was approved by the institutional review board of the University of California at Davis.

Preparation of peptide-agarose conjugates

The PDC peptide amide (IETDKATIGFEVQEE) was synthesized on Rink amide MBHA resin by Fmoc chemistry [19, 20]. Modification of agarose with methyl ketone groups was performed as previously described [10, 20, 21]. Briefly, 5 g of sodium carbonate was added to a solution of 3.2 g of agarose (Type XI: low gelling temperature; Sigma-Aldrich, St. Louis, MO) that was previously melted in 250 mL of deionized water. 100 mg of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) (Sigma-Aldrich) dissolved in 1 mL of dimethylsulfoxide (DMSO) (Sigma-Aldrich) and 0.2 g of sodium bromide (Sigma-Aldrich) were added [22]; stirring at 4 °C, 4.0 mL of sodium hypochlorite (1.3M) (Sigma-Aldrich) solution was slowly added. The mixture was stirred overnight at 4 °C. The solid was removed by filtration and the filtrate was poured into 3 volumes excess of ethanol. The agarose precipitate was obtained by filtration and washed with 70% ethanol. The powder was acidified by 1.0 M hydrochloric acid and precipitated in ethanol again. The white powder was thoroughly washed with 70% ethanol and lyophilized. Oxidized agarose (0.4 g, 0.46 mmol of $-\text{CO}_2\text{H}$) was then dissolved in 50 mL DMSO with heating. A solution of 2,2'-

(ethylenedioxy)-bis(ethylamine) (6.85 mmol) (Sigma-Aldrich) and *N,N*-diisopropylcarbodiimide (DIC) (6.85 mmol) was added to the agarose solution. The mixture was then stirred at room temperature for 5 hours and poured into 5-fold excess of cold (0 °C) ethanol. The resulting precipitate was filtered and washed with ethanol.

The coupling was confirmed by the Chloranil test [23]. The modified agarose was then dissolved in 50 mL of 100% DMSO. A solution of DIC (6.85 mmol), levulinic acid (6.85 mmol), and 1-hydroxybenzotriazole (HOBt) (6.85 mmol) was added to the modified agarose solution. The mixture was stirred at room temperature overnight and poured into 200 mL of cold ethanol and the precipitate was filtered and washed with ethanol. Completion of coupling was confirmed using the ninhydrin test. Methyl ketone-modified agarose (47 mg, 0.04 mmol) was then dissolved in 10 mL of the appropriate Aoa (amino-oxy acetyl)-peptide-solution (4 mM) in a 0.05 M NaOAc/AcOH buffer (pH 4.5) containing 50% DMSO. The mixture was stirred for 5 hours at 65-70 °C. Ketones on modified agarose react selectively with amino-oxy groups on peptides to form oximes under slightly acidic conditions [24, 25]. The conjugation solution was then dialyzed and subsequently lyophilized. Loading of the peptide was calculated by a quantitative ninhydrin test at 570 nm and determined to be 430 μ mol of the PDC-E2 peptide/g of agarose.

Synthesis of mimeotopes and coupling with peptide-agarose conjugate

Xenobiotics and control compounds (lipoic acid and methacrylic acid) were either synthesized by the Department of Chemistry, University of California at Davis or purchased from Sigma-Aldrich. The structure of these compounds is shown in Table 1. Xenobiotics and control compounds were coupled to *N*-hydroxysuccinimide (NHS) resulting in the corresponding NHS ester, which was subsequently coupled to the lysine residue of PDC-E2 on peptide-agarose conjugates as outlined in Figure 1 [20, 26]. NHS esters were coupled to peptide-agarose conjugates by mixing 40 μ g of the peptide-agarose conjugate and 0.4 μ mol of NHS ester in 40 μ L of DMSO. The mixtures were then incubated for 2 hours at room temperature. Complete coupling was confirmed by the Kaiser test [27].

Preparation of microarray and detection of antibody against xenobiotics-modified PDC-E2 peptides

All microarray printing steps were performed in a hepa and carbon filtered clean room at the UC Davis Micro-array Core Facility, Molecular Biosciences (<http://array.ucdavis.edu>). Xenobiotics-peptide-agarose mixtures were transferred to 384-well plates and printed using a Lucidea Array Spotter (Amersham Biosciences) in a 65% humidity controlled environment at room temperature. Microarrays were deposited using 500 μ column and row pitches, and spot diameters averaged 100 μ under these conditions. After spotting, slides were incubated at 70% humidity overnight at room temperature to maximize binding of the spotted material. Microarrays were dried at ambient conditions and stored at 4 °C under argon until use. Compounds were spotted in triplicate and mean values of the binding of the sera to these three spots were calculated to determine and quantitate immunoglobulin (Ig) reactivity. Microarray slides were blocked with Blocker Blotto in tris-buffered saline (TBS) for one hour at room temperature and individual slides were thereafter incubated with diluted (1:250) patient or control sera in blocking buffer containing 0.05% Tween-20 for one hour. After thorough washes with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-T), slides were incubated with Cy3-conjugated goat anti-human IgG and Cy5-conjugated goat anti-human IgM secondary antibodies at a pre-determined optimum dilution of 1:1500 for 45 minutes at room temperature in blocking buffer containing 0.05% Tween-20. Subsequently, slides were washed with PBS-T, PBS, and water. Slides were dried and scanned using a GenePix 4000B Microarray Scanner System.

Enzyme-linked immunosorbent assay (ELISA) for serum AMA levels and total serum IgG/IgM

96-well plates were coated with recombinant human PDC-E2-GST in coating buffer at a concentration of 1 µg/well. The plates were incubated at 4 °C overnight and blocked with PBS-T with 1% BSA at room temperature for one hour. Serum samples were diluted with PBS-T containing 1% BSA and 100 µl dispensed into each well. The plates were incubated at room temperature for one hour and then washed with PBS-T. Secondary antibodies in a volume of 50 µL/well (horseradish peroxidase (HRP)-conjugated goat anti-human IgG (gamma) or HRP-conjugated goat anti-human IgM) were then added at a pre-determined optimum dilution of 1:3000. The plates were incubated at room temperature for one hour and then washed with PBS-T. TMB (BD OptEIA) solutions A and B were mixed at 1:1 ratio and then added to the wells as substrate. The plates were incubated in the dark for color to develop. Sulfuric acid (2N) was diluted with deionized water at 1:1 ratio and added to the wells to stop the reaction. The optical density (OD) was measured using an ELISA plate reader at 450 nm. Total IgM and IgG serum levels were measured by using IMMUNOtek Quantitative Human IgM and IgG ELISA kits (ZeptoMetrix Corporation).

Data analysis

Microarray image extraction and data analysis were performed using GenePix Pro 6.0 Software. Data normalization and statistical analysis were performed using Prism Software (GraphPad). Two-tailed unpaired t-test with Welch's correction was used to statistically analyze the Ig reactivity against xenobiotic-modified PDC-E2 peptides between sera from AMA-positive patients with PBC and controls. Pearson product-moment correlation coefficient was used to analyze the correlation of the data with AMA and total Ig levels.

Results

Ig reactivity against xenobiotics-modified PDC-E2 peptides

The Ig reactivity against the xenobiotics-modified PDC-E2 peptide was initially analyzed using a panel of 8 newly synthesized compounds with a different chemical modification of the lipoic acid disulfide as shown in Table 1. 2-octynoic acid, a compound previously shown to react specifically with sera from PBC patients, as well as lipoic acid were included as positive controls. Methacrylic acid was used as a negative control [10]. These 11 compounds were conjugated to the immunodominant 15-amino-acid-PDC-E2-peptide-agarose conjugate as outlined in Figure 1. The unconjugated-PDC-E2-peptide-agarose was also used as an additional negative control and normalizing factor. These xenobiotics-conjugated-PDC-E2-agarose mixtures were then spotted onto glass slides. The spotted glass slides were incubated with sera from either AMA-positive PBC patients or controls, including AMA-negative patients with PBC, PSC patients, SLE patients, and healthy individuals. The slides were washed and incubated with fluorescence-conjugated secondary antibodies. The slides were then washed and dried. The signals were detected by the microarray scanner. Each individual signal was then normalized with its reactivity to the unconjugated-PDC-E2-peptide-agarose.

Figure 2A demonstrates the titers of IgM reactivity in the sera from AMA-positive PBC patients and healthy controls against the modified PDC-E2 peptides (OASAc, SFm, SAc, SCOEt, SCOPh, SMe, SOME and SO₂Me), lipoic acid (LA, positive control), the previously identified lipoic acid mimeotope 2-octynoic acid (2OA), and methacrylic acid (MA, negative control). The IgM reactivity of sera from AMA-negative patients with PBC, PSC patients, and SLE patients were similar to that seen with reactivity of sera from healthy controls (see Table 2). Overall, there were statistically significant differences in the levels of reactivity of IgM isotype from AMA-positive patients with PBC against LA, 2OA, OASAc,

SFm, SAc, SCOEt, SCOPh, and SO₂Me when compared with other controls. Similarly Figure 2B demonstrates the IgG isotype specific reactivity of sera from PBC patients and healthy controls against the same panel of antigens. The reactivity of sera from AMA-negative PBC, PSC and SLE patients were similar to that seen with sera from healthy controls (see Table 3). There were statistically significant differences in the levels of reactivity of the sera from AMA-positive patients with PBC based on the IgG isotype specific reactivity against the LA, 2OA, OASAc, SAc, SCOEt, and SCOPh modified PDC-E2 when compared with sera from the controls. Thus, while the levels of IgM isotype specific reactivity of the AMA-positive sera from PBC patients against SAc, OASAc, and SCOEt were higher than the reactivity of aliquots of the same PBC sera against LA and 2OA, interestingly, only the level of IgG isotype specific reactivity of the AMA-positive sera from PBC patients against SAc was higher than the reactivity of aliquots of the same PBC sera against LA and 2OA. Signals from MA, SMe, SOME, and SO₂Me were low compared to other compounds.

Correlations between Ig reactivity against xenobiotics-modified PDC-E2 peptide, AMA levels, and total Ig levels

A comparison was made between the AMA ELISA data and the data from microarray to determine the degree of concordance between these two sets of data. Pearson statistical analysis reflected the existence of a significant correlation between levels of IgM AMA and levels of IgM reactivity against LA (Table 4). There was also a significant correlation between levels of IgG AMA and levels of IgG reactivity against LA, SAc, SCOEt, and SCOPh.

To investigate if the Ig reactivity against the xenobiotics-modified PDC-E2 peptide correlated with the total Ig levels, we also performed an ELISA to detect the total IgM and IgG levels in each patient sample. The total IgM and IgG levels were compared with the data from the microarray analysis. Once again Pearson statistical analysis demonstrated that there was a significant correlation between total IgM levels and IgM reactivity against LA (Table 4). In addition, there was also a statistically significant correlation between total IgG levels and IgG reactivity against LA.

Discussion

The results obtained in this study reflect that the modified disulfide forms of lipoic acid reacted with the AMA-positive PBC sera at levels that were significantly higher than the native PDC-E2 itself. These xenobiotic-modified PDC-E2 did not react with sera from a series of other control sera utilized including sera from AMA-negative patients with PBC, PSC, SLE or healthy individuals. This finding is very intriguing because the major function of PDC-E2 is to catalytically transfer an acetyl group from pyruvate to coenzyme A (CoA) to produce acetyl-CoA. This function relies on the lipoic acid molecule on PDC-E2 which, during this process, opens the catalytic disulfide and transfers the acetyl group from the E1 component to CoA by using one of the arms of this open disulfide (Figure 3). This function is crucial during the synthesis of adenosine triphosphate (ATP) and will be discussed below with regards to its importance in the modification of the disulfide of lipoic acid by the xenobiotics.

In this study, we found that among all the modified compounds used in our current experiments, three of them (SAc, OASAc, and SCOEt) had very high levels of reactivity against sera from AMA-positive PBC patients that were even higher than the reactivity against lipoic-acid-conjugated PDC-E2 peptide which is a functional form of native PDC-E2. Furthermore, Pearson product-moment correlation coefficient analysis of our data showed that there were significant correlations ($p < 0.05$) among Ig reactivities against SAc,

OASAc, SCOEt, SCOPh, and 2OA from AMA-positive PBC sera. In other words, the probability to bind to one of the xenobiotic-modified peptides was higher in sera binding to other xenobiotic-modified peptides.

These findings prompted us to investigate the similarity between these three compounds in efforts to determine the potential structural similarities in the nature of the compounds that imparted such higher levels of reactivity. First, efforts were made to determine whether these compounds and/or structurally similar compounds are found in nature, in the laboratory or industry. An examination of the available literature on structurally identical and/or similar compounds failed to show similar compounds. However, a closer study of the nature of these compounds led us to the conclusion that these three compounds are in fact quite similar to what happens naturally during physiological reactions. These thioester modified analogs are known to arise from acylation of reduced lipoic acid. That is, these compounds contain a reduced lipoic acid with the addition of one (OASAc) or two (SAc) acetyl groups or the addition of two propionyl groups (SCOEt). Since there are many electrophilic agents that can chemically modify proteins, for example aspirin (ASA) and acetaminophen (APAP), we speculate that this modified disulfide of lipoic acid may prevent the lipoic-acid-conjugated PDC-E2 from functioning properly as we discuss above. We reason that such modifications are likely to result in the intracellular disruption of ATP synthesis and are thus likely to lead to cell death. Therefore, such chemical reactions may lead to the release of the xenobiotics-modified PDC-E2 and the exposure of this modified self-protein to the immune system of genetically susceptible individuals [28, 29] leading in turn to the breakdown of self-tolerance to native PDC-E2 itself by molecular mimicry and epitope spreading mechanism (Figure 4) [30]. In fact, our laboratory has previously shown that transient AMAs were present in approximately 35% of patients with acute liver failure caused by APAP toxicity [31]. However, ASA or APAP induced animal models of PBC have not been reported in the literature.

APAP metabolism primarily occurs in the liver. Roughly eighty five percent of the metabolites of APAP are conjugations of the aromatic ring to sulfate or glucuronic acid. These inert products are then excreted through the kidneys. The remaining fifteen percent is converted into *N*-acetyl-*p*-benzoquinoneimine (NAPQI) through isozymes of cytochrome p450. NAPQI is a highly-electrophilic metabolite, and is readily intercepted by glutathione. These reactions of glutathione either involve Michael addition to the aromatic ring, or reduction of NAPQI back to APAP. The predominant method of NAPQI detoxification occurs through the former mechanism, resulting in depletion of glutathione molecules from within a cell [32].

In the presence of excess APAP, glutathione depletion occurs. The resulting decrease in cellular glutathione allows for the accumulation of the reactive NAPQI metabolite. NAPQI will rapidly undergo addition reactions that preferentially target the thiol groups of proteins and related cofactors [33, 34]. Previous data [35] have suggested that glutathiolation would decrease the antigenicity of PDC-E2. However, due to cellular depletion of glutathione, very little glutathione would be available for such covalent protection of PDC-E2. This could lead to possible modification of native PDC-E2 by high levels of reactive NAPQI or other electrophilic agents.

Interestingly, the oxidative states of lipoic acid have been shown to affect the immunogenicity of PDC-E2 [36]. The lipoic-acid-conjugated PDC-E2 is more immunogenic if it is in a reduced form, which corresponds to the ring-open form of lipoic acid. This observation supports our findings that select modifications of the disulfide of lipoic-acid-conjugated PDC-E2 moiety makes it more immunogenic. However, in nature this reduced and more immunogenic form of PDC-E2 is less stable than the oxidized and less

immunogenic form of PDC-E2, which corresponds to the closed ring form of lipoic acid. Hence, the modification which we found in this study may not only make the PDC-E2 more immunogenic but also maintains it in an immunogenic form because the disulfide bond cannot re-form. This modification could enhance the potential for a breakdown in self-tolerance to PDC-E2.

One important question that remains un-answered with regards to the pathogenic mechanisms of PBC concerns the specific targeting of biliary epithelial cells (BECs). The results from the studies reported herein provide some clues to this enigma. According to earlier data [36], BECs are different from other cell types in that they have higher levels of glutathione, an anti-oxidant, inside the cells. When cell lineages other than BECs undergo apoptosis or oxidative stress, the GSSG (oxidized form of glutathione):GSH (reduced form of glutathione) ratio increases. Subsequently, the oxidized form of glutathione binds to the lipoic acid on PDC-E2 at the disulfide bond position. It has been hypothesized that this binding masks the PDC-E2 epitope and thus prevents the immune response to PDC-E2 [35]. However, in BECs since there is a relatively higher level of intracellular glutathione, the GSSG:GSH ratio does not increase as much as within other cell types during apoptosis or oxidative stress. Therefore, the masking effect of the oxidized form of glutathione to PDC-E2 cannot occur efficiently. This remaining unmasked PDC-E2 thus becomes prone to immune recognition and subsequent activation of the immune system. Our findings support this notion. That is, if the lipoic-acid-conjugated PDC-E2 is modified at the disulfide bond position, such as in SAc, OASAc, and SCOEt, this modification will further prevent the binding of PDC-E2 to glutathione in BECs. Such modification will increase the likelihood of the exposure of this unmasked form of PDC-E2 in BECs to the immune system and aggravate the breaking of self-tolerance to PDC-E2.

It is also important to note that our laboratory has recently shown that when BECs, but not control cells, undergo apoptosis, PDC-E2 remains immunologically intact [37]. More importantly, when monocyte-derived macrophages from PBC patients were incubated with apoptotic bodies from BECs in the presence of AMAs, there was intense pro-inflammatory cytokine production. This production was inhibited by anti-CD16, an antibody against the Fc (fragment crystallizable) receptor. Our data suggest that the triad of AMAs, biliary apoptoses, and innate immune response plays an important role in disease pathogenesis [38-40]. These recent findings emphasize a unique role of AMAs. Notably, AMAs are recognized as a serologic marker of PBC; they can be detected in individuals long before the manifestation of liver pathology and remain at high titer in patients with PBC even after liver transplantation [41, 42]. Based on the data from this study and our working hypothesis, it is likely that Ig reactivities to modified disulfide forms of lipoic acid, such as OASAc, SAc, SCOEt and SCOPh, could also be present in the individuals who are genetically susceptible to PBC. Lastly, it is interesting to note that the IgG reactivities were specifically reactive against these xenobiotic-modified PDC-E2 peptides and correlated with the levels of AMA more so than the IgM counterpart. These findings may be explained by the affinity maturation and isotype switching process [43]. Hence, our study not only suggests the potential trigger of the breakdown of self-tolerance to PDC-E2 but also reveals the footsteps or the initial process of how AMAs might develop.

Our data suggest that direct alteration of the lipoyl ring – i.e., disruption of the S-S linkage – renders the lipoic acid “activated” and receptive for xenobiotic modification and subsequent AMA recognition. This is of significance in light of the biochemical function of the lipoyl moiety in electron transport that constantly cycles opening and closing of the disulfide. Thus, in genetically susceptible individuals, the prolonged exposure to electrophilic agents, such as acetaminophen and NSAIDs, may initiate and/or enhance the breakdown of self-tolerance to PDC-E2 and eventually lead to PBC.

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Research Highlights

- Ig reactivity to lipoyl mimics on PDC-E2 peptide is studied by microarray and QSAR.
- IgG & IgM of AMA+ PBC but not controls recognized modified S-S bond of lipoic acid.
- The PDC-E2 lipoic acid S-S bond is prone to modification by electrophilic agents.
- Xenobiotic reduction of PDC-E2 lipoic acid could break tolerance to PDC-E2.
- Common electrophiles may initiate AMAs and PBC in genetically susceptible individuals.

Figure 1.
Preparation of xenobiotic-coupled derivatives of the 15-amino-acid-PDC-E2 mimic.

Figure 2. Comparison of IgM (A) and IgG (B) reactivity against xenobiotics-modified PDC-E2 peptide between AMA-positive patients with PBC and healthy controls. Asterisks indicate

significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; two-tailed unpaired t-test with Welch's correction).

Figure 3.
Schematic representation showing the physiological function of lipoylated PDC-E2 during ATP synthesis.

Figure 4. Hypothetical mechanism of electrophile-modified lipoylated PDC-E2 leading to AMA production. Covalent modification at the thiol group of lipoylated PDC-E2 by electrophilic agent (E⁺) inhibits acyl transfer to CoA, disrupts ATP synthesis, and leads to cell death. Subsequently, the immune exposure to modified PDC-E2 in genetically susceptible individuals results in the generation of AMAs by molecular mimicry and epitope spreading. Examples of E⁺ are listed in the left lower panel.

Table 1

Chemical structure and nomenclature of xenobiotics

Structure	Name	Abbreviation
Lipoic acid	LA
Methacrylic acid	MA
2-octynoic acid	2OA
8-(acetylthio)octanoic acid	OASAc
6,8-bis(formylthio)octanoic acid	SFm
6,8-bis(acetylthio)octanoic acid	SAc
6,8-bis(propionylthio)octanoic acid	SCOEt
6,8-bis(benzoylthio)octanoic acid	SCOPh
6,8-bis(methylthio)octanoic acid	SMe
6,8-bis(methylsulfinyl)octanoic acid	SOMe
6,8-bis(methylsulfonyl)octanoic acid	SO ₂ Me

Table 2

IgM reactivity expressed as mean $\pm$ SEM in normalized fluorescence intensity unit against xenobiotic-modified PDC-E2 peptide from AMA-positive patients with PBC, healthy controls, SLE patients, PSC patients, and AMA-negative patients with PBC.

Xenobiotics	AMA-positive PBC	Healthy control	SLE	PSC	AMA-negative PBC
LA	3544 $\pm$ 508.4	130.5 $\pm$ 74.03 ***	-124.0 $\pm$ 136.0 ***	-50.07 $\pm$ 112.2 ***	133.8 $\pm$ 91.40 ***
MA	-26.00 $\pm$ 50.35	-114.6 $\pm$ 62.44	-117.5 $\pm$ 46.07	59.50 $\pm$ 110.2	55.80 $\pm$ 25.28
2OA	3730 $\pm$ 482.8	149.9 $\pm$ 96.47 ***	-342.7 $\pm$ 80.71 ***	-176.9 $\pm$ 102.7 ***	32.70 $\pm$ 87.21 ***
OASAc	12860 $\pm$ 1793	694.7 $\pm$ 189.9 ***	555.7 $\pm$ 314.2 ***	467.1 $\pm$ 211.6 ***	1943 $\pm$ 834.5 ***
SFm	615.5 $\pm$ 245.0	-102.1 $\pm$ 66.58 **	-374.4 $\pm$ 91.26 ***	-133.4 $\pm$ 82.80 **	-42.50 $\pm$ 40.80 *
SAC	12480 $\pm$ 1798	1763 $\pm$ 333.8 ***	1019 $\pm$ 284.9 ***	517.0 $\pm$ 141.6 ***	1378 $\pm$ 563.7 ***
SCOEt	10040 $\pm$ 1491	1587 $\pm$ 475.2 ***	434.8 $\pm$ 195.2 ***	688.2 $\pm$ 203.3 ***	2078 $\pm$ 876.5 ***
SCOPh	4625 $\pm$ 775.9	209.8 $\pm$ 105.0 ***	-167.3 $\pm$ 86.44 ***	64.07 $\pm$ 127.8 ***	61.70 $\pm$ 32.21 ***
SMe	380.9 $\pm$ 153.1	257.8 $\pm$ 138.1	-73.18 $\pm$ 102.4 *	-72.64 $\pm$ 127.6 *	-1.300 $\pm$ 60.33 *
SOMe	304.9 $\pm$ 224.1	576.9 $\pm$ 238.6	17.91 $\pm$ 194.8	-278.3 $\pm$ 173.4 *	262.6 $\pm$ 180.1
SO ₂ Me	-340.6 $\pm$ 69.77	-37.29 $\pm$ 64.33 **	-97.47 $\pm$ 84.57 *	254.9 $\pm$ 222.2 *	201.7 $\pm$ 58.35 ***

Asterisks indicate significant differences between AMA-positive patients with PBC and other controls

* $p < 0.05$;

** $p < 0.01$;

*** $p < 0.001$; two-tailed unpaired t-test with Welch's correction.

IgG reactivity expressed as means $\pm$ SEMs in normalized fluorescence intensity unit against xenobiotics-modified PDC-E2 peptide from AMA-positive patients with PBC, healthy controls, SLE patients, PSC patients, and AMA-negative patients with PBC.

Table 3

Xenobiotics	AMA-positive PBC	Healthy control	SLE	PSC	AMA-negative PBC
LA	1364 $\pm$ 198.6	242.4 $\pm$ 57.81 ***	-171.5 $\pm$ 241.9 ***	56.71 $\pm$ 136.7 ***	-37.00 $\pm$ 352.9 **
MA	-92.39 $\pm$ 35.85	-47.68 $\pm$ 73.35	-208.2 $\pm$ 143.0	-179.8 $\pm$ 80.13	-228.4 $\pm$ 93.90
2OA	512.1 $\pm$ 157.7	-83.68 $\pm$ 125.0 **	-140.8 $\pm$ 100.6 ***	-542.9 $\pm$ 204.8 ***	-776.6 $\pm$ 289.8 **
OASAc	842.9 $\pm$ 196.1	-5.821 $\pm$ 125.3 ***	-143.9 $\pm$ 105.4 ***	-77.64 $\pm$ 134.0 ***	-40.70 $\pm$ 297.2 *
SFm	-99.24 $\pm$ 55.75	-275.1 $\pm$ 107.4	-317.4 $\pm$ 141.2	-298.0 $\pm$ 132.7	-52.70 $\pm$ 118.6
SAc	2756 $\pm$ 640.2	158.8 $\pm$ 235.0 ***	-440.9 $\pm$ 280.6 ***	-78.57 $\pm$ 163.9 ***	11.20 $\pm$ 217.6 ***
SCOEt	1130 $\pm$ 265.7	-155.2 $\pm$ 109.0 ***	-55.32 $\pm$ 136.5 ***	-166.8 $\pm$ 147.4 ***	63.40 $\pm$ 301.5 *
SCOPh	304.7 $\pm$ 93.45	-101.4 $\pm$ 119.4 **	-197.1 $\pm$ 144.0 **	-140.3 $\pm$ 160.1 *	-41.80 $\pm$ 49.82 ***
SMe	-513.7 $\pm$ 99.59	-718.0 $\pm$ 168.0	-975.3 $\pm$ 386.4	-595.1 $\pm$ 222.6	-857.1 $\pm$ 319.3
SOMe	-686.1 $\pm$ 135.3	-1008 $\pm$ 237.2	-1328 $\pm$ 546.6	-1255 $\pm$ 369.3	-1571 $\pm$ 559.3
SO ₂ Me	-509.5 $\pm$ 119.3	-220.0 $\pm$ 179.9	-350.2 $\pm$ 197.9	-44.43 $\pm$ 184.8 *	-175.2 $\pm$ 140.3

Asterisks indicate significant differences between AMA-positive patients with PBC and other controls

* $p < 0.05$;

** $p < 0.01$;

*** $p < 0.001$; two-tailed unpaired t-test with Welch's correction.

Table 4

Correlation between Ig reactivity against xenobiotics-modified PDC-E2 peptide and AMA or total Ig levels in AMA-positive patients with PBC.

	Xenobiotics		AMA		Total Ig			
	IgM	Pearson r	P value	IgG	Pearson r	P value	IgG	Pearson r
LA	0.6392	***	0.0002	0.4475*	0.0149	0.4373*	0.553**	0.0019
MA	-0.2198		0.2519	-0.2069	0.2815	-0.07762	-0.07344	0.705
2OA	0.2609		0.1716	0.06799	0.726	0.2562	-0.2587	0.1754
OASAc	0.283		0.1369	0.3401	0.071	0.2976	0.117	0.08882
SFm	0.06767		0.7273	-0.2147	0.2633	0.1115	-0.09089	0.6391
SAC	0.3506		0.0623	0.3736*	0.0459	0.3376	-0.09289	0.6318
SCOEt	0.2639		0.1666	0.4513*	0.014	0.2387	-0.05193	0.789
SCOPh	0.3194		0.0912	0.4301*	0.0199	0.3092	0.07792	0.6879
SMe	0.002273		0.9907	-0.07569	0.6963	-0.05677	0.02791	0.8857
SOMe	-0.07974		0.681	-0.0997	0.6068	0.07856	-0.01428	0.9414
SO ₂ Me	0.01447		0.9406	-0.02627	0.8924	-0.1085	-0.02411	0.9012

Asterisks indicate significant correlations

* $p < 0.05$;

** $p < 0.01$;

*** $p < 0.001$; Pearson product-moment correlation coefficient.