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Metabolic fate of endogenous molecular damage: Urinary glutathione conjugates of DNA-derived base propenals as markers of inflammation

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Although mechanistically linked to disease, cellular molecules damaged by endogenous processes have not emerged as significant biomarkers of inflammation and disease risk, due in part to poor understanding of their pharmacokinetic fate from tissue to excretion. Here, we use systematic metabolite profiling to define the fate of a common DNA oxidation product, base propenals, to discover such a biomarker. Based on known chemical reactivity and metabolism in liver cell extracts, 15 candidate metabolites were identified for liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) quantification in urine and bile of rats treated with thymine propenal (Tp). Analysis of urine revealed three metabolites (6% of Tp dose): thymine propenoate and two mercapturate derivatives of glutathione conjugates. Bile contained an additional four metabolites (22% of Tp dose): cysteinylglycine and cysteine derivatives of glutathione adducts. A bis-mercapturate was observed in urine of untreated rats and increased approximately three- to fourfold following CCL2-induced oxidative stress or treatment with the DNA-cleaving antitumor agent, bleomycin. Systematic metabolite profiling thus provides evidence for a metabolized DNA damage product as a candidate biomarker of inflammation and oxidative stress in humans.

DNA damage | metabolism | biomarker | mass spectrometry | oxidative stress

Endogenous DNA damage has long been considered a potentially useful source of biomarkers of diseases associated with inflammation and oxidative stress given the strong mechanistic links to the pathophysiology of cancer and the broad spectrum of damage chemistries, such as alkylation, oxidation, deamination, nitration and halogenation (1–6). Major limitations to the development of endogenous DNA damage products as biomarkers involve the choice of a sampling compartment, with invasive sampling of tissues preventing large-scale human studies, and a lack of appreciation for the biological fate of endogenous DNA lesions following their formation in a tissue. The latter problem is reflected in the potential for rapid DNA repair to maintain relatively low steady-state levels of DNA damage even in severely inflamed tissues (1). Although there have been attempts to characterize endogenous DNA damage products released from cells into accessible compartments such as urine, blood or feces (7, 8), the biotransformational fates of the damage products, such as the metabolism after release by DNA repair, have not been rigorously assessed, which has the potential to allow important biomarker candidates to escape detection. One notable exception arises in the recent studies of the metabolic fate of the 3-(2-deoxy-β-D-erythro-pentofuranosyl)pyrimido[1, 2-d]purin-10(3H)-one (M₄dG) adduct arising from reaction of 2-deoxyguanosine (dG) with the endogenous electrophiles malondialdehyde and base propenals. When released from cells presumably by nucleotide excision repair, M₄dG is found at low levels (10–20 fmol/kg) in human urine (9). However, Marnett and coworkers discovered that intravenous (i.v.) administered M₄dG was oxidized by hepatic xanthine oxidase to generate 6-oxo-M₄dG, which was excreted to a small extent in urine and mainly in bile (10–12). On the basis of this example, we developed a logical and systematic approach of in vitro metabolic profiling to predict the in vivo metabolic fate of endogenous DNA lesions and other damage products, using the base propenal products of DNA oxidation to illustrate the approach.

Although most biomarker studies focus on nucleobase damage products, such as 8-oxoG, oxidation of 2-deoxyribose in DNA yields thousands of strand breaks in each human cell on a daily basis (13). Oxidation of 2-deoxyribose produces a spectrum of stable and reactive products such as the electrophilic base propenals (e.g., thymine propenal in Fig. 1) that contain an α,β-unsaturated carbonyl capable of reacting with nucleophiles in DNA, protein and low molecular weight thiols, such as glutathione (GSH) (13). Base propenals are similar in their reactivity to the lipid peroxidation product malondialdehyde that reacts to form M₄dG in DNA and protein carbonyls (7, 13, 14). The α,β-unsaturated carbonyl in base propenals is also a target for nucleophilic attack by the thiol of GSH, which is present in cells at millimolar concentrations (15). These facts make base propenals ideal substrates for glutathione S-transferases (GSTs) (16, 17).

The example of base propenals illustrates the importance of considering the potential for biotransformation of endogenous damage products in their development as biomarkers. The numerous

Significance

Endogenous DNA damage is mechanistically linked to inflammation and cancer, yet the multitude of DNA damage products have not emerged as significant biomarkers partly due to poor understanding of their metabolic fate following formation in tissues. Using a systematic approach of metabolite profiling, we identified 15 candidate metabolites of a common DNA lesion, thymine propenal (Tp), of which three and seven compounds were found in the urine and bile, respectively, of Tp-treated rats. Only one metabolite, a bis-mercapturate derivative, was observed in urine from untreated rats and increased approximately three- to fourfold upon treatment with toxicants producing oxidative stress and DNA oxidation. This metabolite thus represents a strong biomarker candidate for inflammation and oxidative stress.

Author contributions: W.J. and P.C.D. designed research; W.J. and I.R.B. performed research; W.C. contributed new reagents/analytic tools; W.J., W.C., K.T., I.R.B., and P.C.D. analyzed data; and W.J., W.C., K.T., I.R.B., and P.C.D. wrote the paper.

The authors declare no conflict of interest.

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novel and abundant metabolites readily quantified in urine as biomarkers of oxidative stress.

Results

Defining Candidate Biomarkers by Metabolite Profiling in Vitro. The strategy for developing biomarkers based on metabolites of damaged biomolecules involves (i) in silico assessment of metabolic targets and reactive sites in the parent molecule, (ii) in vitro analyses of biotransformation products, and (iii) in vivo validation of candidate metabolites as biomarkers, using chromatography-coupled mass spectrometric (LC-MS) methods to identify and quantify the metabolites. The α,β-unsaturated carbonyl group of base propenals suggested metabolic pathways involving GSH conjugation and aldehyde oxidoreductases/dehydrogenases, so the products of these potential reactions were progressively assessed in a series of in vitro studies.

The first analysis involved a direct reaction of a representative base propenal, Tp, with glutathione (GSH), based on the premise that substrates for glutathione S-transferases (GSTs) also react directly, albeit at a slower rate, with glutathione (GSH). The reaction occurred rapidly ($t_{1/2} = 105$ s, $37 \degree C$; SI Appendix, Fig. S2) and resulted in three major reaction products, as shown in Fig. 1.

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Possible fates of an electrophilic product such as the base propenal include reactions with nucleic acids, proteins and GSH at the cellular site of formation in an inflamed tissue, diffusion or transport into the extracellular space and blood circulation, metabolism in the liver and other organs, and finally excretion in urine or bile. For example, the oxidative stress that gives rise to base propenals and other damage products also up-regulates metabolic and detoxification enzymes such as GSTs, glutathione peroxidase (GPXs) (18), and of relevance to the aldehyde in base propenals, aldehyde dehydrogenases and aldo-keto reductases (19, 20). Therefore, the major biotransformational fates and excretory forms of an endogenous damage product must be assessed in the form of a metabolite profile, with the goal of identifying stable, abundant biomarkers of mechanism and risk. Here we illustrate this approach using thymine propenal (Tp) derived from oxidatively damaged DNA, with the discovery of novel and abundant metabolites readily quantified in urine as biomarkers of oxidative stress.

Results

Defining Candidate Biomarkers by Metabolite Profiling in Vitro. The strategy for developing biomarkers based on metabolites of damaged biomolecules involves (i) in silico assessment of metabolic targets and reactive sites in the parent molecule, (ii) in vitro analyses of biotransformation products, and (iii) in vivo validation of candidate metabolites as biomarkers, using chromatography-coupled mass spectrometric (LC-MS) methods to identify and quantify the metabolites. The α,β-unsaturated carbonyl group of base propenals suggested metabolic pathways involving GSH conjugation and aldehyde oxidoreductases/dehydrogenases, so the products of these potential reactions were progressively assessed in a series of in vitro studies.

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in Fig. 2: a pair of enantiomeric GSH Michael adducts (M-GSH, 1a), a GSH addition/base elimination product (AE-GSH, 2a), and a bis-GSH adduct (bis-GSH, 3a). All product structures were characterized by combinations of collision-induced dissociation (CID) and high-resolution MS, NMR spectroscopy, and comparison with synthetic standards. For example, 1H-NMR was used to confirm the NaBH₄-induced reduction of the aldehyde of 1a, 2a, and 3a to an alcohol (SI Appendix, Table S5). The 1a enantiomers underwent a rapid β-elimination (t₁/₂ ~ 47 min) to form the parent Tp and GSH, or to 2a and thymine (Fig. 2C and SI Appendix, Fig. S1B). Although high-resolution MS of 3a confirmed its molecular formula (SI Appendix, Table S5), 3a was found to undergo rapid β-elimination to 2a (Fig. 2D and SI Appendix, Fig. S1B) with a t₁/₂ of ~8 min, similar to the instability of 1a. A similar instability has been observed in 1, 4-butythiol Michael-type adducts (21). In contrast, 2a was found to be stable (Fig. 2E), with the trans configuration confirmed by 1H-NMR with a coupling constant J_H-H = 15 Hz (SI Appendix, Table S5), in agreement with the previous studies (22). To compare products of direct GSH reaction with GST catalyzed reactions, Tp was added to a mixture of equine GSTs in the presence of GSH. The GST-catalyzed reaction revealed similar levels of 2a compared with the direct reaction but a twofold higher level of 1a (Fig. 3A). These results confirm that thymine propenal is a GST substrate, which is consistent with previous studies (17).

To identify other candidate metabolites, we next assessed the reaction of Tp and GSH in rat liver extracts. Reactions with the microsomal fraction yielded a product spectrum similar to the direct reaction (SI Appendix, Fig. S3A), whereas reactions in the cytosolic fraction were very rapid, with complete consumption of Tp, as well as no detection of any metabolites from the direct reaction, within 15 s of initiating the reaction (SI Appendix, Fig. S3B). As shown in Fig. 4, the spectrum of products from the cytosolic fraction included oxidized Tp (Tpoxide, 5) and alcohol reduction products of M-GSH (M-GSHred, 1b), AE-GSH (AE-GSHred, 2b), bis-GSH (bis-GSHred, 3b), and Tp (Tpred, 4). All of these metabolites proved to be stable to degradation.

Many of the base propenal metabolites identified in vitro represent GSH adducts that would be further processed by γ-glutamyl transferases, cysteinylglycinyl dipeptidase and N-acetyl transferase to cysteinylglycine (CysGly) and cysteine (Cys) adducts for excretion in bile and to mercapturic acid adducts for excretion in urine (23, 24). The predicted structures of these derivatives of Tp-GSH adducts are shown in Fig. 1 (1c–1e, 2c–2e, 3c–3e). Toward the goal of identifying these metabolites in urine and bile, synthetic standards were prepared for 1b–1e, 2b–2e, 3b–3e, 4–6 and LC-MS/MS methods were developed (SI Appendix, Table S1) to quantify all of these species in urine and bile from Tp-treated rats and in rats subjected to oxidative stress.

Identifying Tp Metabolites in Urine and Bile from Tp-Treated Rats.

With the set of predicted Tp metabolites (Fig. 1), we proceeded to assess the metabolism of Tp in rats exposed by i.v. administration through a jugular vein catheter. Sampling of blood and bile was performed through jugular vein and bile duct catheter, respectively, with urine collected over dry ice in 8-h intervals in metabolic cages without food or water to contaminate the collection.

Of the 15 targeted compounds, analysis of urine samples revealed three major metabolites excreted within 8 h of i.v. administration of Tp: the mercapturic acid derivatives 1e (M-NAc\textsuperscript{red}) and 3e (bis-NAc\textsuperscript{red}) and the Tp oxidation product 5 (Fig. 5 and SI Appendix, Table S2). Tp was not detected in the urine nor were the metabolites conjugated with Cys (1d, 2d, 3d, 1c, 2c, 3c, and GSH (1b, 2b, 3b). This observation was not unexpected and is likely due to the high activity of γ-glutamyl transferases, CysGly dipeptidases and N-acetyl transferases in the kidney. Quantitative analysis using external calibration curves based on synthetic standards revealed that 1c, 3e, and 5 accounted for ~6% of the i.v. injected dose (Fig. 5C). Interestingly, bis-NAc\textsuperscript{red} 3e is the only metabolite detected in the urine of saline-treated rats (Fig. 5A and SI Appendix, Table S2) and was present at levels ~39-times lower than the bis-NAc\textsuperscript{red} 3e adduct level detected in Tp-treated rats during 24 h of urine collection (SI Appendix, Table S2).

Given the low recovery of urinary Tp metabolites, we considered the possibility that Tp and its metabolites are excreted in bile. Bile was periodically collected through biliary catheters over 6 h following i.v. injection of Tp and the bile samples were analyzed by LC-MS/MS for the presence of the 15 targeted metabolites. With the highest levels observed in the first 30 min following injection, we detected not only the mercapturic acid metabolites 1e and 3e, but also the GSH, CysGly and Cys conjugation products 1b, 1c, 1d, 3d and 3e (Fig. 6 and SI Appendix, Table S3). Surprisingly, Tp\textsuperscript{oxide} 5 was detected at levels ~36 times higher than the next most abundant metabolite 1b, which contrasts with the low level of 5 in urine (Figs. 5C and 6C). In addition, M-NAc\textsuperscript{red} 1e and bis-NAc\textsuperscript{red} 3e were detected in the saline-treated rats (Fig. 7A and SI Appendix, Table S2). The biliary levels of M-NAc\textsuperscript{red} 3e in Tp-treated rats were ~50-fold higher than the levels in saline controls during the 6 h of bile collection (SI Appendix, Table S3). Although bis-NAc\textsuperscript{red} 3e was
approximately sevenfold higher in bile from Tp-treated rats compared with saline controls (SI Appendix, Table S3). Tp treatment caused urinary levels of 3e to be ~39-fold higher than in saline controls (SI Appendix, Table S2). Again using external calibration curves, quantitative analysis of the biliary excretion products revealed that they accounted for ~22% of the i.v. injected dose.

Finally, analysis of blood samples as early as 5 min after i.v. injection revealed no detectable Tp (limit of detection ~10 fmol). This observation raised questions about the stability of base propenals in blood, so we undertook a series of in vitro experiments to assess the reactivity of Tp with serum components. Addition of Tp to commercial preparations of rat serum recapitulated the observations in the blood of Tp-treated rats, with no detectable Tp in as little as 2–3 min after addition of Tp to the serum. To assess the reactivity of Tp with serum nucleophiles, the second-order rate constants (37 °C) for the reaction of Tp with BSA, CysGly and Cys were found to be 1.4 ± 0.12, 28 ± 1.9, and 33 ± 2.1 M⁻¹s⁻¹, respectively. Using concentrations of the major sulfur nucleophiles in serum (25) (GSH, 3.9 μM; Cys, 183 μM; CysGly, 29 μM; and GluCys, 4.9 μM), we calculated pseudo-first-order rate constants for Tp consumption in serum. The overall first-order rate constant for reaction of Tp with these sulfur components and albumin is estimated to be 7.7 × 10⁻³ s⁻¹, which yields a Tp half-life of ~90 s (SI Appendix, Table S6).

Considering that other serum components will also react with Tp (e.g., immunoglobulins, etc.), it is reasonable to conclude that Tp is rapidly consumed by direct reactions in serum and blood, which would account for our inability to detect it in serum after 2–3 min of incubation or in blood after i.v. injection in rats. It is also possible that there is a catalytic activity that consumes Tp in blood or serum, or some kind of sequestration of the Tp in lipids or proteins that accounts for the inability to detect free Tp in rat blood after injection.

**Tp Metabolites as Biomarkers of Oxidative Stress.** The evidence for Tp metabolism in rats and the detection of a variety of Tp metabolites as urinary and biliary excretion products, including two putative endogenous species present in saline-treated rats, suggested that the readily accessible urinary Tp metabolite could serve as a biomarker of oxidatively damaged DNA and of oxidative stress more broadly. To test this hypothesis, rats were treated by intraperitoneal (i.p.) injection of a DNA-cleaving, base propenal-generating anticancer drug, bleomycin (26), or with carbon tetrachloride (CCl₄) as a toxicant that induces widespread inflammation and oxidative stress (27). Subsequent analysis of urine samples revealed only one metabolite, bis-NAcled 3e, detected in urine from the saline-treated, bleomycin-treated, and CCl₄-treated rats (Fig. 7A). Quantitation using external calibration revealed that the urinary level of bis-NAcled from the bleomycin- and CCl₄-treated rats is ~3- to 4-times higher than that from the saline-treated group (Fig. 7B). Coupled with the observation of increased levels of bis-NAcled in the urine and bile from rats treated with Tp, these results suggest that the increase in bis-NAcled detected in the urine arises from base propenals generated during DNA damage by the stressors.

**Discussion**

It is abundantly clear that local and systemic oxidative stress and inflammation generate reactive chemical species that cause damage to all types of cellular molecules and this pathology is mechanistically linked to disease. However, with the notable exception of myeloperoxidase-derived lesions (28), damaged molecules have not emerged as significant biomarkers of these stresses in the same way that C-reactive protein, for example, has been exploited as a marker for systemic inflammation and cardiovascular disease risk (29). C-Reactive protein does not fulfill all of the criteria for a biomarker because its relationship to inflammation and disease is unknown. DNA damage, on the other hand, has a much firmer link to oxidative stress, inflammation and cancer as a mediator of mutations that cause the disease (30, 31). We argue that, in addition to incomplete knowledge of the damage actually forming in human cells, the lack of appreciation for DNA lesions as biomarkers arises in part from limited understanding of the fate of DNA damage products from the moment of formation in a tissue to release in some form from cells to final excretion from the body in clinically- and epidemiologically-relevant sampling compartments. Here we
Targeted analysis of 15 predicted Tp metabolites in urine from rats treated with thymine propenal (Tp, 6). Following i.v. administration of Tp or saline, urine was collected on dry ice over 4-h time periods (0–4, 4–8, and 8–16 h) and subsequently resolved into less polar (A) and more polar (B) fractions by HPLC, followed by LC-MS/MS analysis of each fraction. A and B illustrate the analysis with urine from the 4–8 h collection. Compounds were identified by comparison with synthetic standards (A and B, Top) and by fragmentation patterns during multiple reaction monitoring (MRM) (A and B, Middle and Bottom). LC-MS/MS analysis of the less polar fraction (A) revealed the presence of compounds 1b–1e, 3c, and 5 in urine from rats treated with Tp (A, Middle), and compound 3c in urine from rats treated with saline (A, Bottom). The more polar compounds 2c, 2d, 3c, and 3d were not observed in urine from either Tp- or saline-treated rats (B, Bottom). C) Quantitation of 1e, 3c, and 5 in urine from rats treated with Tp, using external calibration curves generated with synthetic standards. The structure for each compound was confirmed by comparison with synthesized standards as described in SI Appendix, with structural details shown in SI Appendix, Table S5.
**Fig. 6.** Targeted analysis of 15 predicted Tp metabolites in bile from rats treated with thymine propenal (Tp, 6). Following i.v. administration of Tp or saline, bile was collected at various times over 6 h and subsequently resolved into less polar (A) and more polar (B) fractions by HPLC, followed by LC-MS/MS analysis of each fraction. A and B illustrate the analysis with bile collected at 0.25 h after administering Tp. Compounds were identified by comparison with synthetic standards (A and B, Top) and by fragmentation patterns during multiple reaction monitoring (MRM) (A and B, Middle and Bottom). LC-MS/MS analysis of the less polar fraction (A) revealed the presence of compounds 1b–1e, 3e, and 5 in bile from rats treated with Tp (A, Middle), and compounds 1e and 3e in bile from rats treated with saline (A, Bottom). Of the more polar compounds 2c, 2d, 3c, and 3d, only 3d was observed in bile from saline-treated rats (B, Bottom). (C) Quantitation of 1b–1e, 3d, 3e, and 5 in bile from rats treated with Tp, using external calibration curves generated with synthetic standards. The structure for each compound was confirmed by comparison with synthesized standards as described in SI Appendix, with structural details shown in SI Appendix, Table S5.

\(\gamma\)-glutamyltransferase activity in rat liver, relative to the high level in rat kidney, is the predominant route for initial metabolism of GSH conjugates of Tp, whereas the kidney is the major site for slower \(N\)-acetylation of the resulting Cys conjugates to form the mercaptauic acid products observed in urine. This type of interorgan transfer of GSH conjugate metabolites during mercapturic acid processing is well established (34), though the established hepatic capacity for mercapturic acid formation appears to be minimal for Tp metabolism in rats.

We must now ask whether the metabolites lacking the thymidine nucleobase, 3a–e, are possibly derived from metabolism of endogenous molecules other than Tp or the other base propenals, such as malondialdehyde or acrolein (lipid peroxidation products). This scenario seems unlikely given our observations that 3a–d appeared only for Tp, 3e increased significantly with Tp administration and 3e increased significantly following treatment with bleomycin, an agent that is highly specific for cleaving DNA to produce base propenals (26). The background production of 3e is unlikely to involve malondialdehyde or acrolein, because malondialdehyde is metabolized mainly to CO\(_2\) (35, 36), whereas acrolein is mainly excreted as the monoacetylcysteine adduct in vivo (37, 38). Even if malondialdehyde metabolism to CO\(_2\) is not quantitative, malondialdehyde does not form GSH conjugates under biologically relevant conditions (39), due to a nonelectrophilic beta-carbon caused by resonance effects with the adjacent oxygen and the fact that the oxygen is a very poor leaving group (39). This is the same reason that malondialdehyde only reacts with G to form M\(_2\)G at extremely high concentrations, whereas base propenals react 1,000 times more efficiently: Malondialdehyde is relatively inert to nucleophilic attack (40). Although labeling of Tp with radioactive or stable isotopes could be used to prove the identity of 3e arising from Tp injections, the presence of 3e in bleomycin- and CCL\(_4\)-treated rats will ultimately be nearly impossible to determine.

Although kidney cannot be ruled out as a source of Tp\(^{\text{OX}}\) (5), the fact that it is excreted into bile at levels significantly higher than urine (SI Appendix, Tables S2 and S3) suggests that the oxidized Tp is formed in the liver and excreted mainly in bile, with trace amounts in urine analogous to the appearance of 1e and 3e in bile. The high level of Tp\(^{\text{OX}}\) 5 (molecular weight 196 g/mol) in bile is at odds with the general rule of biliary clearance for compounds with molecular weights higher than 300 g/mol. It is therefore highly likely that the negative charge of the carboxylate at physiological pH makes Tp\(^{\text{OX}}\) a substrate for organic anion transporters or organic anion transporting peptides in bile canaliculari (41).

Despite the identification of seven metabolites in urine and bile, these potential biomarker candidates accounted for only 28% of the injected dose of Tp, which raises the question about the fate of the bulk of the Tp. Certainly there may be other metabolic pathways such as conjugation with glucuronic acid, sulfate or amino acids. The inability to detect Tp in serum in vitro or in blood immediately following i.v. injection of Tp in rats suggests the possibility of a metabolic activity in blood that intercepts Tp, a nucleophilic reaction with serum components, or sequestration of Tp by a high-affinity binding site in a protein. The latter would bias our analyses because proteins were removed by ultrafiltration. Ultimately, an understanding of the complete fate of base propenals will rely on tracking with radiolabeled compounds.

A major requirement for biomarker development is the existence of a dose–response relationship between the marker and the pathology, in this case formation of base propenals in response to inflammation and oxidative stress. The observation of bis-NAC\(^{\text{oxd}}\) 3e in the urine of saline-treated rats made it a strong candidate for a base propenal-derived biomarker of oxidative stress. To induce the oxidative stress, rats were treated with CCL\(_4\), which is well established to cause acute and chronic tissue injury.
and inflammation (42) by virtue of hepatic metabolism to trichlormethyl and peroxy trichloromethyl radicals (43). One piece of evidence that CCl₄ would lead to formation of base propenals involves the observation by Marnett and coworkers that the level of M₄DG increased approximately twofold in rats treated with base propenal-generating bleomycin (26). The increase in urinary bis-NAC<sub>red</sub> 3e in oxidatively stressed rats suggests that base propenal metabolites may represent biomarkers of inflammation, with strong implications for biomarkers of disease risk.

**Materials and Methods**

**Materials.** The following chemicals were commercially obtained and used without further purification: glutathione (GSH); potassium monohydrogen phosphate (KH₂PO₄); potassium dihydrogen phosphate (KH₂PO₄); liver microsome from female Sprague–Dawley rats; liver cytosol from human; β-nicotinamide adenine dinucleotide phosphate, reduced, sodium salt (NADPH); glutathione S-transferases from equine liver, were purchased from Sigma-Aldrich Chemical. Deionized water was further purified with a Milli-Q system (Millipore) and was used in all experiments.

**Chemical Standards.** Standards for all compounds (1a–1e, 2a–2e, 3a–3e, 4, 5, and 6) were synthesized and structurally characterized as described in detail in SI Appendix, with structural characterizations shown in SI Appendix, Figs. S3–S8 and Table S5.

**Instrumentation.** UV-visible spectroscopy measurements were made on an HP8452 diode-array spectrometer (Agilent Technologies). HPLC analyses with UV spectroscopic detection were carried out on Agilent 1100 series with binary pumps and degassers. Full scan and collision-induced dissociation (CID) mass spectra of in vitro reaction products were obtained from an Agilent Technologies 6430 ion trap LC/MS coupled to Agilent HPLC 1200 series. Quantitation data are obtained from multiple reaction monitoring mode (MRM) from Agilent Technology 6430 QQQ coupled to Agilent HPLC 1290 series.

**HPLC Methods.** Four HPLC methods were applied in the experiment and described as follows.

**HPLC method 1.** HPLC columns specified elsewhere in Materials and Methods were equilibrated with 100% solvent A (0.1% formic acid in water) and 0% B (acetonitrile) at a flow rate of 0.2 mL/min at 30 °C. The solvent was programmed as follows: a linear gradient from the starting solvent to 5% (vol/vol) B in 30 min; a linear gradient increasing from 5 to 46% (vol/vol) B for 12 min; increasing to 100% B in 0.1 min, holding for 10 min; decreasing to 0% B in 0.1 min; and re-equilibrating at initial conditions for 15 min.

**HPLC method 2.** A ZORBAX Eclipse RRHD, XDB-C18 (2.1 × 100 mm, 1.8-μm particle size; Agilent Technologies) was equilibrated with 99.5% solvent A (0.1% formic acid in water) and 0.5% B (acetonitrile) at a flow rate of 0.25 mL/min at 20 °C. The solvent was programmed as follows: a linear gradient from the starting solvent to 13% (vol/vol) B in 13 min; increasing to 100% B in 0.1 min, holding for 10 min; decreasing to 0.5% B in 0.1 min; and reequilibrating at initial conditions for 5 min.

**HPLC method 3.** A ZORBAX Eclipse, XDB-C8 (4.6 × 250 mm, 5-μm particle size; Agilent Technologies) was equilibrated with 99.5% solvent A (0.1% formic acid in water) and 0.5% B (acetonitrile) at a flow rate of 0.3 mL/min at 20 °C. The solvent was programmed as follows: a linear gradient from the starting solvent to 7% (vol/vol) B in 12 min; increasing to 100% B in 0.1 min, holding for 10 min; decreasing to 0.5% B in 0.1 min; and reequilibrating at initial conditions for 15 min.

**HPLC method 4.** A Dionex Acclaimed Polar Advantage, C18 (2.1 × 150 mm, 3-μm particle size; Thermo Scientific) was equilibrated with 100% solvent A (0.1% formic acid in water) and 0% B (acetonitrile) at a flow rate of 0.25 mL/min at 20 °C. The solvent was programmed as follows: an isocratic elution of 100% A for 6 min; a linear gradient to 3% (vol/vol) B in 13 min; a linear gradient from 3 to 20% (vol/vol) B in 10 min; increasing to 100% B in 0.1 min, holding for 10 min; decreasing to 0% B in 0.1 min; and reequilibrating at initial conditions for 15 min.

**Direct Reaction of Thymine Propenal with Glutathione.** Tp (0.1 mM) was reacted with 1 mM GSH at 37 °C in 50 mM phosphate buffer, pH 7.4, for 1 h. The reaction mixture was then analyzed by LC-MS with HPLC Method 1 using HP8452 diode-array spectrometer (Agilent Technologies). HPLC analyses with UV spectroscopic detection were carried out on Agilent 1100 series with binary pumps and degassers. Full scan and collision-induced dissociation (CID) mass spectra of in vitro reaction products were obtained from an Agilent Technologies 6430 ion trap LC/MS coupled to Agilent HPLC 1200 series. Quantitation data are obtained from multiple reaction monitoring mode (MRM) from Agilent Technology 6430 QQQ coupled to Agilent HPLC 1290 series.
trifluoroacetic acid (final concentration) at 0 °C. GSTs were removed using a 10 kDa spin filter. The filtrate was then analyzed by HPLC with UV detection (Agilent 1100 series) using HPLC Method 4 with a Dionex Acclaimed Polar Advantage C18 column (2.1 × 150 mm, 3-μm particle size; Thermo Scientific). Structural characterization of the metabolites is described in SI Appendix.

**Metabolite Stability.** M-GSH 1a, AE-GSH 2a, and bis-GSH 3a were purified from reactions (described earlier) using HPLC Method 1 with a Dionex Acclaimed Polar Advantage, C18 column (2.1 × 150 mm, 3-μm particle size; Thermo Scientific), with fractions containing each species collected on ice. Each purified compound was then incubated in 50 mM phosphate buffer, pH 7.4, at 37 °C for 0, 15, 30, 60, and 120 min for M-GSH 1a; 0, 2, 3, 4, 6, 8, and 12 h for AE-GSH 2a; and 1, 3, 6, and 10 min for bis-GSH 3a. Reactions were quenched by addition of 0.05% trifluoroacetic acid and products analyzed by HPLC with UV detection for 1a and 2a (method described above) and by ion trap LC-MS for 3a (Agilent LC/MSD ion trap mass spectrometer). Structural characterization of the metabolites is described in SI Appendix.

**Reaction with Rat Liver Microsomes.** Tp (0.1 mM) was added to a solution of 1 mM GSH, 1 mM NADPH, and 20 mg/mL micromodal fraction in 50 mM phosphate buffer, pH 7.4, at 37 °C for 1 h. The reaction mixture was then de-proteinized by filtration with 10 kDa spin filter before LC-MS analysis using HPLC Method 1 with a Dionex Acclaimed Polar Advantage C18 column (2.1 × 150 mm, 3-μm particle size; Thermo Scientific). Control experiments were conducted similarly but NADPH was not added. Structural characterization of the metabolites is described in SI Appendix.

**Reaction in Rat Liver Cytosol.** Tp (0.1 mM) was added to a solution of 1 mM GSH, 1 mM NADPH, and 20 mg/mL cytosolic fraction in 50 mM phosphate buffer pH 7.4, at 37 °C for 1 h. The reaction mixture was then analyzed by LC-MS using HPLC Method 1 with a Dionex Acclaimed Polar Advantage C18 column (2.1 × 150 mm, 3-μm particle size; Thermo Scientific). Control experiments were conducted similarly but NADPH was not added. The reaction mixture was de-proteinized by filtration with a 10 kDa spin filter before LC-MS analysis. Structural characterization of the metabolites is described in SI Appendix.

**LC-MS Analysis of in Vitro Thymine Propenal Metabolites.** LC-MS analyses of direct GSH reactions, GST-catalyzed reactions, and reactions in liver extracts were performed using HPLC Method 1 with a Dionex Acclaimed Polar Advantage C18 column (2.1 × 150 mm, 3-μm particle size; Thermo Scientific) on an Agilent 1200 HPLC operated at a flow rate of 0.2 mL/min and coupled to an Agilent LC/MSD ion trap mass spectrometer operating in positive ion mode with nitrogen as the nebulizing and drying gas (20.0 psi, 10.0 L/min, 325 °C). The analyses were performed with the mass spectrometer set to scanning mode, with a scan range from m/z 160–1000. The metabolites were then reanalyzed with the mass spectrometer set in manual CID mode to obtain fragmentation patterns for each of the compounds.

**Administration of Thymine Propenal to Sprague-Dawley Rats.** Animal experiments were performed in accordance with the protocols approved by MIT Committee on Animal Care (CAC) and with the NIH Guide for the Care and Use of Laboratory Animals (44). Female Sprague-Dawley rats (225–250 g) with catheters surgically implanted into the jugular vein and bile duct were obtained from Charles River Laboratories and housed in shoebox cages. Before the experiment the animals were transferred into metabolic cages and fasted for 8 h before the beginning of dosing. For each experiment, three animals were dosed with 0.25 mL of sterile saline as vehicle. Tp (0.1 mM) was added to 0.25 mL of sterile saline, all by injection into the jugular vein catheter. Rats were housed in clean metabolic cages to collect urine over the following intervals: predose (8 h), 0–8, 8–16, 16–24 h. Urine was collected in sterile polypropylene tubes placed in dry ice to reduce adventitious chemical and biological reactions. Bile was collected through the bilial catheter at the following time points following Tp administration: 0.25, 0.5, 1, 2, 4, and 6 h. Urine and bile samples were stored at −80 °C before analysis.

**Sample Workup.** A sample of urine (50 μL) or bile (3 μL for the Tp-treated group and 20 μL for the saline-treated group) was filtered through a 10-kDa spin filter to remove proteins. The filtrate was then further resolved by HPLC Method 1 with a ZORBAX Eclipse XDB C8 analytical column (4.6 × 150 mm, 5-μm particle size; Agilent Technologies) with the elution divided into two fractions: a polar fraction collected earlier (7–12 min) and less polar fraction collected later (17–30 min). Each fraction was lyophilized and the residue reconstituted in water before LC-MS/MS analysis using HPLC Method 2. The mass spectrometric method is described below.

**Administration of Bleomycin and Carbon Tetrachloride.** Animal experiments were performed in accordance with the protocols approved by MIT Committee on Animal Care (CAC) and with the NIH Guide for the Care and Use of Laboratory Animals (44). Female Sprague-Dawley rats (225–250 g) were obtained from Charles River Laboratories and housed in shoebox cages. Before the experiment, the animals were transferred into metabolic cages and fasted for 8 h before dosing. Bleomycin (~5 mg/kg) was prepared in sterile saline and administered by i.p. injection in a volume of 0.25 mL, whereas 800 mg/kg CCl4 was administered in corn oil vehicle by i.p. injection. Six rats were used in this experiment: two dosed with sterile saline; two dosed with bleomycin; and two dosed with CCl4. All animals were housed in metabolic cages through the duration of the experiment to collect urine over the following intervals: predose (8 h), 0–4, 4–8, 8–16, and 16–24 h. Urine was collected in sterile polypropylene tubes placed in dry ice to reduce artifacts. Samples were stored at −80 °C before analysis.

**LC-MS/MS Analysis of in Vivo Metabolites.** LC-MS/MS analyses of urine and bile metabolites were conducted with sterile saline as vehicle. For the polar fraction, samples were resolved using HPLC Method 3 with a ZORBAX Eclipse XDB C8 analytical column (4.6 × 250 mm, 5-μm particle size; Agilent Technologies), whereas for the less polar fraction, samples were resolved using HPLC Method 2 with a ZORBAX Eclipse RRHD, XDB-C18 column (2.1 × 100 mm, 1.8-μm
particle size; Agilent Technologies). In all cases, the HPLC was coupled to an Agilent 6430 series QQQ tandem quadrupole mass spectrometer operating in positive ion mode, with nitrogen as the nebulizing and drying gas (15 psi, 10 L/min, 325°C). Analyses were performed with the mass spectrometer set to multiple reaction monitoring (MRM) mode with the fragmentation voltages and collision energies shown in Table S1.

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38. Committee on Care and Use of Laboratory Animals (1996) Guide for the Care and Use of Laboratory Animals (Natl Inst Health, Bethesda), DHHS Publ No (NIH) 85-23.