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Protein Targets of Reactive Metabolites of Thiobenzamide in Rat Liver In Vivo

Keisuke Ikehata^{†,§}, Tatyana G. Duzhak[†], Nadezhda A. Galeva[‡], Tao Ji^{†,¶}, Yakov M. Koen[†], and Robert P. Hanzlik^{†,*}

[†]Department of Medicinal Chemistry University of Kansas, Lawrence, KS 66045

[‡]Mass Spectrometry Laboratory University of Kansas, Lawrence, KS 66045

Abstract

Thiobenzamide (TB) is a potent hepatotoxin in rats, causing dose-dependent hyperbilirubinemia, steatosis, and centrilobular necrosis. These effects arise subsequent to and appear to result from the covalent binding of the iminosulfinic acid metabolite of TB to cellular proteins and phosphatidylethanolamine lipids (Ji, et al., *Chem. Res. Toxicol.* 2007, 20, 701–708). To understand better the relationship between the protein covalent binding and toxicity of TB we investigated the chemistry of the adduction process and the identity of the target proteins. Cytosolic and microsomal proteins isolated from the livers of rats treated with a hepatotoxic dose of [*carboxyl*-¹⁴C]-TB contained high levels of covalently bound radioactivity (25.6 and 36.8 nmol eq./mg protein, respectively). These proteins were fractionated by 2-dimensional gel electrophoresis, and radioactive spots (154 cytosolic and 118 microsomal) were located by phosphorimaging. Corresponding spots from animals treated with a 1:1 mixture of TB and TB-*d*₅ were similarly separated, the spots were excised, and the proteins were digested in-gel with trypsin. Peptide mass mapping identified 42 cytosolic and 24 microsomal proteins, many of which appeared in more than one spot on the gel; however, only a few spots contained more than one identifiable protein. Eighty six peptides carrying either a benzoyl- or a benzimidoyl adduct on a lysine side chain were clearly recognized by their *d*₀/*d*₅ isotopic signature (sometimes both in the same digest). Because model studies showed that benzoyl adducts do not arise by hydrolysis of benzimidoyl adducts, it was proposed that TB undergoes *S*-oxidation twice to form iminosulfonic **4** (PhC(=NH)SO₂H) which either benzimidoylates a lysine side chain or undergoes hydrolysis to **9** (PhC(=O)SO₂H) and then benzoylates a lysine side chain. The proteins modified by TB metabolites serve a range of biological functions and form a set that overlaps partly with the sets of proteins known to be modified by several other metabolically-activated hepatotoxins. The relationship of the adduction of these target proteins to the cytotoxicity of reactive metabolites is discussed in terms of three currently popular mechanisms of toxicity: inhibition of enzymes important to the maintenance of cellular energy and homeostasis, the unfolded protein response, and interference with kinase-based signaling pathways that affect cell survival.

Introduction

Thiobenzamide (TB, **1**)¹ is acutely hepatotoxic in rats, causing dose-dependent cholestasis, hyperbilirubinemia, steatosis (fatty liver), and centrilobular necrosis (1,2). To elicit these

*To whom correspondence should be addressed. E-mail: rhanzlik@ku.edu..

[§]Current address: 3–096 CNRL Natural Resources Engineering Facility, University of Alberta, Edmonton, Alberta, Canada T6G 2W2

[¶]Current address: Drug Disposition, Lilly Research Laboratories, Indianapolis, IN 46285

Courier/Postal address: KU-MedChem 4048 Malott 1251 Wescoe Hall Drive Lawrence, KS 66045–7582

responses thiobenzamide requires metabolic activation through a series of two sequential *S*-oxidations, as shown in Figure 1. These oxidations are catalyzed by both the microsomal flavin-containing monooxygenase and one or more isoforms of cytochrome P450 (3,4). The intermediate *S*-oxide or sulfine metabolite TBSO (**2**) is somewhat more reactive than thiobenzamide, but it can be synthesized and isolated by chromatography. Compared to thiobenzamide, TBSO is more potent and faster-acting as a hepatotoxin. On the other hand the *S,S*-dioxide or sulfene metabolite TBSO₂(**3**) is an extremely reactive intermediate that behaves as an electrophilic acylating agent (5), probably through the intermediacy of its iminosulfinic acid tautomer (**4**). Thus, treatment of rats with [¹⁴C]-TB leads to extensive covalent binding of radioactivity, not only to proteins, but also to lipids in the phosphatidylethanolamine (PE) class (6). In the latter case the adducts are exclusively amidines of general structure **5**.

The covalent binding of chemically reactive metabolites to cellular macromolecules is thought to be an obligatory triggering event in the mechanism of cytotoxicity of numerous small molecule toxins such as acetaminophen, diclofenac, halothane, bromobenzene, and many others (7-9). The accumulated evidence in support of this hypothesis is extensive and derives from several classical types of studies. For example, covalent binding and toxicity are typically dose-dependent, and inducing or inhibiting xenobiotic metabolism respectively potentiates or mitigates both binding and toxicity. Likewise depletion of glutathione, which can trap many reactive metabolites chemically before they damage cellular macromolecules, potentiates both covalent binding and toxicity. Both in vivo and in isolated cells, the occurrence of covalent binding peaks earlier in time than cellular damage, and in vivo, covalent binding is largely confined to injured areas within sensitive tissues. The fact that the toxic potency of many chemicals can be manipulated through structural changes as simple as deuteration of a critical CH bond (10), or a change in the electronic properties of a substituent (11), is further indication that the chemical events of metabolic activation and covalent binding are intimately (i.e., *causally*) involved in the production of cellular injury by simple chemicals that do not otherwise affect vital metabolic pathways or known pharmacological receptors.

In the case of thiobenzamide and its analogs, the connection between chemical reactivity and cytotoxicity is affirmed by extensive structure-activity relationships linking the electronic and steric effects of substituents to toxic potency. For example, electron-donating substituents in the meta- or para position of thiobenzamide greatly enhance its toxic potency, with Hammett rho values ranging from -4 to -2 (1,12). Ortho substitution, which sterically blocks the ability of the *S,S*-dioxide intermediate to acylate nucleophiles, also blocks covalent binding and toxicity without blocking metabolism (13). In contrast, *N*-methylation increases potency considerably but the toxicity is directed to the lung rather than the liver (14-16). This latter example illustrates a potential liability of perturbational approaches to investigating mechanism, namely, that the perturbation may change the mechanism qualitatively rather than simply modulating it quantitatively.

While the chemical events involving metabolic activation and covalent binding of reactive metabolites are essential to the mechanism of toxicity, it is likely that a number of subsequent events not directly involving the protoxin or its reactive metabolites *per se* are nevertheless required in the chain of events leading to observable toxicity. In particular, much interest has been centered on identifying the individual proteins that become adducted by chemically

¹Abbreviations used: 2DGE, 2-dimensional gel electrophoresis; CYP2E1, cytochrome P450 2E1; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione *S*-transferase; ICAT, isotope coded affinity tag; Keap1, Kelch ECH associating protein 1; MALDI-TOF-MS, Matrix-assisted laser desorption ionization time of flight mass spectrometry; LFABP, liver fatty acid binding protein; MAPK, mitogen-activated protein kinase; MM, molecular mass; NAPQI, *N*-acetyl-*p*-benzoquinonimine; Nrf2, nuclear factor-E2-related factor; PE, phosphatidylethanolamine; PPI, protein-protein interaction; PVDF, poly(vinylidene difluoride); ROS, reactive oxygen species; TB, thiobenzamide; TBSO, thiobenzamide *S*-oxide (sulfine); TBSO₂, thiobenzamide *S,S*-dioxide (sulfene); TPDB, reactive metabolite target protein database; UPR, unfolded protein response.

reactive metabolites. For example, the work of several research groups has lead to the identification of more than 30 hepatic target proteins for acetaminophen metabolites (17-19), and our laboratory has identified more than 40 hepatic protein targets of bromobenzene reactive metabolites (20,21). In this manuscript we describe the identification of 63 protein targets for thiobenzamide metabolites in rat liver, and compare them to those identified as targets of acetaminophen and bromobenzene. This comparison encourages the hope that as additional information becomes available, it may be possible to discern a group of target proteins whose covalent modification constitutes part of common downstream pathway that culminates in cytotoxicity.

Experimental Procedures

Materials

Thiobenzamide (TB), benzoyl chloride, methyl benzimidate hydrochloride and *N*(α)-acetyllysine were purchased from Aldrich (Milwaukee, WI). [*carboxyl*- ^{14}C]-TB (4.37 Ci/mol) and TB-*d*₅ were prepared as described (6). Other reagents were as described previously (6, 20).

N(α)-Acetyl-*N*(ϵ)-benzimidoyl-lysine

Triethylamine (101 mg, 1.0 mmol), methyl benzimidate hydrochloride (85.8 mg, 0.5 mmol) and *N*(α)-acetyl-L-lysine (188.2 mg, 1.0 mmol) were combined in 6 mL ethanol and heated to reflux for 2 h. The solvent was removed under vacuum and the residue was purified by column chromatography (silica gel) eluting with methanol to give 102 mg (70%) of the title compound. TLC *R*_f 0.90 (10% H₂O/MeOH). ^1H NMR (400 MHz, CDCl₃) δ 7.57–7.72 (5H, m), 4.16 (1H, m), 3.47 (2H, m), 1.99 (3H, s), 1.69–1.76 (4H, m), 1.49 (2H, m). ^{13}C NMR (100 MHz, CD₃OD) δ 23.2, 24.4, 28.5, 33.7, 44.5, 56.2, 129.3, 130.7, 131.1, 134.9, 166.1, 172.8, 179.3. *m/z* (HRMS, ESI): found 292.1533 [*M*+*H*]⁺, C₁₅H₂₂N₃O₃ requires 292.1661.

N(α)-Acetyl-*N*(ϵ)-benzoyl-lysine

N(α)-Acetyl-L-lysine (1.33 mmol, 250 mg) was dissolved in 5 mL of 1 N NaOH in a 50 mL screw-cap culture tube, cooled with an ice bath and stirred magnetically while benzoyl chloride (250 μL , 2.17 mmol) was added dropwise. The mixture was stirred for 1 h on ice and 2 h at room temperature, after which the reaction mixture was acidified with 6 N HCl and extracted with benzene and ethyl ether and then with ethyl acetate. The latter extracts were combined, dried over MgSO₄ and concentrated in vacuo to give a clear colorless oil. The oil was dissolved in warm water (7 mL) and lyophilized to give a white powder (252 mg, 0.86 mmol). TLC *R*_f 0.90 (10% H₂O/MeOH). ^1H NMR (400 MHz, NaOD/D₂O) δ 1.09 (m, 2H), 1.26 (m, 2H), 1.37 (m, 1H), 1.58 (m, 1H), 1.68 (s, 3H), 3.02 (dd, 2H), 3.80 (t, 1H), 7.17 (m, 2H), 7.26 (m, 1H), 7.38 (d, 2H). ^{13}C NMR (100 MHz, NaOD/D₂O) δ 22.10, 22.87, 28.17, 31.26, 39.78, 55.34, 127.12, 128.91, 132.17, 33.80, 170.86, 173.69, 179.73.

Treatment of Animals and Preparation of Liver Subcellular Fractions

All animal husbandry protocols 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. Briefly, male Sprague-Dawley rats (210–230 g, Charles River Laboratories, Wilmington, MA) were pre-treated with sodium phenobarbital (80 mg/kg; ip; 3d.), then injected with a single ip dose of [^{14}C]-TB (0.86 mmol/kg) or [*d*₀/*d*₅]-TB (1:1 mol/mol; 1.16 mmol TB/kg) in corn oil as described (6). Five hours after the injection the animals were anesthetized by CO₂ narcosis and killed by decapitation. The liver microsomes were then

isolated exactly as described (6). The cytosolic fraction (100,000 supernatant) was clarified by re-centrifugation and dialyzed as described earlier (20). The subcellular fractions were aliquoted and stored at -80°C .

Analysis of Protein-Bound Radiolabel—in liver fractions was performed as described (20). Briefly, aliquots of final microsomal suspension or dialyzed cytosol from the livers of ^{14}CTB treated rats were precipitated with 10% trichloroacetic acid. The precipitates were washed successively with acetone, methanol/water (80:20 v/v), acetone and diethyl ether, dried by rotary evaporation, dissolved in 1 mL of M NaOH and neutralized with 1 M HCl. Radioactivity was then measured by liquid scintillation counting and protein was determined by Bradford assay using a standard kit (Bio-Rad).

Two-dimensional Electrophoresis (2DGE) and Phosphorimaging—were performed essentially as described previously (21).

In-gel Digestion and Mass Spectral Analysis of Tryptic Digests

Digestion of proteins in excised gel pieces with trypsin and analyses of the resulting peptide mixtures by matrix assisted laser desorption-ionization time-of-flight (MALDI-TOF) MS were carried out as described (20,21). Proteins in excised gel pieces were digested with trypsin and the digest samples were analyzed by matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) MS on a Proteomics Analyzer 4700 (Applied Biosystems, Foster City, CA). The samples were desalted and concentrated on C_{18} ZipTips (Millipore, Bedford, MA), eluted with 2 μL of a saturated solution of α -cyano-hydroxycinnamic acid in aqueous 50% acetonitrile/0.1% trifluoroacetic acid directly onto a sample plate and allowed to crystallize. Mass spectra were acquired in positive ion reflectron mode. Peptide mass acquisition was performed over the m/z range 700–3000. Mass spectra were externally calibrated using a standard mixture of known peptides covering the entire mass range, which typically resulted in mass accuracy within 50 ppm or better. The calibration was verified using internal m/z peaks arising from trypsin autolysis.

Protein Database Searching

The observed monoisotopic peptide masses were compared to respective theoretical masses for all proteins available from UniProt database (<http://us.expasy.org>) using the Mascot searching engine with a probability based scoring algorithm (<http://www.matrixscience.com>). A maximum mass error of 50 ppm and one missed cleavage was allowed in the search. Pyridylethylation of cysteine residues as well as the presence of methionine sulfoxide was considered. Where available, the database information on specific posttranslational modifications (e.g. N-terminal acetylation) was taken into consideration. The identification was considered positive when the highest-scoring protein entry showed MOWSE score higher than 56 ($p < 0.05$) and the number of matches not less than 4. Typically, the most probable candidates showed scores > 80 ($p < 0.005$), with more than 7 peptides matching at mass error within 20–30 ppm. For select digest samples, the results of the automated search were then checked by visual inspection of the observed mass spectra and verified by searching the UniProt or the NCBI nr (<http://www.ncbi.nlm.nih.gov>) protein databases with the aid of Protein Prospector (<http://prospector.ucsf.edu/>). Select protein digest samples were submitted also to LTQ-FT MS/MS analysis as described below.

Detection of Adducts in Protein Digests

The digests of TB-adducted proteins from rats treated with 1:1 mixture of TB/TB- d_5 were expected to contain peptides whose mass spectra would show the d_0/d_5 isotopic signature, i.e. at least one pair of m/z peaks having similar intensity but differing by 5 mass units.

Accordingly, we searched mass spectra of digests of all identified protein spots for the presence of any 2 peptide ions five units apart from each other. Once such a pair was detected, the masses of both peaks in the pair were compared to all theoretical *unmodified* tryptic peptide masses for the identified protein. Next, we subtracted 103 or 104 units (i.e., the calculated mass addition from a putative TB-derived benzimidoyl or benzoyl moiety, respectively) from the mass of the smaller ion (d_0) in the observed pair and, again, compared *the resulting mass* to the theoretical *unmodified* peptide mass list. Finally, the presence of the adduct was considered established if all of the following criteria were met: i) in the mass spectrum, there was a pair of ions 5 units apart; ii) neither of these ions could be matched to any unmodified theoretical peptide; iii) the smaller ion in the pair was matched to a theoretical peptide after correction of the observed mass for the addition of benzimidoyl or benzoyl moiety; iv) the matching (theoretical) peptide contained at least one missed cleavage at lysine. For select adducted peptides, the identity of adducts was then verified by MS/MS analysis of both parent-ion peaks in a d_0/d_5 -pair as described below.

LC-MS experiments for protein identification

Samples were introduced into a LTQ-FT hybrid linear quadrupole ion trap Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (ThermoFinnigan, Bremen, Germany) via capillary liquid chromatography. Peptides were separated on a reverse-phase column (0.32×50 mm, MicroTech Scientific) at a flow rate 10 $\mu\text{L}/\text{min}$ with a linear gradient rising from 5 to 65% (v/v) acetonitrile in 0.06% (v/v) aqueous formic acid over a period of 55 min using an LC Packings Ultimate Chromatograph (Dionex, Sunnyvale, CA). Data dependent acquisition LC-MS experiments were performed using Xcalibur 1.4 software (Thermo Scientific). Survey mass spectra were acquired in the FT-ICR over a mass range (m/z) 300–2000 using full MS target automated gain control (AGC) value of 50,000 accumulated ions, or an ion accumulation time of 1 s, with a resolution $R=25,000$ at m/z 400. The three most intense ions were selected and sequentially fragmented in the linear ion trap by collision-induced dissociation using a target AGC value of 3000. Ion selection threshold was 1500 counts. Dynamic exclusion duration was 200 sec with early expiration if ion intensity falls below S/N threshold of 2. The ESI source was operated with spray voltage of 2.8 kV, a tube lens offset of 170 V and a capillary temperature of 200 °C. All other source parameters were optimized for maximum sensitivity of the YGGFL peptide MH^+ ion at m/z 556.27. The instrument was calibrated using an automatic routine based on a standard calibration solution containing caffeine, peptide MRFA, and Ultramark 1621 (Sigma). Raw experimental files were processed using BioWorks 2.0 software followed by peptide/protein identification using Sequest and Mascot and X!Tandem database-searching programs with the Swiss-Prot database. The peptide assignments obtained were validated using a statistical method of the Scaffold 1.7 software (Proteome Software Inc.).

Results and Discussion

Covalent Binding of Thiobenzamide Metabolites to Rat Liver Proteins

The covalent binding of [^{14}C]-TB metabolites to rat liver cytosolic proteins was found to be 25.6 nmol eq./mg protein. The corresponding value for microsomal proteins was 36.8 nmol eq./mg protein, as reported in our earlier study of covalent binding of TB metabolites to microsomal lipids (6). These values are higher by a factor of 8 to 10 than those typically reported for other small molecule hepatotoxins such as acetaminophen or bromobenzene (6,22). An average adduct density of 36.8 nmol eq./mg protein means that a representative protein of 36.8 kDa would carry, on average, 1 molecule of adduct per molecule of protein. Several factors probably contribute to the very high labeling densities observed with TB. First, TB is an excellent substrate for *two* different monooxygenase systems in hepatocytes, FMO and P450, yet both form the same reactive metabolite, TBSO_2 . Second, TBSO_2 is especially selective among nucleophiles, and since the elimination to form a nitrile metabolite requires basic

conditions (Figure 1), this route is likely to be relatively slow at pH 7.4 in cells. Thus, side reactions that might compete with acylation of amine groups on proteins and phospholipids are minimized. Finally, there are no known enzyme systems that specifically detoxify iminosulfinic acids (or any other type of acylating metabolite). The resulting high degree of protein labeling not only enabled us to observe a large number of target proteins, it also enabled us to observe numerous adduct-bearing peptides during the course of protein identification work (see below).

Separation and Identification of Thiobenzamide Target Proteins

Aliquots of cytosolic or microsomal proteins were separated by 2DGE. Two replicate gels were run for both [^{14}C]-labeled protein fractions, and 5–6 replicate gels were run for corresponding protein fractions prepared from animals treated with [d_0/d_5]-TB. The former were transblotted onto PVDF membranes which were then exposed to a phosphorimage plate to locate radioactive spots. Proteins on the other gels were located by Coomassie staining. The protein spot patterns were highly reproducible and resembled closely those observed in our earlier study of bromobenzene target proteins (20,21). Representative images of the Coomassie-stained blots and the corresponding phosphorimages are shown in Figures S1 and S2. As indicated in Table 1, the cytosolic protein fraction resolved into >700 protein spots, 154 of which contained measurable radioactivity, while the microsomal fraction resolved into >200 spots, 118 of which were radioactive. Also as observed in our previous study with bromobenzene, ^{14}C -adduct density varies widely among different protein spots; some low-abundance proteins become highly labeled, while some abundant proteins acquired little radioactivity.

Spots on gels from animals treated with [d_0/d_5]-TB that corresponded to radioactive spots on the companion gels were excised prior to digestion. For low-abundance proteins, spots excised from several gels were pooled. The excised protein spots were digested in-gel with trypsin and the resulting peptide mixtures analyzed by MALDI-TOF MS. The data were then matched against the complete Swiss-Prot database using the Mascot peptide mass fingerprinting program and Applied Biosystems software. The results were then checked and verified using Protein Prospector (UCSF) and complete Swiss-Prot or NCBI databases. A summary of the results of protein identification is presented in Table 1; a complete listing of all the non-redundant proteins identified is presented in Table 2.

From the mass spectral data obtained from digests of the 154 radioactive spots of cytosolic protein we were able to make 127 identifications with a high degree of confidence. One hundred spots contained apparently single proteins, while 23 spots clearly contained two identifiable proteins and four spots contained three. As observed in our previous investigations of bromobenzene target proteins, some individual TB target proteins appear in multiple spots on 2DGE (viz. Table 2). When this occurred, it usually involved spots of the same apparent molecular mass that differed in their apparent pI. In rare cases a single protein could be found in several spots differing in both MM and pI. Thus, these 127 identifications of cytosolic TB targets comprised only 42 individual proteins. Analogous observations were made with the microsomal protein fraction. Of the 118 spots analyzed, 64 appeared to contain a single identifiable protein while 7 clearly contained two, but these 71 identifications comprised only 24 individual proteins.

Observation of Adduct-Bearing Peptides

It has generally been difficult to observe adducted peptides in tryptic digests, especially for samples obtained from animals treated in vivo. This difficulty arises from several factors (23). First, some pro-toxins give rise to multiple reactive metabolites. Bromobenzene is perhaps an extreme example, giving rise to four quinone and two epoxide metabolites. Second, most

proteins have multiple nucleophilic sites across which the reactive metabolites may be distributed. In addition, tryptic digestion, especially when carried out in-gel, may not result in complete release of adducted peptides, resulting in incomplete coverage of the protein sequence and failure to observe the portion that contains the adduct. Finally, as noted above, the overall level of adduction is often quite low (especially in vivo), with an average of only 1 adduct per 10 or more molecules of a given protein. Thus, this type of adduction situation is very different from the affinity-labeling of enzyme active sites, which usually results in essentially stoichiometric modification of a single active-site residue by a single molecular species.

Another factor making it difficult to observe adducted peptides is that they do not correspond in mass to predicted peptides (which, by definition, are not adducted). One can easily search mass spectra specifically for peptides whose mass corresponds to that of an expected tryptic peptide plus a mass increment predicted for a putative reactive metabolite, but true adduct peaks are often of very low intensity and surrounded by other peaks of comparable or even higher intensity. Thus the mere observation of a signal at an expected mass does not provide convincing evidence for claiming an adduct has been observed. One way that an adducted peptide can be recognized with greater confidence is from MS/MS data that reveals a non-standard mass interval in the peptide sequencing ladder. Another is through the “isotopic signature” of the adduct moiety, whether intrinsic as in the case of bromine (23,24), or extrinsic as in the case of deuterium or other stable labels (6,10,25-28). With a $[d_0/d_5]$ isotopic signature built into the TB we administered to the animals, we simply inspected the mass spectrum of the digest of each protein spot manually for the appearance of pairs of peaks of equal intensity separated by 5 mass units. This approach worked very well when we analyzed the lipid adducts from animals treated with $[d_0/d_5]$ -TB (6), and it has worked equally well with adducted proteins, as discussed below.

The protein in spot 4, identified as RNase UK114, was the first in which we observed the expected isotopic signature of a $[d_0/d_5]$ -labeled peptide adduct (see Figures 2A and 2B). From the identity of the protein (deduced from numerous non-labeled peaks in the spectrum) it was easy to deduce that the d_0/d_5 peaks at m/z 1435.8 and 1440.8, which do not match predicted tryptic peptides of this protein, were adducts of the peptide $^{107}\text{AAYQVAALPKGSR}^{119}$. This 1331.7 Da peptide contains one missed cleavage at lysine-116 due to the blocking of the lysine sidechain by an adduct of mass 104 or 109. This is precisely the mass shift expected for a *benzoyl* ($\text{PhCO}-$) moiety (i.e., **6** in Figure 1). As shown in Table 2, the protein RNase UK114 was also found in several other protein spots, including spot 24 whose digest mass spectrum also showed a d_0/d_5 doublet peak (Figure 2C and 2D). In this spot the adducted peptide is the same, but the observed mass shift is 103 and 108 instead of 104 and 109, corresponding to a *benzimidoyl* ($\text{PhC}(\text{NH})-$) adduct rather than a benzoyl adduct (i.e., **7** in Figure 1). We will return to this point later, but this observation of two types of adducts of the same peptide is not unique. As indicated in Table 3, we observed $[d_0/d_5]$ -adducted peptides in a total of 37 different protein spots comprising 7 different proteins, 6 cytosolic and 1 microsomal. The spectra of several other adducted peptides are shown in Figure 3, and the full details of all adducted peptide observations are given in Table S1 (see supporting information).

The ability to use isotopic signatures to detect adducted peptides is sensitive to interference from other naturally-occurring isotopes and to the signal-to-noise (S/N) ratio of the data (24). Both the natural abundance isotope peaks and the d_0/d_5 signature peaks are very clear in the spectra of Figures 2B, 2C, 3A and 3B, but as the S/N ratio decreases the noise begins to distort the d_0/d_5 intensity ratio from its original value of 1:1. Nevertheless, as illustrated in Figure 3C and 3D, the d_0/d_5 signature can still be seen fairly clearly at S/N ratios as low as 3. These observations of adducted peptides are important, because when proteins are identified as radioactive spots on a gel, it is potentially ambiguous as to whether the protein identified is actually the adducted protein, or merely an abundant bystander protein that co-migrated with

a minor adducted protein. The observation of identifiable adducted peptides eliminates this ambiguity.

As mentioned above, adducted peptides can also be detected using MS/MS and observing a non-standard gap in the sequencing ladder. An example of this is shown in Figure 4. Panel A shows (as a doubly-charged ion) the same d_0/d_5 peptide as seen in Figure 2B, while panels B and C show the singly-charged ions from MS/MS sequencing of both the d_0 and d_5 forms of the peptide, respectively. The spectra look quite similar except for the presence (in Panel C) of 5 deuterium atoms in y-ions equal to y''_4 or larger, and b-ions equal to b_{10} or larger. The large gap from y''_3 to y''_5 , like that from b_8 to b_{10} , corresponds to loss of the proline-benzoyllysine moiety as a unit. In Figure 5, Panel A shows a peptide derived from liver fatty acid binding protein (LFABP), while Panels B and C show the singly-charged ions from MS/MS sequencing of both the d_0 and the d_5 forms of peptide, respectively. Again, deuterium appears only in y-ions equal to or larger than y''_7 and in b-ions equal to b_4 or larger, in agreement with the proposed sequence and adduction site.

Mechanism of Protein Adduct Formation

The oxidative metabolism of thioacetamide (**1**, $R = CH_3$) has been shown to lead to the formation of N- ϵ -acetyllysine residues in proteins (i.e., **6**, $R = CH_3$) (29). The observation that TB metabolism generates both benzimidoyl and benzoyl adducts on lysine sidechains (and sometimes both within a single protein), stands in contrast to the fact that *only* benzimidoyl adducts are found in the PE lipid fraction of the same livers. Since protein analysis and adduct characterization involve more extensive sample processing than lipid analysis, it is conceivable that benzoyl adducts might arise by chemical degradation (hydrolysis) of initially-formed benzimidoyl protein adducts. To test this we synthesized both N- ϵ -benzimidoyl- and N- ϵ -benzoyl-N- α -lysine and exposed them to pH 3.5, 7.5 or 8.5 for 24 h at 37 °C followed by 7 d at room temperature, but HPLC/MS analysis showed that no chemical change occurred. Similarly, in studies of the metabolism and protein covalent binding of thioacetamide, Dyroff and Neal (30) synthesized [3H]-acetimidoyl-modified bovine serum albumin, subjected it to extensive enzymatic hydrolysis, and observed N- ϵ -acetimidoyllysine with only traces of N- ϵ -acetyllysine in the hydrolysate. Thus, benzimidoyllysine adducts are not precursors to benzoyllysine adducts.

The formation of benzimidoyl adducts **7** undoubtedly occurs by the direct reaction of **4**, the iminosulfonic acid tautomer or $TBSO_2$, with lysine side chains on proteins. To account for the formation of benzoyl adducts we suggest that **4** reacts with water to form a tetrahedral intermediate that can either lose H_2SO_2 to form amide **8**, or lose NH_3 to form α -ketosulfonic acid **9** which then benzoylates proteins. The absence of benzoyl adducts of PE lipids may indicate that the lipid environment is unfavorable, relative to the more highly aqueous cytosol, for the required hydrolysis of **4** to **9**. Finally, the mechanisms shown in Figure 1 also account for the formation of the major soluble metabolites of thioamide compounds, which include amides **8** and nitriles **10**.

Protein Targets of Thiobenzamide Metabolites and Their Role in Cytotoxicity

The TB target proteins identified in Table 2 serve a broad spectrum of biological functions ranging from binding proteins and structural proteins to stress response proteins, chaperones and enzymes. Comparable diversity of target protein function has also been observed for numerous other cytotoxic chemicals(31). The main purpose in identifying reactive metabolite target proteins is to attempt to provide a mechanistic basis for the strong association between protein covalent binding and toxicity. Of course, not all covalent binding leads to cytotoxic consequences. For example, physiological processes such as the perception of sharp taste and/or pungent odor often involves the covalent binding of reactive chemicals to Transient Receptor

Potential ion channels which then modifies their conduction properties (32,33). Examples of xenobiotic compounds that undergo metabolic activation and protein covalent binding with no apparent cytotoxic consequences include p-bromophenol (34), m-acetamidophenol (35) and mycophenolic acid (36). Nevertheless, as more target proteins are identified for more agents whose reactive metabolites *do* cause cytotoxicity, it is worthwhile to speculate on potential general mechanisms that might connect covalent binding to cytotoxicity. The literature on the topic of cell death is vast, but in the context of cytotoxicity induced by chemically-reactive metabolites, three mechanistic hypotheses have attracted particular attention. As discussed below, they focus on 1) inhibition of critical enzymes, 2) the unfolded protein response (UPR), and 3) dysregulation of intracellular signaling pathways.

Enzyme inhibition by reactive metabolites

Among thiobenzamide target proteins the largest single group contains enzymes of intermediary metabolism, all of which were identified in the cytosolic fraction, although some also occur in mitochondria where they participate in the Krebs-TCA cycle. Since this latter pathway provides the primary source of ATP energy used for maintenance of cellular homeostasis and integrity, significant inhibition of its enzymes can have rapid and grave consequences for cell viability (37). Thus, mitochondrial enzymes have long been of much interest as potentially consequential targets of chemically reactive metabolites (38).

It is not known if adduction by TB metabolites inhibits any of the enzymes listed in Table 2, but even if it did, the consequences would not likely be as rapidly fatal to cells as inhibition of their corresponding mitochondrial forms of these enzymes for at least two reasons. First, the reactions catalyzed by the cytosolic enzymes are not as directly coupled to the production of energy and maintenance of cellular integrity. Second, as for many other protoxins, the fractional labeling of target proteins by TB metabolites is low and the binding is distributed across multiple sites on the protein, some of which may not lead to enzyme inactivation. For example, Dietze et al. (39) showed that treatment of mice with [¹⁴C]-acetaminophen leads to the covalent adduction of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), accompanied by significant decreases in hepatic levels of GAPDH activity. They also showed that NAPQI, the reactive metabolite of acetaminophen, inactivates GAPDH in vitro by modifying its active site sulfhydryl group. The reactive metabolite of TB also targets GAPDH, but unlike NAPQI, TBSO₂ has a strong preference for acylating lysine side chains rather than acylating sulfhydryl groups (Figure 1). Considering the enzymes and other proteins in Table 2, and making the generous assumption that adduction does impair function, it is difficult at best to conclude that adduction of any of them, individually, would be likely to cause acute cytotoxicity. In other words, none of these targets would appear to constitute an “Achilles heel” of vulnerability on the part of the cell.

Could toxicity be the cumulative result of adducting multiple cellular proteins to a small degree, thereby causing multiple small problems that eventually overwhelm the cell? LoPachin and coworkers (40) used an ICAT labeling approach to identify striatal proteins having sulfhydryl groups that become alkylated by acrylamide (in a rat model in vivo) during chronic exposure at a dose rate known to lead to cumulative neurotoxicity. One of the 58 target proteins they observed at all three endpoints (7, 14 or 21 days of exposure) was GAPDH, but there are very few other proteins in common between their target list and that of Table 2 above. After considering the adduction of specialized proteins unique to nerve tissue, as well as metabolic enzymes common to most tissues, LoPachin et al. concluded that their evidence implicated “a diffuse pathophysiological mechanism” for the cumulative chronic toxicity of acrylamide. Conceivably, such a diffuse, non-specific mechanism could underlie the relatively rapid, acute cytotoxicity of thiobenzamide and that of other metabolically-activated protoxins. However, much more information about the effects of adduction on the functional abilities of reactive

metabolite target proteins will be needed to support a non-specific mechanism of toxicity involving “Death by a thousand cuts.”

The unfolded protein response (UPR)

Another major group of thiobenzamide target proteins includes heat shock and stress response proteins that function as chaperones or enzymes associated with protein folding. These targets are found almost exclusively in the ER. This organelle not only directs the folding and post-translational modification of newly synthesized polypeptides into functional proteins, it also monitors protein quality and responds to a variety of stresses that lead to malformed or improperly folded proteins (41,42). Such stresses include heat or osmotic shock, ionizing radiation, expression of folding-defective mutant proteins, nutrient deprivation, inhibition of glycosylation, abnormal glycation and oxidant challenge. To this list we would also add covalent modification of ER proteins by chemically-reactive metabolites or electrophilic products of lipid peroxidation.

The UPR is an evolutionarily conserved mechanism that is activated by misfolded or abnormal proteins in the ER (43,44). The ER protein BiP/GRP78 is a “master regulator” of the UPR (45). Its binding to unfolded or abnormal proteins unmask the transmembrane sensor proteins PERK, ATF6 and IRE1 which then signal the nucleus to block the translation of most proteins but increase the synthesis of stress response proteins. Among the latter are BiP/GRP78, GRP94, protein disulfide isomerase, calreticulin and Hsp70, all of which are target proteins for multiple chemically reactive metabolites (46). The upregulation of these and other stress response proteins helps the cell cope with misfolded proteins by re-folding them correctly, or by enhancing ER-associated degradation of malformed proteins via ubiquitination and proteasomal hydrolysis. When these defense mechanisms are overwhelmed, or when BiP expression is reduced by siRNA (44), apoptosis and/or autophagy ensue (47).

Commonality of target proteins among different reactive metabolites

As noted above, certain ER proteins are common targets for reactive metabolites of multiple protoxins. To date, more than 200 different proteins have been reported to be targets for 21 different metabolically activated small molecule toxins (31). For many of these agents the number of known target proteins is actually rather small (<8). As indicated in Table 4, the five agents for which the largest number of target proteins have been identified are acetaminophen, bromobenzene, butylated hydroxytoluene, naphthalene, and TB. Collectively these five agents have 158 known target proteins, some of which are also targeted by other cytotoxic agents. This is elucidated more fully in Table 5, which indicates that one protein, protein disulfide isomerase A1, is a known target for seven different reactive metabolites. In addition, three other proteins are common targets for five different reactive metabolites, four are common targets for four reactive metabolites, and one is a common target for three reactive metabolites. Could this modest degree of commonality among protein targets of multiple reactive metabolites be an early emerging sign of a common mechanistic pathway leading to reactive metabolite-induced cytotoxicity? If so, how might such a pathway operate?

Interference with cell signaling

It is well known that within living cells, proteins interact both extensively and specifically with other proteins. Some of these protein-protein interactions (PPIs) clearly subserve signaling functions. For example, numerous studies have implicated the mitogen activated protein kinase (MAPK) family of signaling pathways in regulating cellular responses (survival, proliferation or apoptosis) to pro-inflammatory mediators such as TNF- α or IL-1 β , and to oxidative stress (48-51). The MAPK signaling pathways are cascade processes propagated by PPIs and regulated and amplified by protein phosphorylation and dephosphorylation effected by specific protein kinases and phosphatases (49,52). These signaling cascades culminate in the activation

of transcription factors whose associated gene products determine whether the cell survives or undergoes apoptosis. The principal MAP kinases include the extracellular signal-regulated kinases 1 and 2 (ERK1/2), the c-Jun N-terminal kinases 1, 2, and 3 (JNK1–3), and the kinase p38 (53–55). In the context of oxidative stress-induced cytotoxicity the MAP kinases JNK1/2 and ERK1/2 are of particular importance (50,56–60).

The oxidative stresses that activate these signaling pathways all produce reactive oxygen species (ROS) in various ways. Examples include redox cycling of menadione (50,60,61) or paraquat (62), reductive metabolism of CCl₄ by CYP2E1 (63), activation of plasma membrane NADPH oxidases by hydrophobic bile acids (59), NADPH oxidase activity due to uncoupling or simply overexpression of CYP2E1 (64), and oxidant molecules such as hydrogen peroxide or tert.-butyl hydroperoxide (50). ROS such as hydroxyl radical (generated by the Fenton reaction of H₂O₂ with adventitious iron) and the Cl₃C-O-O• radical (derived from CCl₄ metabolism) are potent oxidants that are well known for their ability to damage biological macromolecules including DNA, proteins and lipids (65). Such damage has often been correlated with, and assumed to cause, loss of cell viability in much the same way that covalent binding of electrophilic reactive metabolites to proteins has been associated with causing cell death. However, it is important to note that electrophilic products derived by ROS-induced lipid peroxidation, such as 4-hydroxy-2-nonenal, 4-oxo-2-nonenal and acrolein, are very aggressive at covalently modifying proteins (66,67), and are clearly very cytotoxic in their own right (68–70). Furthermore, lipid peroxidation products directly activate a number of kinase-mediated signaling pathways including ERK1/2, JNK and p38 (70–72). This “direct” activation could very well be mediated through their covalent binding to cellular proteins and ensuing alterations in PPIs.

Could interference with endogenous signal transduction pathways be a mechanism or factor contributing to the cytotoxicity associated with electrophilic metabolites of protoxins that *do not* generate ROS? Information on this subject is scant, but covalent modification of proteins by electrophiles, whether derived by oxidative bioactivation of xenobiotics or by xenobiotic-initiated peroxidation of endogenous lipids, could clearly alter protein-protein interactions and, perhaps, thereby interfere with signaling cascades. For example, the cytosolic transcription factor nuclear factor-E2-related factor (Nrf2) normally complexes to the Kelch ECH associating protein 1 (Keap1), which itself binds to and resides on actin filaments (73,74). Keap1 is a sulfhydryl-rich protein, but covalent modification of its sulfhydryl groups by many types of electrophiles allows Nrf2 to dissociate and migrate to the nucleus to initiate transcription (75). Cytosolic GSTM1–1, a target of acrylonitrile (76), bromobenzene (20) and thiobenzamide metabolites (Table 2), suppresses the activation of the cytosolic transcription factor c-Jun by apoptosis signal-regulating kinase 1 (ASK-1), a kinase related to JNK, in mouse hepatocytes (77). Conceivably, covalent adduction of GSTM1–1 could lead to JNK activation and cell injury. Likewise, the activity of the c-Jun N-terminal kinase JNK is kept low in non-stressed cells by its complexation to GST-pi, but this suppression is released by ethacrynic acid, a Michael acceptor that S-alkylates GST-pi (78). NAPQI, the reactive metabolite from acetaminophen also targets GST-pi, but it is not known if it also releases the suppression of c-Jun activity.

It seems unlikely that ethacrynic acid, a xenobiotic compound unrelated to normal cellular metabolism, would be *unique* in its ability to influence JNK signaling. It seems equally unlikely that *all* sulfhydryl alkylating agents would necessarily have the same effect as ethacrynic acid. Thus, it should not be surprising that not all covalent binding of electrophiles causes toxicity. In other words, even when the same proteins are targeted by different reactive metabolites, the adducts are necessarily different and may therefore have different biological consequences.

While there is yet little direct evidence that modulation of signal transduction pathways plays a role in the cell death associated with electrophilic (non-ROS) metabolites of xenobiotic agents, we have recently found indirect evidence that points strongly toward such a role. From the more than 200 reactive metabolite target proteins in the TPDB, we selected 28 of the most commonly targeted proteins and used a bioinformatics approach to search for proteins with which they interact. We hoped that despite the modest extent of commonality among target proteins, there might be greater commonality among the proteins with which they interact in the cell, and that examining these interactions might afford new insights into mechanisms of cytotoxicity. Druckova et al. (79) recently used a bioinformatics-based approach to analyze the protein targets of the reactive metabolite of teucrin A in rat liver. In our analysis we found² that 21 of these 28 target proteins had a total of 165 interacting partner proteins, and that this group of 186 proteins participated in a total of 538 PPIs. When these 186 proteins were sorted into gene ontology (GO) categories, the categories for apoptosis, protein folding and unfolded protein binding were highly significantly overpopulated (compared to statistical expectations). Similarly, sorting the 186 target and partner proteins into KEGG biochemical pathways revealed statistically significant enrichment in 8 pathways, with the MAPK signaling pathway having the most significant enrichment. These results suggest that modification of cellular proteins by electrophilic metabolites may indeed affect the UPR and/or MAPK signaling pathways, and that activation or inhibition of these pathways may underlie the cytotoxic effects of electrophilic reactive metabolites. Much additional work will be required to test these hypotheses, but we believe that bioinformatics approaches will be very useful in pointing out new directions for research into mechanisms of reactive metabolite cytotoxicity.

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Supplementary Material

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Figure 1.
Biotransformation of thiobenzamide.

Figure 2.

MALDI-TOF mass spectra of tryptic digests of protein spots 4 and 24 from two dimensional electrophoretic separation of liver cytosolic proteins from rats treated with deuterated thiobenzamide ($d_0/d_5 = 1:1$). Both spots have the same apparent MM but spot 4 has a lower pI than spot 24. Based on peptide mass mapping, both spots were determined to contain RNase UK114 as the only identifiable protein. Panels A and D show full mass spectra of digests from spots 4 and 24, respectively. Panels B and C show expanded views of spectra A and D, respectively. Based on the identity of the protein, its predicted tryptic peptides, and the d_0/d_5 isotope pattern, the peak at mass 1435.81 in panel B represents the *benzoyl* adduct of the peptide $^{107}\text{AA}^{\text{D}}\text{YQVAALPK}^*\text{GSR}^{119}$ (where * indicates the modified lysine), while the peak

at 1434.79 in panel C represents the *benzimidoyl* adduct of the same peptide. Neither of these masses correspond to any tryptic peptide from the unadducted protein, and neither mass occurs without the other in any spot determined to contain this protein.

Figure 3.

Effects of signal-to-noise (S/N) ratio on ability to observe d_0/d_5 isotopic signatures in MALDI-TOF mass spectra of tryptic digests of liver cytosolic proteins from rats treated with deuterated thiobenzamide ($d_0/d_5 = 1:1$). A) $S/N \geq 10$. The spectrum shows the benzimidoyl-modified peptide $^{204}\text{GVFTK}^*\text{ELPSGK}^{214}$ from the digest of spot 56 (peroxiredoxin 6). B) $S/N \sim 4-5$. The spectrum shows the benzoyl-modified peptide $^{81}\text{AVVK}^*\text{M(O)EGDNK}^{90}$ from the digest of spot 12 (LFABP). C) $S/N < 3$. The spectrum shows the benzimidoyl-modified peptide $^{40}\text{LK}^*\text{ETEYNVR}^{48}$ from the digest of spot 100 (arginase). D) $S/N < 3$. The spectrum shows three different benzoyl-modified peptides in a single digest of spot 12 (LFABP): $^{34}\text{DIK}^*\text{GVSEIVHEGK}^{46}$ at m/z 1514.78, $^{21}\text{AM(O)GLPEDLIQK}^*\text{GK}^{33}$ at m/z

1519.77, and $^{85}\text{M}(\text{O})\text{EGDNK}^*\text{M}(\text{O})\text{VTTFK}^{96}$ at m/z 1536.70 (where * indicates the modified lysine).

Figure 4.

ESI-MS/MS analysis of digest of cytosolic protein spot 4 (RNase UK114). Panel A) Partial mass spectrum showing d_0/d_5 forms of benzoyl peptide $^{107}\text{AAYQVAALPK}^*\text{GSR}^{119}$ as doubly-charged ions. Both the d_0 and d_5 ions ($m/2+ = 718.4$ and 720.9 , respectively) showed the same MS/MS behavior and sequence information (Panels B and C, respectively).

Figure 5.

ESI-MS/MS analysis of digest of cytosolic protein spot 25 (LFABP). Panel A) Partial mass spectrum showing d_0/d_5 forms of benzoyl peptide $^{81}\text{AVVK}^*\text{MEGDNK}^{90}$ as doubly-charged ions. Both the d_0 and d_5 ions ($m/2^+ = 597.8$ and 600.3 , respectively) showed the same MS/MS behavior and sequence information (Panels B and C, respectively).

Table 1

Summary of Thiobenzamide Target Protein Isolation and Identification.

	Cytosol	Microsomes
Spots observed on gel	>700	>200
Radioactive spots	154	118
Spots identified as		
single proteins	100	64
two proteins	23	7
three proteins	4	0
Total Identifications ^a	127	71
Individual target proteins	42 ^b	24 ^c

^aThree proteins (serum albumin, GSTM1, apolipoprotein 1) were identified in both microsomal and cytosolic fractions. Thus the total number of unique proteins identified is 63.

^bOne of these is a microsomal protein (mEH), and two are secreted proteins (albumin, STTPO).

^cIncludes 5 cytosolic proteins or their precursors, and 6 secreted proteins or their precursors.

Table 2
Proteins Identified as Targets of Thiobenzamide Metabolites in Rat Liver Microsomes and Cytosol.

Protein Name	Protein ID ^a	Protein MW ^b	Protein pI ^b	Source ^c	Spot numbers ^d
binding/carrier proteins					
Acyl-CoA-binding protein (ACBP)	P11030	9890	8.78	C	28, 41
Alpha-1-antitrypsin precursor	P17475	46422	5.7	C	111, 1
Apolipoprotein A-I precursor (Apo-AI)	P04639	30174	5.52	C, M	53, 54 (C); 35(M)
Calreticulin precursor (CRP55)	P18418	48281	4.33	M	25–27, 48, 49, 71
Fatty acid-binding protein, liver (L-FABP)	P02692	14368	7.79	C	11–13, 25, 27, 37, 43–47
Major urinary protein precursor (MUP)	P02761	21249	5.85	M	17, 1, 18–22
Nucleobindin 2 precursor (DNA-binding protein NEFA)	Q9185	50269	5.02	M	74
Phosphatidylethanolamine-binding protein (PEBP)	P31044	20867	5.48	C	49, 50
Serotransferrin precursor (Splice isoform 1)	P12346	80412	6.94	M	113
Serum albumin precursor	P02770	72351	6.09	C, M	123, 136–144, 153 (C); 106–110 (M)
Transferrin precursor (Prealbumin)	P02767	15920	5.77	M	14
cytoskeleton, structural proteins					
Fibrinogen beta chain precursor	P14480	55424	7.89	M	96
Tubulin beta-5 chain	P69897	50479	4.78	C	85
receptors, signal transduction					
Alpha-2-macroglobulin receptor-associated protein precursor	Q99068	42006	6.85	M	61, 62
intermediary metabolism					
3-Alpha-hydroxysteroid dehydrogenase	P23457	37949	6.67	C	93, 101
3-Hydroxyanthranilate 3,4-dioxygenase	P46953	33087	5.57	C	83
Alcohol dehydrogenase	P51635	36772	6.81	C	102
Alpha-enolase	P04764	47597	6.16	C	112–118
Arginase-1	P07824	35266	6.76	C	97–100, 129
Beta-ureidopropionase	Q03248	45064	6.47	C	119
Carbonic anhydrase 3	P14141	29807	6.97	C	72, 150
D-dopachrome decarboxylase	P80254	13204	6.15	C	7, 8, 22
Fructose-bisphosphate aldolase B	P00884	40302	8.67	C	108–110
Fumarate hydratase	P25093	46471	6.67	C	127, 128, 130
Glucose-6-phosphate dehydrogenase	P04797	36207	8.44	C	103, 104, 106
Guanylate kinase N-methyltransferase	P10868	26784	5.69	C	62
Isocitrate dehydrogenase, cytoplasmic	P41562	47334	6.53	C	125
Lactoylglutathione lyase	Q6P7Q4	20990	5.12	C	49, 4
Malate dehydrogenase, cytoplasmic	O88989	36644	6.16	C	89
NG,NG-dimethylarginine dimethylaminohydrolase 1	O08557	32010	5.76	C	86, 87
Phosphoglycerate kinase 1	P16617	44395	7.52	C	131–134
similar to biliverdin reductase B	Q923D2	22290	6.29	C	58–60
xenobiotic metabolism					
Cytochrome P450 2B1/2B2	P00176–00–01–00	56482	7.29	M	68
Cytochrome P450 2B2 (Splice isoform 2)	P04167–01–00–00	57484	6.79	M	68
Epoxide hydrolase 1	P07687	52863	8.59	C	151
Glutathione S-transferase Mu 1	P04905	26081	8.42	C	62, 1, 65, 67, 77, 79–81
Glutathione S-transferase Mu 2	P08010	25870	7.3	C	69
redox regulation					
Catalase	P04762	60219	7.15	C	148
cytochrome b5	P00173	11269	5.26	M	13, 15–17
Peroxioredoxin-1	Q63716	22516	8.27	C	61
Peroxioredoxin-4	Q920V5	31408	6.18	M	38, 40
Peroxioredoxin-6	Q35244	24777	5.65	C	52, 56

Protein Name	Protein ID ^a	Protein MW ^b	Protein pI ^b	Source ^c	Spot numbers ^d
Superoxide dismutase [Cu-Zn]	P07632	16086	5.89	C	14–17
Thioredoxin	P11232	12165	4.8	C	1–3
protein folding/heat shock/stress response					
150 kDa oxygen-regulated protein precursor (Orp150)	Q63617	111640	5.11	M	117, 118
78 kDa glucose-regulated protein precursor (GRP 78)	P06761	72618	5.07	M	65, 66, 100–103
Endoplasmic reticulum protein ERp29 precursor (ERp31)	P52555	28662	6.23	M	37, 39
Heat shock cognate 71 kDa protein	P63018	71247	5.37	M	104, 105
DnaJ (Hsp40) homolog B-11	Q6TUG0	40995	5.92	M	51, 52
Peptidyl-prolyl cis-trans isomerase A	P10111	18152	8.37	C	34, 35
Peptidyl-prolyl cis-trans isomerase B precursor	P24368	22893	9.42	M	28
Protein disulfide-isomerase precursor	P04785	57651	4.82	M	72, 73, 76, 77
Protein disulfide-isomerase A3 precursor	P11598	57428	5.88	M	80–88, 91
Protein disulfide-isomerase A6 precursor (CaBP1)	Q63081	48879	5	M	64, 75
Similar to FK506-binding protein 2 precursor	P45878	21037	10.17	M	12
Stress-induced-phosphoprotein 1	O35814	63686	6.4	C	145–147
protein degradation					
Proteasome subunit alpha type 1	P18420	30024	6.15	C	84
Ubiquitin	P62989	8560	6.56	C	40
nucleic acid metabolism					
Cytidylate kinase	Q4KM73	26342	8.19	C	55
Nucleoside diphosphate kinase B	P19804	17482	6.92	C	31, 32
Ribonuclease UK114	P52759	14269	7.77	C	4, 13, 19–24, 27, 37, 39, 43–47
Other					
Calumenin precursor (Crocalbin)	O35783	37183	4.4	M	63
Pulmonary surfactant-associated protein D precursor (SP-D)	P35248	38168	6.78	C	135

^a Swiss-Prot accession numbers.^b Data furnished by the program Mascot used to search the UniProt Databank.^c Liver fraction where the protein was observed: C, cytosol; M, microsomes.^d Protein spots are numbered as in Figures S1 (cytosol) and S2 (microsomes).

Table 3

Summary of Adduct-Bearing Peptides Observed.^a Summary of Adduct-bearing Peptides Observed by MALDI-TOF MS. Protein spots from animals treated with deuterated thiobenzamide that corresponded to radioactive spots from animals treated with [¹⁴C]-thiobenzamide were excised, digested and examined by MS with manual data examination as illustrated in Figure 3.

Number of	Cytosol	Microsomes
spots with d ₀ /d ₅ peptides	35	2
unique proteins with d ₀ /d ₅	6	1
benzoyl peptides	35	2
benzimidoyl peptides	51	0

^a For a detailed listing see Supporting Information.

Table 4

Numbers of Identified Target Proteins for Reactive Metabolites of Acetaminophen (APAP), Bromobenzene (BB), Butylated Hydroxytoluene (BHT), Naphthalene (NAPH) and Thiobenzamide (TB).^a

	APAP	BB	BHT	NAPH	TB
APAP	33	7	3	4	7
BB	7	46	5	6	25
BHT	3	5	36	6	7
NAPH	4	6	6	17	6
TB	7	25	7	6	62

^aNumbers on the diagonal are for individual agents. The off-diagonal numbers indicate the number of known target proteins common to any pair of chemicals. The number of non-redundant proteins represented here is 158. Data taken from TPDB (ref 46).

Table 5

Common Protein Targets of Reactive Metabolites.^a

^a Acetaminophen (APAP) targets are from mice; targets for bromobenzene (BB), butylated hydroxytoluene (BHT), naphthalene (NAPH), thiobenzamide (TB) or other reactive metabolites are from rats. Data taken from TPDB (ref 46).

^b Numbers indicate the number of other reactive metabolites known to target the indicated protein.

^c There are several closely related isoforms of tropomyosin; accession numbers are shown only for two of them.

Target Protein	SwissProt ID	APAP	BB	BHT	NAPH	TB	Other ^b
protein disulfide-isomerase A3	P27773, P11598	+	+	-	+	+	1
tropomyosin	P21107-2, P04692-5 ^c	+	+	+	+	-	1
heat shock 70 kDa protein 8	P63018	-	+	+	+	+	1
alpha-enolase	P04764	-	+	+	-	+	1
aldehyde dehydrogenase (mitochondrial; ALDH1)	P47738, P11884	-	+	-	-	-	2
ribonuclease UK-114	P52759	+	+	-	-	+	1
glyceraldehyde-3-phosphate dehydrogenase	P16858, P04797	+	-	-	-	+	2
protein disulfide-isomerase A1	P09103, P04785	+	+	-	+	+	3
carbonic anhydrase 3	P16015, P14141	+	+	-	-	+	1