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Covalent Modification of Lipids and Proteins in Rat Hepatocytes, and In Vitro, by Thioacetamide Metabolites

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Abstract

Thioacetamide (TA) is a well-known hepatotoxin in rats. Acute doses cause centrilobular necrosis and hyperbilirubinemia while chronic administration leads to biliary hyperplasia and cholangiocarcinoma. Its acute toxicity requires its oxidation to a stable *S*-oxide (TASO) that is oxidized further to a highly reactive *S,S*-dioxide ($TASO_2$). To explore possible parallels between the metabolism, covalent binding and toxicity of TA and thiobenzamide (TB) we exposed freshly isolated rat hepatocytes to [^{14}C]-TASO or [$^{13}C_2D_3$]-TASO. TLC analysis of the cellular lipids showed a single major spot of radioactivity that mass spectral analysis showed to consist of *N*-acetimidoyl PE lipids having the same side chain composition as the PE fraction from untreated cells; no carbons or hydrogens from TASO were incorporated into the fatty acyl chains. Many cellular proteins contained *N*-acetyl- or *N*-acetimidoyl lysine residues in a 3:1 ratio (details to be reported separately). We also oxidized TASO with hydrogen peroxide in the presence of dipalmitoyl phosphatidylenthanolamine (DPPE) or lysozyme. Lysozyme was covalently modified at five of its six lysine side chains; only acetamide-type adducts were formed. DPPE in liposomes also gave only amide-type adducts, even when the reaction was carried out in tetrahydrofuran with only 10% water added. The exclusive formation of *N*-acetimidoyl PE in hepatocytes means that the concentration or activity of water must be extremely low in the region where $TASO_2$ is formed, whereas at least some of the $TASO_2$ can hydrolyze to acetylsulfinic acid before it reacts with cellular proteins. The requirement for *two* sequential oxidations to produce a reactive metabolite is unusual, but it is even more unusual that a reactive metabolite would react with water to form a new compound that retains a high degree of chemical reactivity toward biological nucleophiles. The possible contribution of lipid modification to the hepatotoxicity of TA/TASO remains to be determined.

Introduction

Compounds that possess a thiocarbonyl group (>C=S) have been found to serve a wide range of roles in medicine, agriculture and industry. Most of these compounds fall into one of three broad structural classes: thioamides, thioureas, and thiosemicarbazones. For example, quazepam (**1**) is used as a hypnotic agent for insomnia while methimazole (**2**) is used to treat hyperthyroidism. Other examples include the antitubercular agents ethionamide

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Supporting Information Available: Table of observed masses of six tryptic peptides of lysozyme treated with $TASO/H_2O_2$, the MS/MS spectra of these peptides, and MS/MS reference spectra of a lysozyme tryptic peptide bearing acetimidoyl vs acetyl adducts. This material is available free of charge via the Internet at http://pubs.acs.org.

(**3**) and thiacetazone (**4**), the pneumotoxic rodenticide alpha-naphthylthiourea (**5)**, and the herbicide Prefix (**6**). Tolrestat (**7**) was developed as an aldose reductase inhibitor for glaucoma but was withdrawn because of liver toxicity. 1,3-Diphenylthiourea (**8**) is used in the production of synthetic rubber and is often responsible for hypersenitivity reactions to rubber products. [1] Thiosemicarbazone derivatives related to **4** continue to attract attention as promising anti-cancer agents. Nearly all thiocarbonyl compounds are metabolized at least in part, and sometimes almost exclusively, by a general pathway known as oxidative desulfurization.[2]

As noted below, intermediates in this pathway are often highly reactive and prone to react covalently with cellular proteins. In many cases this leads to cytotoxicity and/or immunological sensitization. Two thiocarbonyl compounds are of particular note in this context: thiobenzamide (TB[1], **9a**) and thioacetamide (TA, **9b**), both of which are widely used to model hepatotoxicity and to investigate the connection between biotransformation and toxicity.[3–10] The metabolic activation of thiobenzamide is known to require two sequential *S*-oxidations.[11] The first produces an isolable *S*-oxide or "sulfine" metabolite (TBSO, **10a**), while the second produces a highly reactive S,S-dioxide or "sulfene" metabolite ($TBSO_2$, **11a**) that tautomerizes to benzimidoylsulfinic acid (**12a**) as shown in Scheme 2. The latter is capable of benzimidoylating cellular nitrogen nucleophiles including protein lysine side chains 12 and PE lipids 13. $TBSO_2$ can also undergo base-catalyzed elimination of its sulfur group (via tautomer **12a**) to form benzonitrile (**13a**) or react with water to form benzamide (**14a**), both of which are stable metabolites. On the other hand $TBSO_2$ can also react with water to form benzoylsulfinic acid (**15a**) which can then react further to benzoylate protein lysine side chains as in structure **16a**. Finally, $TBSO_2$ can react directly with protein lysine side chains or with the amine group of phosphatidylethanolamine (PE) lipids to form the corresponding benzimidoyl or amidino adducts (**17a** or **18a**, respectively). Interestingly, metabolism of TB does not lead to the formation of *N*-benzoyl-PE lipids.[13] Thioacetamide is also known to undergo metabolic activation and covalent binding to protein lysine side chains via intermediates **10b**, **11b**, and **12b,** [14, 15] so it might also be expected to modify PE lipids via *N*-acetylation or *N*-acetimidoylation. A number of different *N*-acyl- PE lipid derivatives are known to form endogenously,[16–19] but *N*-imidoylated-PE species such as **18** are apparently not naturally occurring.

The work described in this paper had two primary objectives. One was to determine whether TA metabolism also leads to covalent modification of PE lipids in rat hepatocytes, and if so to quantitate and characterize the nature of the modification(s). The second was to characterize mass spectrometrically the adducts formed in vitro between $TASO_2$ and a model protein as a prelude to analyzing proteins adducted in vivo by thioacetamide metabolites. Here we report that incubation of rat hepatocytes with [^{14}C]-TASO, and separately with a mixture of TASO and [$^{13}C_2D_3$]-TASO, leads to substantial covalent labeling of both cellular lipids and cellular proteins. Mass spectral analysis shows that the lipid adducts are exclusively *N*-acetimidoylated PE lipids; *N*-acetyl-PE lipids are not formed, nor are they detectable in untreated cells. In contrast, when generated in solution from TASO and hydrogen peroxide, $TASO_2$ reacts with PE to give *N*-acetyl-PE but not *N*-acetimidoyl PE, and it reacts with the model protein lysozyme forming acetyl but not acetimidoyl adducts on protein-NH_2 groups. Although it is not clear whether lipid

[1]**Abbreviations:** AGC, automatic gain control; CID, collision induced dissociation; DPPE, dipalmitoyl phosphatidylethanolamine; ESI, electrospray ionization; MA, methyl acetimidate; NL, neutral loss; PC, phosphatidyl choline; PE, phosphatidylethanolamine; PI, phosphatidyl inositol; PS, phosphatidyl serine; TA, thioacetamide; TASO, thioacetamide S-oxide; $TASO_2$, thioacetamide S,S-dsioxide; TB, thiobenzamide; TBSO, thiobenzamide S-oxide; $TBSO_2$, thiobenzamie S,S-dioxide; THF, tetrahydrofuran; TLC, thin layer chromatography.

modification by $TASO_2$ contributes to the toxicity of TA and TASO, it is clear that its chemistry inside the hepatocyte is very different from its chemistry in simple aqueous solution.

Experimental Procedures

1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). ^{14}C-TASO and $^{13}CD_3$ $^{13}C(SO)NH_2$ were synthesized according to a literature procedure 20. All other chemicals and solvents were obtained from Sigma Aldrich (St. Louis, MO). ^{1}H and ^{13}C NMR spectra were recorded on Bruker AV III 500 MHz spectrometer and spectra were referenced to the residual NMR solvent peak. Column chromatography was performed using silica gel. Thin-layer chromatography was performed either on Whatman LK5DF plates (www.whatman.com) or on Silica Gel GF plates (2.5 × 10 cm, 0.25 mm, Analtech, www.ichromatography.com) with fluorescence detection under UV light at 254 nm. The solvent system used for TLC was $CHCl_3$/MeOH/NH_4OH (80/20/3). Lipids were visualized using iodine vapors or by spraying the plate with 10% $CuSO_4$ solution in 10% H_3PO_4 followed by heating. Radioactivity on the TLC plate was detected by exposing it to a Molecular Dynamics phosphor screen (3 days at room temperature) and scanning the screen with a Typhoon Molecular Imager scanning unit (GMI, Inc.; www.gmi-inc.com).

N-Acetyl-DPPE (19)

A mixture of acetic anhydride (3 mg, 0.030 mmol), DPPE (20 mg, 0.030 mmol) and triethylamine (6 mg, 0.060 mmol) in chloroform was stirred at room temperature overnight. The reaction mixture was diluted with ethyl acetate, washed with brine and the organic portion was dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the residue was purified by column chromatography over silica gel starting with 10% MeOH in chloroform. Compound **19** (Scheme 3) was eluted using 50% MeOH in chloroform. TLC R_f 0.48 (80:20:3 $CHCl_3$/MeOH/NH_4OH). ESIMS: $[M\text{-}H]^-$ = 732.5153 ± .0022 (n=5); calcd 732.5185.

N-Acetimidoyl-DPPE (20)

Methyl acetimidate (MA) hydrochloride (0.003 g, 0.030 mmol), DPPE (0.020 g, 0.030 mmol) and triethylamine (0.006 g, 0.060 mmol) were combined in ethanol and heated to reflux for 2 h. After cooling to room temperature, ethyl acetate was added and the mixture was washed with brine solution. After drying the organic portion over anhydrous Na_2SO_4, the solvent was removed under vacuum and the residue was purified by column chromatography over silica gel starting with 10% MeOH in chloroform. Compound **20** was eluted using 50% MeOH in chloroform. TLC R_f 0.37 (80:20:3 $CHCl_3$/MeOH/NH_4OH). ESIMS: $[M\text{-}H]^-$ = 731.5329 ± .0036 (n=7); calcd 731.5345.

In vitro reactions of TASO/H_2O_2 with DPPE

A solution of DPPE (5 mg) in chloroform (3 mL) was dried to a film using a rotary evaporator. Water (1 mL) was added and the flask was intermittently swirled vigorously over 60 min while heating at 70 °C with a water bath. A solution of TASO (varying mole ratio to DPPE) in 20 μL water was added directly to the resultant milky suspension of micelles and liposomes (at either 70 °C or after cooling to room temperature), followed immediately by 10 μL of an aqueous solution of H_2O_2 (titrated to contain slighly less than one equivalent compared to TASO), and the mixture was stirred for 16 hours at room temperature. An aliquot was then taken for direct analysis by LC/MS.

In vitro reactions of hen egg lysozyme with acylating reagents

Reactions of hen egg lysozyme with TASO/H_2O_2 or methyl acetimidate or acetic anhydride were conducted as follows. To a solution of lysozyme (10 mg) in water (1 mL) was added 130 μL of a solution of TASO in water (10 mg/mL) followed by addition of 26 μL of a 0.05% solution of aqueous hydrogen peroxide. Methyl acetimidate hydrochloride (8 mg) was dissolved in water (400 μL), 7 mg of triethylamine was added via syringe, and a 15 μL aliquot of this solution was added immediately to a solution of lysozyme (5 mg) in water (1 mL). Acetic anhydride (17 μL) was added directly to a solution of lysozyme (10 mg) in water (1 mL). After standing at room temperature for at least 2 h all three solutions were analyzed by whole-molecule ESIMS using direct flow injection through a C_4 reverse-phase desalting column. For peptide analysis aliquots were reduced with dithiothreitol and alkylated with iodoacetamide in 0.1% SDS aqueous solution, precipitated with cold ethanol at −20 °C overnight and digested with trypsin in 0.2 M ammonium bicarbonate at 37°C.[21]

In vitro reactions of TASO/H_2O_2 with DPPE and hen egg lysozyme

A solution of DPPE (5 mg) in chloroform (3 mL) was dried to a film using a rotary evaporator. Water (1 mL) was added and the flask was intermittently swirled vigorously over 60 min while heating at 70 °C with a water bath. A solution of TASO (varying mole ratio to DPPE) in 20 μL water was added directly to the resultant milky suspension of micelles and liposomes (at either 70 °C or after cooling to room temperature), followed immediately by 10 μL of an aqueous solution of H_2O_2 (titrated to contain slighly less than one equivalent compared to TASO), and the mixture was stirred for 16 hours at room temperature. An aliquot was then taken for direct analysis by LC/MS. Reactions of hen egg lysozyme (5 mg in 1 mL water) with TASO/H_2O_2 or methyl acetimidate or acetic anhydride were conducted similarly at room temperature. After stirring for 2 hr the solutions were analyzed directly by whole-molecule mass spectrometry. Aliquots were also reduced with dithiothreitol, alkylated with iodoacetamide in a 0.1% SDS aqueous solution, precipitated with cold ethanol at −20 °C overnight and digested with trypsin in 0.2M ammonium bicarbonate at 37°C.

Preparation and Incubation of Isolated Hepatocytes

Male Sprague-Dawley rats from Charles River Laboratories were housed in a temperature and humidity controlled room with a 12 h light/dark cycle and *ad libitum* access to food and water. All animal husbandry procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication, Volume 25, 1996, http://grants1.nih.gov/grants/guide/notice-files/not96-208.html). Experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Kansas. Hepatocytes from non-induced male Sprague-Dawley rats (175–275 g) were isolated using a previously described protocol 22. After isolation the cells were transferred to incubation medium (Williams' medium E supplemented with 1% (v/v) Pen-Strep, 1% (v/v) ITS (Insulin-Transferrin-Selenium), 100 nM dexamethasone, and 2 mM L-glutamine (all from Gibco-Invitrogen, www.invitrogen.com).

For metabolism studies TASO (10 mM, 0.57 Ci/mol) was incubated with 2.2×10^7 freshly isolated rat hepatocytes (>90% viability) suspended in the same medium (2.2 mL) in 50 mL round bottom screw-cap culture tubes. Before adding the hepatocytes to the tubes the required amounts of TASO were deposited by evaporation from a methanol solution. Incubations were shaken at 100 Hz at 37°C under an atmosphere of O_2/CO_2 (95: 5) for 4 h. For identification of lipid adducts, a mixture of [$^{13}C_2D_3$]-TASO/TASO (10:6; total TASO, 10 mM) was substituted for radiolabeled TASO, and incubations were conducted identically. The cells from duplicate 450 μL aliquots of incubations were pelleted by centrifugation and

the pellets were subjected to Folch extraction[23] using 19 volumes of MeOH/$CHCl_3$ (2:1 v/v).

Mass Spectrometry

Molecular weight confirmations of **19** and **20** were obtained from spectra acquired by electrospray ionization on a LCT Premier (Waters Corp., Milford MA) time of flight mass spectrometer. Mass scale correction for exact mass determinations were made with the lock mass feature in the MassLynx data system using a reference compound presented with an auxiliary sprayer. Collision induced dissociation (CID) spectra of standards (Figure 2) were acquired using ESI on a Q-Tof-2 instrument (Micromass Ltd., Manchester UK). Argon was the collision gas and the collision energy was varied from 15–35eV to obtain a distribution of fragments from low to high mass.

Lipid type, or "head group specific", CID spectra by precursor or neutral loss scans were acquired on a Quattro Ultima "triple" quadrupole tandem mass spectrometer (Micromass Ltd., Manchester UK) using ESI. The resolution of quadrupoles 1 and 3 were tuned to 0.6 u FWHH. The collision gas (Ar) density was set to attenuate the precursor by 20% (6e-4 mBar on gauge in collision supply line), a density optimized for precursor scans. Samples were dissolved in MeOH/$CHCl_3$/300mM NH_4OAc (665:300:35) and sprayed through a stainless steel needle set at 2.8 kV. Ions were sampled through the cone set to 35 V. Lipid "head group specific" tandem mass spectrometry scans in positive and negative modes for profiling membrane lipids were pioneered by Brugger 24 using ESI on a triple quadrupole type instrument. Since then this approach has been widely adopted for lipid content profiling including rat liver[25] and adapted to the detection of anticipated xenobiotic lipid metabolites.[13]

Intact protein ESI spectra were acquired on a SYNAPT G2 hybrid quadrupole/ion mobility/Tof mass spectrometer (Waters Corp., Milford, MA). The instrument was operated in a sensitivity mode with all lenses optimized on the MH+ ion from the leuenkephalin. The sample cone voltage was 30 eV. Argon was admitted to the trap cell that was operated at 4eV for maximum transmission. Spectra were acquired at 9091 Hz pusher frequency covering the mass range from 100 to 3000 u and accumulating data for 1.5 seconds per cycle. Time to mass calibration was made with NaI cluster ions acquired under the same conditions. Mass spectra of [Glu^1]-fibrinopeptide B were acquired in parallel scans and doubly charged ions at m/z 785.8426 were used as a lock mass reference. Samples were desalted on a reverse phase C_4 column, 1cm, 1mm I.D. (Vydac, 300 Å pore size, 5 μm particles packed by Micro-Tech Scientific) using a NanoAcquity chromatographic system (Waters Corp.). The solvents were A (99.9% H_2O, 0.1% formic acid) and B (99.9% acetonitrile, 0.1% formic acid). A short gradient from 1 to 70% B in 4 minutes with a flow rate of 20 μL/min was used. MassLynx software (Waters Corp., version 4.1) was used to collect the data. Raw multiply charged protein mass spectra were converted into molecular weight spectra using MaxEnt1 or Transform utility of MassLynx.

Digested protein samples were analyzed by LC-MS/MS using a NanoAcquity chromatographic system (Waters Corp.) coupled to an LTQ-FT mass spectrometer (ThermoFinnigan, Bremen, Germany). Peptides were separated on a reverse-phase C_{18} column, 15cm, 300μm I.D. (Thermo Acclaim PepMap300, 300 Å 5 μm). A gradient was developed from 1 to 40% B in 50 minutes, ramped to 95% B in 4 minutes and held at 95%B for 5 minutes at a flow rate of 10 μL/min with solvents A and B identical to those used for intact protein MS analysis. The NanoAquity UPLC Console (Waters Corp., version 1.3) was used to control the injections and gradients. Data-dependent acquisition method for the mass spectrometer (configured version LTQ-FT 2.2) was set up using Xcalibur software (ThermoElectron Corp., version 2.0). Full spectrum survey scans were acquired at a

resolution of 50,000 with an Automatic Gain Control (AGC) target of 5×10^5. Five most abundant ions were fragmented in the LTQ with AGC target of 2×10^3 or maximum ion time of 300 ms.

Results

Synthesis and characterization of modified PE standards

The oxidative biotransformation of thioacetamide was expected to generate chemically reactive metabolites potentially capable of *N*-acetylating and/or *N*-acetimidoylating PE lipids (Scheme 2). To facilitate the search for these adducts we prepared both *N*-acetyl-DPPE (**19**) and *N*-acetimidoyl- DPPE (**20**) as reference standards. On thin layer chromatography (Figure 1) these materials behaved analogously to *N*-benzoyl- and *N*-benzimidoyl-DPPE as described by Ji et al.[13] The mass spectra of these compounds (Figures 2A and 2B) show that they fragment easily at the glycerol-phosphate bond (Figure 2). In the case of the amidines the phosphate-containing fragment retains a majority of the charge, but for the less-basic amides the charge is carried almost exclusively by the diacylglycerol (DAG)-derived fragment. Thus, it is convenient to search for *N*-acetimidoyl-PE derivatives using MS/MS combined with precursor-ion scanning for *m/z* 183 (corresponding to the amidine-phosphate fragment shown in Figure 2A). In contrast a neutral loss scan for *m/z* 183 reveals the presence of *N*-acetyl-PE lipids with great sensitivity. Analogous scans can detect, selectively and sensitively, several common types of *endogenous* phospholipids including phosphatidyl choline (PC), -ethanolamine (PE), -serine (PS), and - inositol (PI), as shown in Figure 3 for a Folch extract of lipids from unteated control rat hepatocytes. Collectively, these scans reveal the major types of fatty acid moieties present in the phospholipids of rat hepatocytes.[24, 25] This information provides important background for interpreting mass spectra of xenobiotic lipid adducts as discussed below.

Lipid adducts from TASO-treated rat hepatocytes

To look for lipid adducts of TASO formed in living cells, freshly isolated rat hepatocytes (2.2×10^7 cells in 2.2 mL medium) were incubated with [^{14}C]-TASO (10 mM) for 4 h at 37 °C. After centrifuging the cells a Folch extract of lipids was made and found to contain 0.55 mg lipids/10^6cells. When examined by TLC alongside synthetic standards of **19** and **20** as well as lipids extracted from untreated control cells, a new spot slightly more polar than TASO appeared under staining with iodine vapor (M1 in Figure 1). The R_f of this material was identical to that of *N*-acetimidoyl-DPPE but distinct from that of *N*-acetyl-DPPE, and it was not formed if cells were first heated to 70 °C to inactivate the enzymes that oxidize TASO. Phosphorimaging of the TLC plate (Figure 1), followed by scraping and counting the radioactive zones, showed that the new spot contained 65% of the total radioactivity on the TLC plate; traces of TASO (and also TA) were present in the extract and are barely visible in the phosphorimage as well. The radioactivity in the new spot corresponded to a lipid covalent binding level of 67 nmol-equiv./mg cellular lipid (i.e., 37 nmol-equiv./million cells). Assuming that PE comprises ca. 20% of the total phospholipid extract, and that the average PE has a MM of 760 g/mol, this corresponds to an adduct density of 0.25 (i.e., one PE molecule in four has been adducted), which is very close to what we observed for adduction of PE lipids by thiobenzamide metabolites.[13] For comparison, the protein covalent binding (which will be described in detail in a separate publication) corresponded to 20 nmol-equiv./mg protein (i.e, 20 nmol-equiv./million cells).

In separate experiments rat hepatocytes were incubated with a mixture of TASO and [$^{13}C_2D_3$]-TASO (mole ratio M/M+5 = 10:6) and the lipids were extracted and analyzed by MS/MS to search for *precursors* of *m/z* 183 and *m/z* 188 (corresponding to M and M+5 forms of $(HO)_2P(O)OCH_2CH_2NHC(=NH_2^+)CH_3$ (*viz.* Figure 2A). The precursor ion scan

for 183 (Figure 4A) shows a strong "phospholipid-like" pattern resembling some of the scans of endogenous lipids (particularly the PEs) shown in Figure 3D. Thus the various major mass peaks in Figure 4A all correspond to logical fatty acid side chain combinations found among the endogenous PEs of hepatocytes, except for the addition of 41 Da for the *N*-acetimidoyl group. The corresponding *precursor* scan for *m/z* 188 (Figure 4B) shows a pattern nearly identical to that in Figure 4A except that the peaks are all shifted +5 mass units and their intensity is only about 60% that of Figure 4A, which is as expected for the isotope ratio built into the TASO sample used in the incubation. Comparable peaks were completely absent from similar (*m/z* 183 or 188) precursor ion scans of lipids extracted from untreated control cells (data not shown). Thus, *N*-acetamidine derivatives of PE lipids are clearly formed during the metabolism of TASO by isolated hepatocytes, but are not present in untreated control cells.

A similar search for *N*-acetyl-PE lipids from TASO-treated and untreated hepatocytes was made using the appropriate neutral loss (NL) scans for *m/z* 183 and 188 (Figures 5A–D). In this case, the NL183 scan (Figure 5A) shows a quasi lipid-like pattern, but the masses of the major peaks do not agree well with the endogenous PE lipid composition indicated in Figures 3D or 4A. This strongly suggests that the material giving rise to this spectrum is not derived from endogenous PE lipids. A glance at the corresponding NL188 scan in Figure 5B confirms this, because the spectrum is very weak and looks nothing like the NL183 scan in Figure 5A (whereas the profile in Figure 4B is nearly identical to that in Figure 4A except for the mass offset of 5u). Furthermore, the NL 183 scan of lipid extracted from *untreated* hepatocytes (Figure 5C) looks almost identical to the scan of the lipid from the treated cells (Figure 5A), suggesting that TASO treatment did not affect the materials giving rise to these scans. This conclusion is supported by the absence of corresponding peaks in the M+5 spectra (Figure 5D) which again indicates that the materials do not contain metabolites of the labeled TASO. The peak pattern in Figures 5A and 5C actually resemble somewhat the peak pattern of the survey scan of the PC lipids shown in Figure 3C, and the major peak masses indicated in Figures 5A and 5C (which have ca. 1% of the intensity of the PC scan in Figure 3) correspond to the empirical composition $[PC - H + Na]^+$. This would explain why the masses do not shift after the cells are treated with dual-labeled TASO. From all of this we conclude that *N*-acetyl-PE lipids are essentially absent from the lipid extract of both untreated and TASO-treated hepatocytes. If *N*-acetyl-PE lipids exist in hepatocytes at all, it is only at very low levels, and they clearly are *not* formed during the metabolism of TASO. As a final comment on the amidino-lipid adducts that are formed when cells are treated with TASO, it is clear that there is no discrimination among the endogenous PE lipids with respect to the lengths of their fatty acyl side chains, and that no carbons or hydrogens from TASO are incorporated into the fatty acyl chains of the PE lipids.

Reaction of $TASO_2$ with PE lipids in vitro

To investigate the chemical reactivity of $TASO_2$ toward PE lipids in vitro we oxidized an aqueous solution of TASO (10 equiv.) with hydrogen peroxide (9 equiv) in the presence of DPPE (which was present mainly as liposomes and micelles). This lead to low yields (ca. 5%) of *N*-acetyl-DPPE (**19**) but no observable *N*-acetimidoyl- DPPE (**20**) was formed. Despite the low yield, this result is striking because during the oxidative metabolism of TASO in vivo, only **20** is formed. Since **20** is very stable with respect to hydrolysis, the formation of only **19** and not **20** in vitro means that $TASO_2$ must hydrolyze completely to generate acetylsulfinic acid (**15b** in Scheme 1) prior to reacting with DPPE. When the reaction was carried out in 10% aqueous tetrahydrofuran (THF) solution the yield of **19** increased to 64%, but still no amidino adduct **20** was formed. The exclusive formation of *amidino*-PE lipids (i.e. **18b** in Scheme 1) in living cells must therefore reflect an

intracellular environment in which the thermodynamic activity of water is very low compared even to 10% aqueous THF.

Reaction of $TASO_2$ with a model protein in vitro

To investigate the chemical reactivity of $TASO_2$ toward proteins we oxidized TASO with aqueous hydrogen peroxide in the presence of lysozyme as a model protein. Lysozyme has six lysines and a free amino terminus that could potentially react with acylating agents. It also has two methionines that could react with hydrogen peroxide, four disulfides and no free cysteine -SH groups. In an initial experiment lysozyme (5 mg in 1 mL water) was treated with TASO and H_2O_2 (1.5 and 0.75 mol per mole lysine residue, respectively) at room temperature for 120 min (i.e., to completion of reaction). Direct analysis of the solution by ESI mass spectrometry revealed largely unchanged lysozyme (14,305.5 ± 1 Da; Figure 6A) plus a small new peak (ca. 5%) at M+41 or M+42 that could be either a mono acetimidoyl adduct, a mono-acetyl adduct or a mixture of both (Figure 6B). In addition there was a larger peak (ca. 20%) at M+16 and a much larger peak (ca. 35%) at M+96. Exposing lysozyme to progressively larger excesses of $TASO/H_2O_2$ produced progressive increases primarily in the peaks at M+16, M+32, M+98 and M+196 (data not shown).

The ions at M+98 and M+196 were presumed to arise as non-covalent adducts of lysozyme with one or two molecules of sulfuric acid generated by the oxidative desulfurization of TASO with hydrogen peroxide. The formation of such adducts under ESI conditions is well precedented for basic proteins such as lysozyme (pI 9.3) and ribonuclease A (pI 8.64).[26–28] We confirmed this by showing that simply adding Na_2SO_4 (5 eq./mol lysine) to lysozyme in solution generates very strong non-covalent adduct ions at M+98 and M+196 (75% and 25% of M, respectively; Figure 6C). For further analysis lysozyme treated with $TASO/H_2O_2$ was subjected to tryptic digestion and the digest analyzed by LC-MS/MS. This confirmed that the M+16 peaks in the whole molecule spectra were likely due to oxidation of Met-12 and/or Met-105, as the respective tryptic peptides were clearly present in the digest (data not shown). As indicated in Table 1, peptides containing acetylated lysines corresponding to all six lysine residues were also present (see Supporting Information). We also observed a bis-acetylated derivative of the peptide KVFGR corresponding the N-terminal five residues of lysozyme. However, we observed no evidence for peptides bearing acetimidoyl adducts, which are clearly distinguished from acetyl peptides in both MS1 and MS2 modes (see Supporting Information).

It is of course conceivable that the observed acetyl modifications resulted from the hydrolysis of an acetimidoyl precursor (see Scheme 2, structures **16** and **17**). To rule this out we prepared acetimidoylated lysozyme using only a slight excess of methyl acetimidate hydrochloride. As shown in Figure 6D, methyl acetimidate very efficiently modifies lysozyme, producing a series of species containing up to seven adducts of 41 u each. This would suggest that MA is capable of modifying all six lysines plus the N-terminal amine group of lysozyme. Tryptic digestion and LC-MS/MS analysis of a sample of lysozyme treated with 1.5 mol MA per mol lysine side chain showed that all six lysines were capable of being adducted. We observed only a mono-acetimidoyl adduct of the N-terminal peptide KVFGR; it eluted as a single peak on the chromatogram, but it was not clear from the CID spectrum if it arose from modification of the lysine side chain or the amino terminus. Importantly, however, no acetylated peptides were observed in reactions with methyl acetimidate, indicating that the amidino modification was stable to handling (including tryptic digestion) and was not a precursor to the acetyl adducts formed via treatment with $TASO/H_2O_2$. For comparison in regard to efficiency as a protein lysine modifying reagent, lysozyme was also treated with up to 50 equivalents of acetic anhydride per lysine residue and then submitted to whole molecule mass spectrometry (Figure 6E) as well as tryptic

digestion and LC-MS/MS. Even when added at this great molar excess, acetic anhydride was not as efficient as MA at modifying lysine residues in lysozyme (Table 1). However, all six lysines and the N-terminal amino group were observed to be acetylated in the digest, and for both modifications there was a preference for the K97 over the adjacent K96 in lysozyme.

Discussion

Thioacetamide is an interesting if not unusual hepatotoxin. It is a small, polar, water soluble molecule yet it is capable of causing a variety of noxious biological effects depending on the dose and duration of its administration. Rats fed with TA at 0.025% of their diet for 6 months exhibit decreased growth rates, decreased survival time and prominent cirrhotic changes in their liver.[29] At 0.1% of the diet survival is less than one month, the livers show bile duct proliferation and the hepatocytes show grossly enlarged nucleoli. Rats given TA in their drinking water (0.03%) for 20 weeks exhibit changes that progress from biliary hyperplasia to cholangiocarcinoma in a way that models the human disease progression [30]

Rees et al.[31] investigated the disposition and metabolism of [^{3}H]-TA in rats and observed that less than 1% of the dose was excreted unchanged, while >90% was converted to acetic acid and entered the acetate pool. At the same time tritium was incorporated into proteins > phospholipids > nucleic acid. Liver and kidney slices converted TA into acetamide and radiolabeled phospholipids. From this it was concluded that TA was desulfurized to acetamide, hydrolzed to acetate and biosynthetically incorporated into fatty acids for phospholipid synthesis. However, the results of our lipid analysis by mass spectrometry clearly show that the acyl moiety derived trom [$^{13}C_2D_3$]-TA is adducted onto the head group of PE lipids and is not incorporated into their fatty acyl chains.

Dyroff and Neal[14, 15] characterized the covalent binding of [^{14}C]-TA to liver proteins of rats, and to BSA when incubated with rat liver microsomes and $^{18}OH_2$. Enzymatic digestion of the proteins released [^{14}C]-ε-acetyllysine, and GC/MS analysis of the latter showed that the oxygen atom of the acetyl group originated from water and not molecular oxygen. Since amidines are not easily hydrolyzed to amides,[13, 15] the reactive metabolite of TA (i.e., $TASO_2$, **11b/12b** in Scheme 2) must react first with water to form a new reactive metabolite, acetylsulfinic acid (**15b**), which then acylates lysine side chains. The requirement for two stages of two-electron oxidation to produce a reactive metabolite from a thioamide group is unusual among metabolically activated toxins, but it is even more unusual that a reactive metabolite would react with water to form a new compound that retains a high degree of spontaneous chemical reactivity toward biological nucleophiles.

In subsequent studies of the covalent binding of thiobenzamide metabolites in rat liver we were surprised to observe extensive modification of cellular lipids as well as proteins. We identified 65 different target proteins and observed numerous adducts on ε-amino groups of lysine residues in peptide digests. The adducts included both *N*-benzoyl and *N*-benzimidoyl groups in the ratio of ca. 3:1 12. In contrast the microsomal lipid adducts consisted exclusively of *N*-benzimidoyl derivatives of PE lipids 13. In the present study we observed analogous results from hepatocytes incubated with thioacetamide, i.e., modified lipids contained only *N*-acetimidoyl- PE derivatives whereas the proteins contained both amide and amidine adducts (*unpublished results, manuscript in preparation*). To investigate this discrepancy further we performed chemical oxidations of TASO in the presence of model lipids and proteins and analyzed the products by mass spectrometry.

The oxidation of TASO with hydrogen peroxide in the presence of DPPE under aqueous conditions where liposomes and micelles are present resulted in a low yield of modified

DPPE containing only amide adducts. Using a larger excess of TASO/HOOH or a higher reaction temperature improved the yield modestly but again only amide adducts were formed. This suggests that in the presence of water as a *solvent*, both the hydrolysis of $TASO_2$ to **15b** and the hydrolysis of **15b** to acetic acid are faster than the reaction of $TASO_2$ with DPPE. Since most biological systems are only 60% water, we carried out the same reaction in THF containing only 10% water to see if that would favor amidine formation. Reducing the water content significantly improved the overall yield of adduct, but again only the amide adduct was formed.

The exclusive formation of amidine-type DPPE adducts in TASO-treated hepatocytes and TB-treated rats implies that upon enzymatic formation and release in living cells, the reactive metabolite (i.e., **11** or its tautomer **12** does not have sufficient time, or does not encounter sufficient *local* water, to hydrolyze to **16** before reacting with the amine groups of PE lipids. This does not appear to be the case for proteins, however, as with both TB[12] and TA (*unpublished results, manuscript in preparation*), three-fourths of the lysine adducts formed in cells are amides. The enzymes that oxidize thioamides (i.e., FMO and/or cytochromes P450) are both located in the endoplasmic reticulum, a membranous structure that occupies 15% of the cell volume. The ER membrane consists of ca. 70% protein and 30% lipids with PCs (55%) and PEs (20–25%) predominating. Its lumen also contains numerous soluble proteins, some of which are targets for reactive metabolites including those of TB and TA.[32] Clearly the behavior of thioamide-derived reactive metabolites in the interior of a living cell is not well modeled by their behavior in aqueous solution.

The toxicity of compounds that generate electrophilic metabolites is usually associated with the covalent binding of the metabolites to cellular macromolecules. Those that covalently modify DNA are often mutagenic or even carcinogenic, while those that covalently modify proteins are often associated with cytotoxic effects. Lipids are not usually thought of as targets for electrophilic metabolites, although several precedents involving the amine group of PE phospholipids exist. For example phosgene, formed by oxidative metabolism of chloroform,[33] forms lipid adducts by crosslinking two molecules of PE through their amine groups in a urea linkage. Similarly the oxidative metabolite of halothane trifluoroacetylates the amine group of PE lipids[34], while the reactive metabolite generated by the action of cysteine conjugate β-lyase on S-(1,1,2,2-tetrafluoroethyl)-L-cysteine generates difluorothioacetyl-PE adducts[35] and, as noted above, the *S,S*-dioxide metabolite of thiobenzamide benzimidoylates the amine group of PE lipids in microsomal membranes.[12]

While lipids are not well known as *targets* of reactive electrophiles, they are well known as *precursors* of reactive electrophiles including malondialdehyde, 4-hydroxynonenal, 4-oxononenal and related γ-ketoaldehydes formed from PGH_2 or certain autoxidized arachidonyl esters (i.e., levuglandins or isoprostanes, respectively).[36] These lipid-derived species are quite reactive toward biological nucleophiles including the amine group of proteins and PE lipids, with which they react to form *N*-substituted pyrroles.[18, 37, 38] Interestingly, the pyrrolic adducts isoprostane-derived isoketals have been shown to be directly cytotoxic in HEK293 cells and human umbilical vein endothelial cells.[38]

Long-chain *N*-acyl PEs occur naturally in bacteria, yeast, plants and vertebrates from fish to mammals.[18] Their concentration is low in human tissues (<0.1% of phoshphlipids)[39] and plasma (20–200 nM).[40] They are synthesized in response to stresses such as ischemia, dehydration and glutamate-induced excitotoxicity.[17, 19, 40] They also serve as precursors of N-acylethanolamines including anandamide, the endogenous ligand for the cannabinoid receptor, via phospholipase D hydrolysis.[16, 17, 40, 41] *N*-Acylation converts a PE from a zwitterionic lipid to an anionic lipid altering intramolecular hydrogen bonding and conformation around the head group. If the acyl chain is longer than about C_8 it can bend

around and insert into the bilayer raising its chain melting temperature.[41] *N*-Acetyl PEs are good substrates for phospholipase A_2 but as the *N*-acyl chain lengthens to C_{16} the rate of hydrolysis falls to zero. It has not been determined if *N*-acetyl-, *N*-acetimidoyl, *N*-benzoyl- or *N*-benzimidoyl PE lipids can be hydrolyzed by phospholipase D, nor is anything known about the overall clearance of such metabolites from the cell or organism, or their potential for exerting toxic effects. This area is prime for investigation using cultured cells, stable isotopes and mass spectrometry.

Although the existence of N-terminal acetylation as a post-translational modification of cellular proteins has long been known, mass spectrometry has recently revealed that a plethora of proteins undergo reversible *N*-acetylation at *internal* positions (i.e. the "acetylome"), and that these PTMs have important consequences for protein function in diverse cellular processes including the cell cycle.[42] Given that a hepatotoxic dose of TA leads to extensive protein acetylation (as well as acetimidoylation) it is not difficult to imagine that this could interfere with endogenous signaling and regulatory mechanisms in a way that would compromise the cell.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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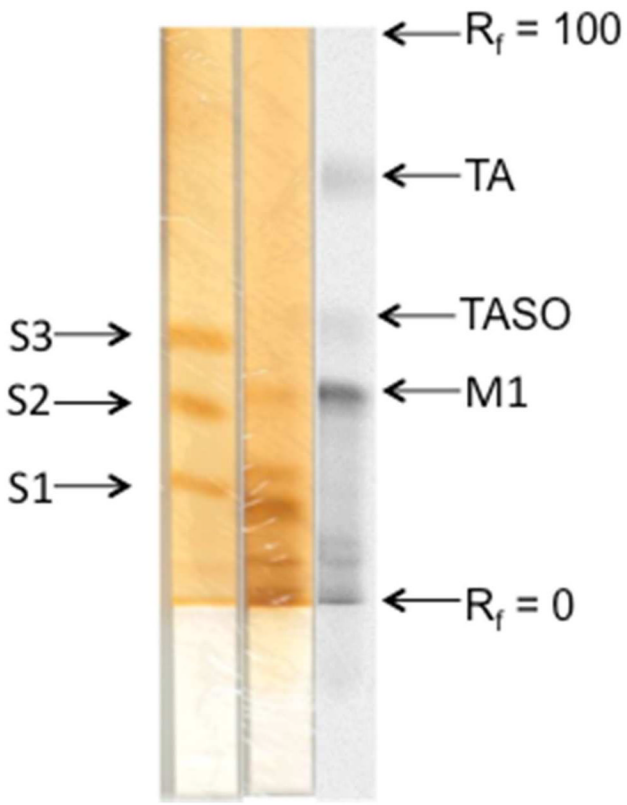


Figure 1.

TLC analysis of lipid standards and lipid extract from TASO-treated hepatocytes. Lane 1, standards of *N*-acetyl-DPPE (S3, R_f = 0.48), *N*-acetimidoyl-DPPE (S2, R_f = 0.37) and DPPE (S1, R_f = 0.20); solvent = $CHCl_3$/MeOH/NH_4OH, 80:20:3 v/v. Lane 2, lipid extract of hepatocytes after incubating 4 h with [^{14}C]-TASO. Lane 3, phosphorimage of lane 2.

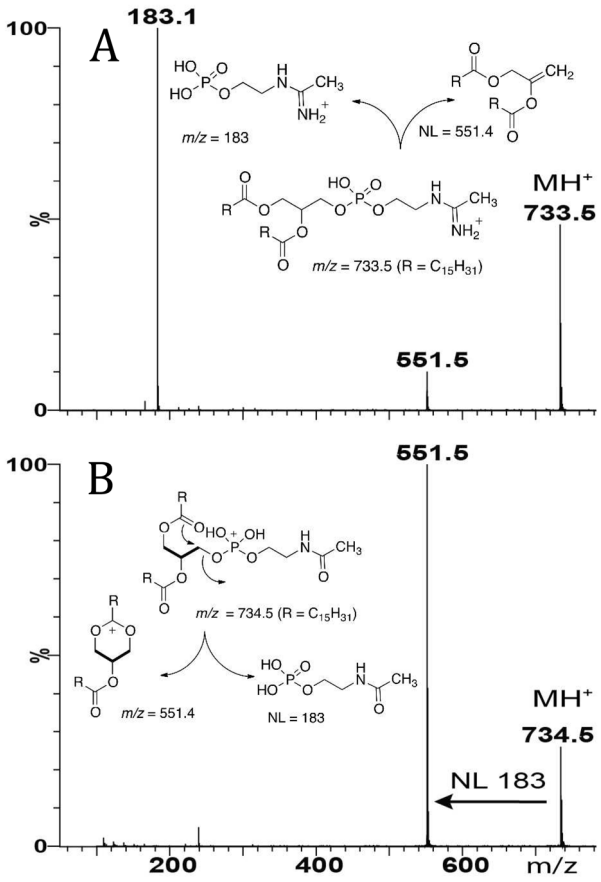


Figure 2.

ESI mass spectra of A) *N*-acetimidoyl-DPPE and B) *N*-acetyl-DPPE standards showing the relevant fragmentation. For the more basic amidine the charge is carried by the head group derived fragment (*m/z* 183), while for the less basic amide the charge is carried by the diacylglycerol-derived fragment (*m/z* 551).

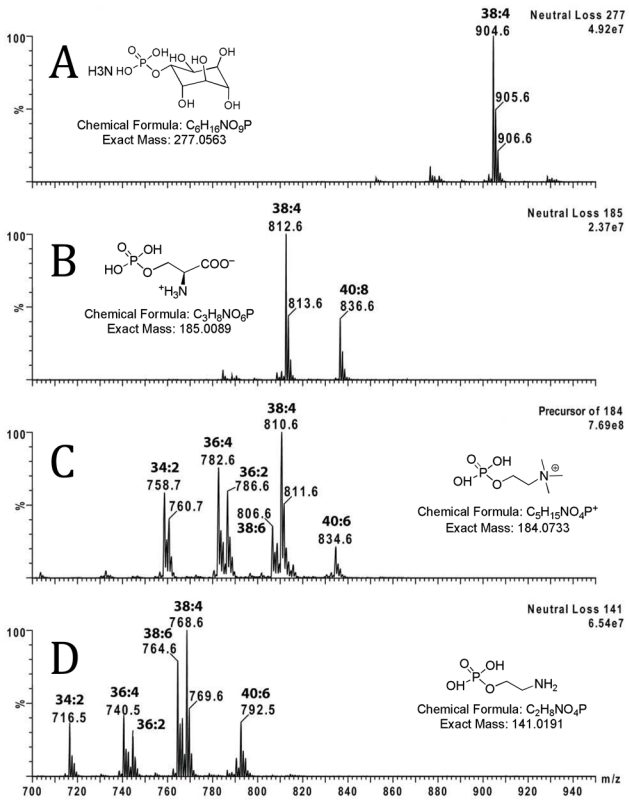


Figure 3.

Class-specific phospholipid survey scans applied to a lipid extract of untreated hepatocytes. A) Neutral loss of 277 scan for phopshatidylinositols. B) Neutral loss of 185 scan for phosphatidylserines. C) Scan for precursors of 184 for phosphatidylcholines. D) Neutral loss of 141 scan for phosphatidylethanolamines. Insets show the respective polar group involved in the diagnostic fragmentation. Major peaks are labeled by *m/z* and the total number of carbons and double bonds in the two acyl sidchains. In all cases, 38:4 is the major diacylglycerol moiety.

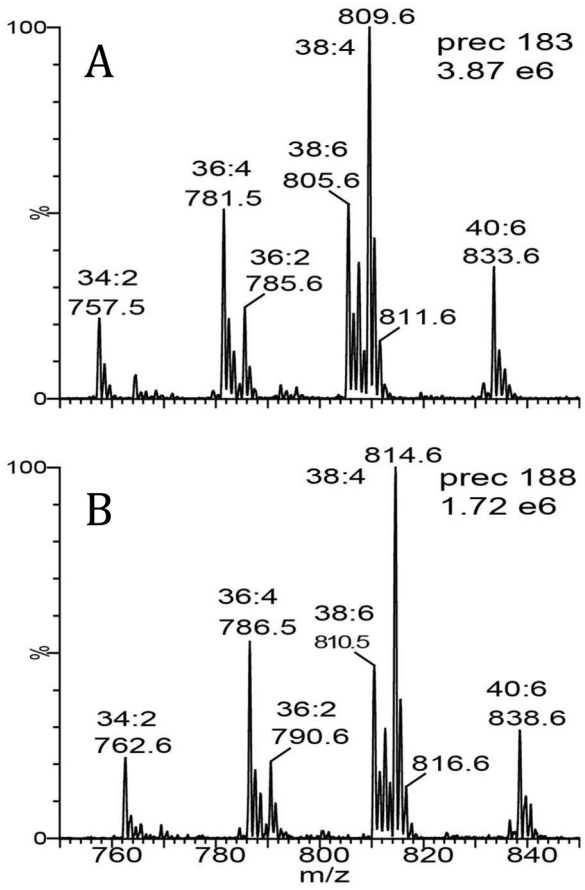


Figure 4.

Survey scans for *N*-acetimidoyl PE lipids in lipid extract of hepatocytes treated with a mixture of TASO and [$^{13}C_2D_3$]-TASO (10:6 mole ratio). Panel A, precursors of *m/z* 183. Panel B, precursors of *m/z* 188. Major peaks are labeled by *m/z* and the total number of carbons and double bonds in the two acyl sidchains.

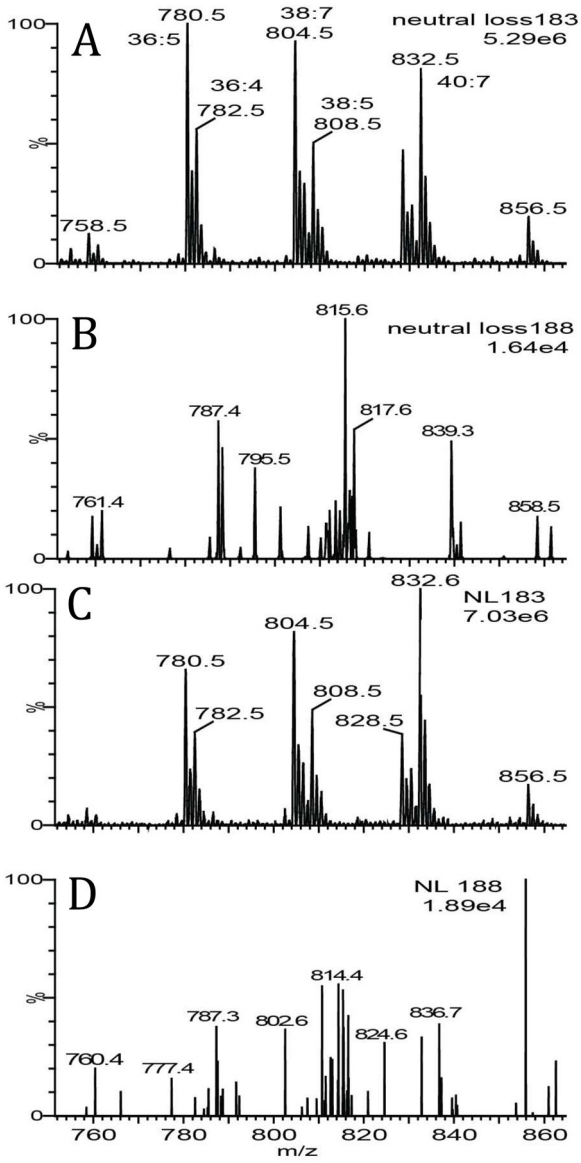


Figure 5.

Survey scans for *N*-acetyl PE lipids in lipid extract of hepatocytes treated with a mixture of TASO and [$^{13}C_2D_3$]-TASO (10:6 mole ratio). Panel A, neutral loss of *m/z* 183. Panel B, neutral loss of *m/z* 188. Panels C and D are analogous scans made from lipid extract of untreated hepatocytes. Major peaks are labeled by *m/z* and the total number of carbons and double bonds in the two acyl sidchains. See text for discussion.

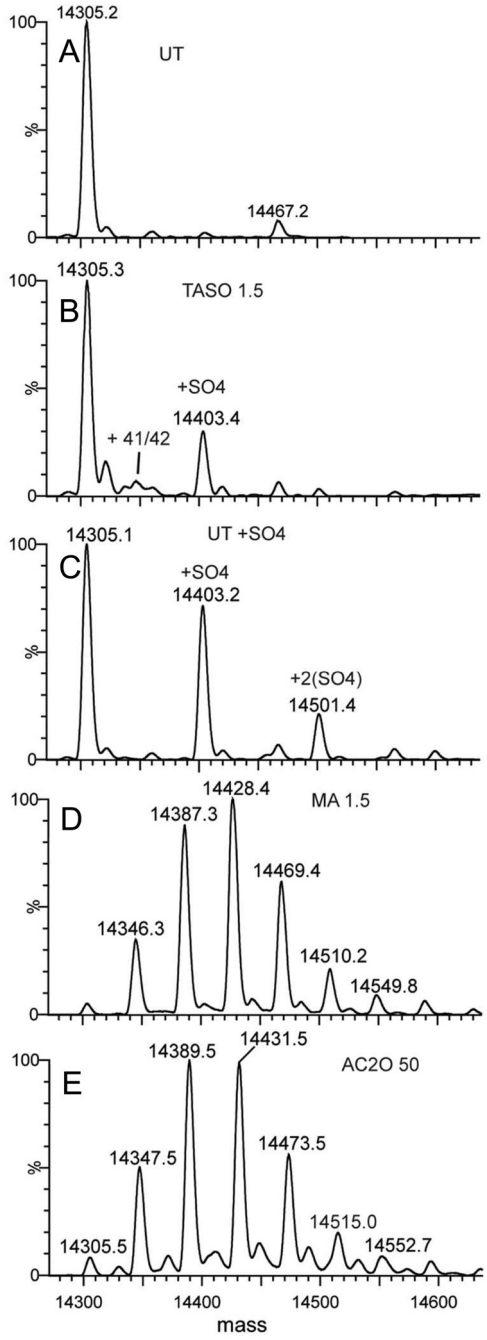
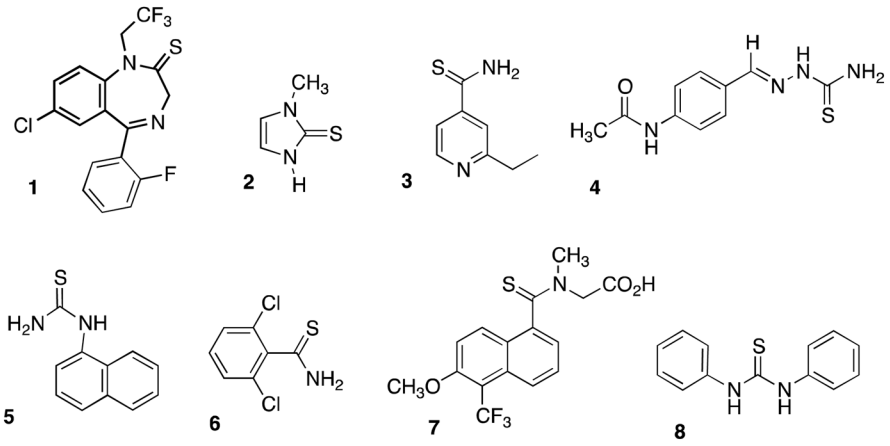
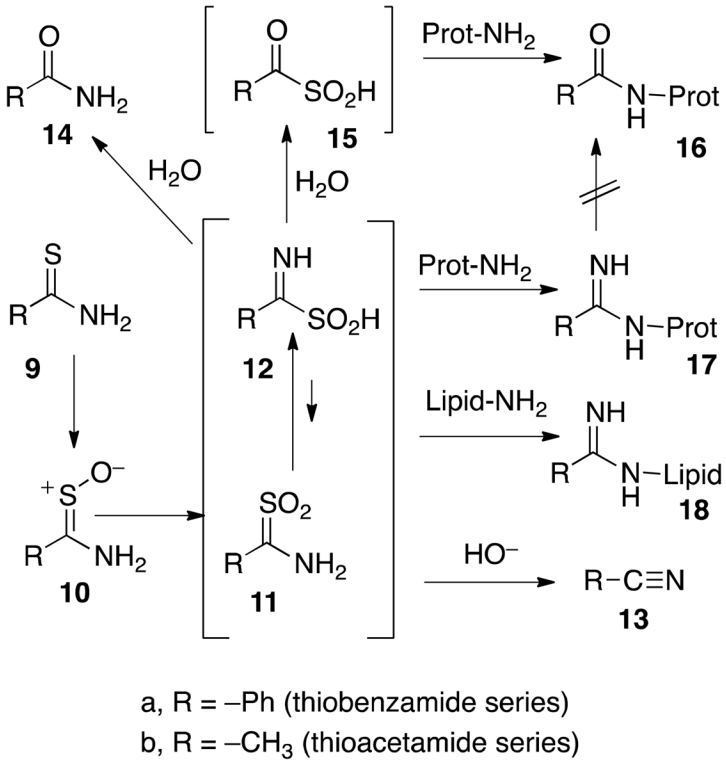


Figure 6.

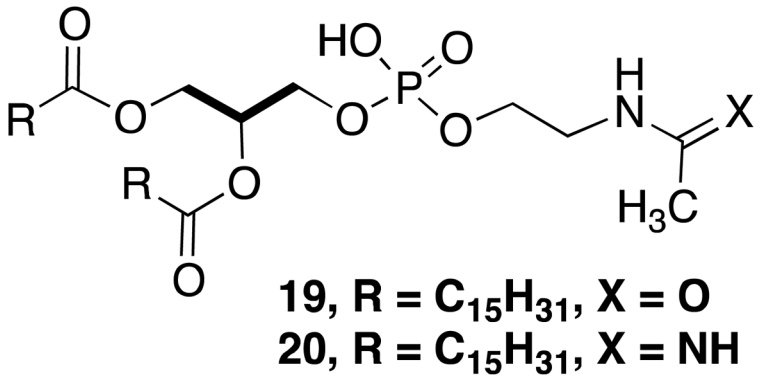
ESI mass spectra of lysozyme, either unreated (A), or treated with with 9 equiv. TASO/ HOOH (B), 30 equiv. sodium sulfate (C), 9 equiv. methyl acetimidate (D), or 300 equiv. acetic anhydride (E). All samples were prepared in deionized water.



Scheme 1.
Representative structures of biologically active thioarbonyl compounds.



Scheme 2.
Bioactivation of thioamide derivatives.



Scheme 3.
Structures of modified DPPE standards.

Table 1

Modification of lysine residues on lysozyme by acylating reagents.

Reagent (mol/mol lysine)	Adduction[a]	Observed Modifications[b]
TASO/H_2O_2 (1.5/0.75)	<0.05	All lysines, amides only
methyl acetimidate (1.5)	2.8	All lysines, amidines only
acetic anhydride (50)	2.1	All lysines, amides only

[a] Weighted average of number of adducts per molecule of protein by whole molecule MS.

[b] Modifications observed by LC-MS/MS analysis of tryptic digest. See Supporting Information.