Chemical Research in Toxicology

Article

pubs.acs.org/crt

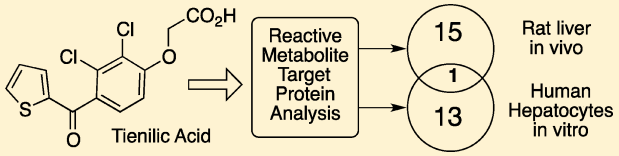
Identification of Protein Targets of Reactive Metabolites of Tienilic Acid in Human Hepatocytes

Yakov M. Koen,[†] Diganta Sarma,[†] Todd D. Williams,[‡] Nadezhda A. Galeva,[‡] R. Scott Obach,[§] and Robert P. Hanzlik*,[†]

[†]Department of Medicinal Chemistry and [‡]Mass Spectrometry Laboratory, University of Kansas, Lawrence, Kansas 66045, United States

[§]Pharmacokinetics, Dynamics and Metabolism Department, Pfizer Global Research and Development, Groton, Connecticut 06340, United States

ABSTRACT: Tienilic acid (TA) is a uricosuric diuretic that was withdrawn from the market only months after its introduction because of reports of serious incidents of drug-induced liver injury including some fatalities. Its hepatotoxicity is considered to be primarily immunoallergic in nature. Like other thiophene compounds, TA undergoes biotransformation to a *S*-oxide metabolite which then reacts covalently with cellular proteins. To identify protein targets of TA metabolites, we incubated [^{14}C]-TA with human hepatocytes, separated cellular proteins by 2D gel electrophoresis, and analyzed proteins in 36 radioactive spots by tryptic digestion followed by LC-MS/MS. Thirty-one spots contained at least one identifiable protein. Sixteen spots contained only one of 14 nonredundant proteins which were thus considered to be targets of TA metabolites. Six of the 14 were also found in other radioactive spots that contained from 1 to 3 additional proteins. Eight of the 14 had not been reported to be targets for any reactive metabolite other than TA. The other 15 spots each contained from 2 to 4 identifiable proteins, many of which are known targets of other chemically reactive metabolites, but since adducted peptides were not observed, the identity of the adducted protein(s) in these spots is ambiguous. Interestingly, all the radioactive spots corresponded to proteins of low abundance, while many highly abundant proteins in the mixture showed no radioactivity. Furthermore, of approximately 16 previously reported protein targets of TA in rat liver (Methogo, R., Dansette, P., and Klarskov, K. (2007) *Int. J. Mass Spectrom., 268*, 284−295), only one (fumarylacetoacetase) is among the 14 targets identified in this work. One reason for this difference may be statistical, given that each study identified a small number of targets from among thousands present in hepatocytes. Another may be the species difference (i.e., rat vs human), and still another may be the method of detection of adducted proteins (i.e., Western blot vs C-14). Knowledge of human target proteins is very limited. Of more than 350 known protein targets of reactive metabolites, only 42 are known from humans, and only 21 of these are known to be targets for more than one chemical. Nevertheless, the demonstration that human target proteins can be identified using isolated hepatocytes in vitro should enable the question of species differences to be addressed more fully in the future.



INTRODUCTION

Hepatotoxicity poses a major challenge for the pharmaceutical industry in several ways.[1] It is a significant cause of attrition among candidates in drug development pipelines, where preclinical testing is effective at weeding out *intrinsic* hepatotoxins (i.e., compounds that show reproducible, dose-dependent hepatotoxicity in several species). Drug-induced liver injury (DILI) can also appear unexpectedly after a compound has entered large scale clinical testing or usage. While infrequent, DILI often leads to the regulatory issuance of warning labels or even restrictions on usage (e.g., benzbromarone, bosentan, felbamate, flutamide, nevirapine, ticlopidine, tolcapone, trovafloxacin, valproic acid, and zileuton).[2−4] Such toxicity is often associated with the covalent binding of reactive metabolites to cellular proteins.[5−8]

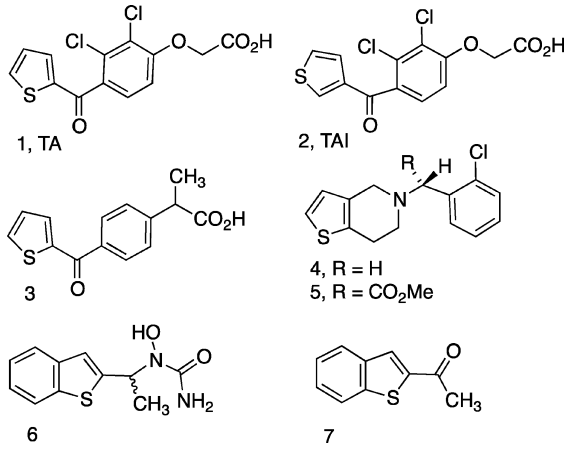
In other cases, severe drug-induced liver injury may be experienced by a very small number of individuals among a large population of patients who experience no adverse effects from the drug. Such *idiosyncratic* drug-induced liver injury (IDILI) usually leads to withdrawal of the drug from the market (e.g., benoxaprofen, bromfenac tienilic acid, troglitazone, and zomepirac). Mechanistic understanding of IDILI is limited, but covalent binding of reactive metabolites to cellular proteins is a common finding, and for several IDILI drugs, an immunological component appears to be involved.[3,9−12]

Tienilic acid (TA, structure **1** (Scheme 1)) is a uricosuric diuretic that was introduced into clinical practice in the U.S. in 1979, but after only a few months, it was withdrawn because of reports of serious incidents of hepatotoxicity including some fatalities.[11,13] TA is a thiophene compound, a close isomer of which (TAI, structure **2**) shows direct intrinsic hepatotoxicity in animals.[14,15] TA is a suicide substrate for CYP2C9,[16,17] but its reactive metabolites can also bind covalently to other hepatocellular proteins to a small extent,[18−20] which may explain why its toxicity in humans has an immunological

Received: March 7, 2012
Published: March 30, 2012

   dx.doi.org/10.1021/tx300103j | *Chem. Res. Toxicol.* 2012, 25, 1145−1154

Scheme 1. Structures of Tienilic Acid (1), Its Isomer (2), and Other Thiophene Compounds



component. TAI, in contrast, does not inactivate CYP2C9, but its metabolites bind extensively to other microsomal proteins.[20] It has even been suggested that the greater incidence of TA hepatotixicity in the U.S. compared to France might have been due to the presence of a greater amount of TAI as an impurity in the TA preparation available in the U.S.[15] The history of TA is long and complicated, but in many ways, it still epitomizes the situation faced by newly marketed drugs. For that reason and because it presaged IDILI, it is useful to review it briefly.

The hepatotoxicity of TA observed in open clinical usage was quite unexpected because it had not shown any propensity to cause liver injury in preclinical safety studies.[14] However, once its association with hepatitis-like liver injury was described in the clinical literature, subsequent studies with perfused livers from phenobarbital-induced rats[21] showed that TA (5−50 μM in perfusate) decreased bile flow and BSP clearance to 50% and 20% of control, respectively, while causing a small release of AST (up to 2 times control) into the perfusate. These findings suggest that at therapeutic concentrations, TA does have direct effects on the liver and on liver function. More recent investigations[22] showed that TA is indeed hepatotoxic in rats after glutathione depletion by treatment with buthionine sulfoximine, a glutamate antimetabolite that inhibits γ-glutamylcysteine synthase. This observation, together with the fact that glutathione conjugates of TA are formed in vitro and in vivo,[14,22−24] constitutes presumptive evidence for a role of reactive metabolites in the hepatotoxicity of TA, even if TA does not ordinarily show the frank, acute, and dose-dependent hepatotoxicity often seen with prototypical precursors of reactive metabolites such as acetaminophen or bromobenzene.

The observation of clinical liver injury due to TA prompted a number of investigations into the mechanism of its toxicity and that of other thiophene-containing compounds. A specific focus of many of these studies was on the role of epoxidation vs S-oxidation of the thiophene ring; the latter reaction seemed plausible but was not well precedented in organic chemistry or enzymology.[25] Through a combination of drug metabolism and chemical synthetic studies, it was established that thiophene *S*-oxides were indeed produced metabolically and that they had considerable reactivity toward nucleophiles, particularly thiol nucleophiles.[23,25−27] Subsequently, TA metabolites were also demonstrated to bind covalently to proteins in rat liver[26] and human liver microsomes,[28] and to proteins of yeast transformed to express human cytochrome P450 2C9(2C10)[16] but not yeast transformed with P450 enzymes from families 1A or 3A.[29]

Quite separately, immunological studies of the sera of patients with hepatitis or kidney disease of various kinds had revealed the presence of autoantibodies that could react with either liver or kidney microsomes (anti-LKM). Examination of many anti-LKM samples eventually led to the recognition of a subgroup of these sera called anti-LKM2; surprisingly, these sera came exclusively from patients who were suffering from tienilic acid-induced hepatitis.[13] Eventually, the microsomal antigen for these antisera was identified as CYP2C9,[30] an enzyme for which TA was later shown to be a moderately efficient suicide substrate.[16,31]

Other thiophene-containing compounds have also been shown to be suicide substrates for P450 enzymes, or to form reactive metabolites that covalently bind to proteins, or both. For example, (±)-suprofen (**3**), an antiinflammatory agent that is structurally similar to TA, is also a suicide substrate for CYP2C9.[32,33] Ticlopidine (**4**) and clopidogrel (**5**) are prodrugs that undergo bioactivation via oxidation at C-5 of the thiophene ring to form a thiolactone en route to formation of the pharmacologically active ring-opened thiol carboxylic acid;[34−36] similar ring oxidation is also known for TA.[28] Zileuton (**6**) is a mechanism-based inactivator of CYP1A2. A degradation product of zileuton, 2-acetylbenzothiophene (2-ABT, **7**),[37] undergoes oxidation by P450 3A and 2E enzymes to an *S*-oxide that covalently binds to Cys-34 of human serum albumin,[38] reacts spontaneously with NAC and GSH, and inactivates GSTM1−1 and GSTP1−1 in vitro.[39] 2-ABT is also cytotoxic to MCL5 cells that are transformed to express P450 enzymes but not to nontransfected control cells.[40] The role of drug metabolizing enzymes, particularly cytochrome P450s, in inducing hepatotoxicity via chemically reactive intermediates is well established.[12] As noted above, the hepatitis caused by tienilic acid is considered to be immunoallergic in nature, and protein covalent binding is also a prerequisite first step for a small molecule to elicit an immune response. Thus, even though the net covalent binding of TA to liver proteins is relatively low[5,6,8] compared to some other paradigm hepatotoxicants such as bromobenzene or acetaminophen,[41] it is likely an important step in TA-induced liver injury.

In view of the growing list of proteins known to be targets for chemically reactive metabolites of other hepatotoxic chemicals,[42] we felt it would be interesting to identify the target proteins to which TA metabolites bind and to compare them to the targets of other hepatotoxicants. In this article, we report the identification of 14 definite and 29 potential protein targets of tienilic acid in human hepatocytes. This information complements a recent report by Methogo et al. of the identification of proteins adducted by tienilic acid metabolites in rat liver in vivo.[43]

EXPERIMENTAL PROCEDURES

Materials. Tienilic acid and [^{14}C]-tienilic acid (Amersham GE HealthCare, Piscataway, NJ, 59 Ci/mol, 98% radiochemical purity) were available from a previous study.[6] A pool of human hepatocytes representing a total of six individual donors was created by combining cryopreserved cell samples obtained from Celcis (Baltimore, MD), InvitroTech (Baltimore, MD), and CellzDirect (Durham, NC). Williams' Medium E was obtained from Gibco-Invitrogen (Grand Island, NY). Trypsin (sequencing grade) was obtained from Roche (Indianapolis, IN). Sequenal grade urea and CHAPS were obtained from Pierce (Rockford IL). 4-Vinylpyridine and EDC hydrochloride were obtained from Sigma (St. Louis, MO). Tris, SDS, glycine, Sequi-

blot PVDF membranes (0.2 μm), broad range IEF Standards, and Precision Protein Standards were obtained from Bio-Rad (Hercules, CA). All other electrophoresis supplies including immobilized pH gradient Dry-Strips and 18.5 × 24.5 cm SDS−polyacrylamide gels were obtained from GE-HealthCare (Piscataway, NJ). HPLC grade solvents and analytical grade inorganic salts were obtained from Fisher (St. Louis, MO). Deionized water (resistivity 18.2 MΩ/cm) was used for the preparation of all solutions and buffers. Dipalmitoyl phosphatidylethanolamine (DPPE) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL).

Synthesis of the Tienilic Acid Amide Conjugate of DPPE (8). Tienilic acid (5 mg, 15 μmol) and dry dimethylformamide (1 mL) were combined in a small flask and cooled with an ice bath. EDC hydrochloride (3 mg, 16 μmol), HOBt (2 mg, 15 μmol), and triethylamine (4 μL, 30 μmol) were added in that order, the ice bath was removed, DPPE (10 mg, 15 μmol) was added, and the reaction was continued overnight at room temperature. For workup, brine was added, and the mixture was extracted with ethyl acetate several times. The extract was back-washed with brine and then sodium bicarbonate solution and dried over anhydrous sodium sulfate. The crude product was purified by chromatography over silica gel eluting with MeOH/$CHCl_3$ (20:80 v/v). Yield ca. 4 mg. R_f ~0.3 (MeOH/$CHCl_3$, 20:80 v/v). MS: $[M - H]^-$ = 1002.4 (calcd 1002.46).

Hepatocyte Incubations. For the incubation of [^{14}C]-TA, the specific activity of the substrate was adjusted to 36.2 Ci/mol by adding unlabeled TA. The substrate (596 nmol) was pipetted into a 50 mL Erlenmeyer flask, solvent was removed in vacuo, and a suspension of 9.5 × 10^6 viable hepatocytes in 4.0 mL of Williams' medium E was added to give a final TA concentration of 150 μM. The flask was flushed with O_2/CO_2 (95:5), sealed with a ground glass stopper, and incubated with gentle agitation at 37 °C for 2 or 4 h. After incubation, the cells were isolated by centrifugation (500 rpm for 3 min), washed twice with PBS, pelleted, and frozen (−80 °C) until analysis. A second incubation was performed similarly but on a larger scale (i.e., 28.8 × 10^6 cells from the same pool in 12 mL of medium containing 155 μM unlabeled TA) to increase the overall yield of adducted protein.

Preparation of Cell Lysates. Human hepatocytes incubated with [^{14}C]-TA were lysed with an isoelectric focusing (IEF) sample buffer (IPG strip rehydration solution, RHS) containing 9 M urea, 4% CHAPS, a mixture of ampholites (IPG buffer, pH 3−10NL), 65 mM DTT, and a trace of bromophenol blue. Briefly, to the cell pellet obtained from the incubation with [^{14}C]-TA (65 mg wet weight) was added 450 μL of RHS, the mixture was then gently mixed by vortex, then kept overnight at room temperature to complete solubilization. Insoluble materials were then removed by centrifugation (15000*g*, 15 min) and the resulting clear supernatant aliquotted. An aliquot containing 6 × 10^5 dpm ^{14}C was further diluted with RHS (1:2, v/v) and immediately infused into an IPG strip. After isoelectric focusing, development in the second dimension, and transblotting, the blot was subjected to phosphorimaging as described below. In addition, small aliquots (1500−2000 dpm) were submitted to one-dimensional SDS−PAGE, followed by phosphorimaging.

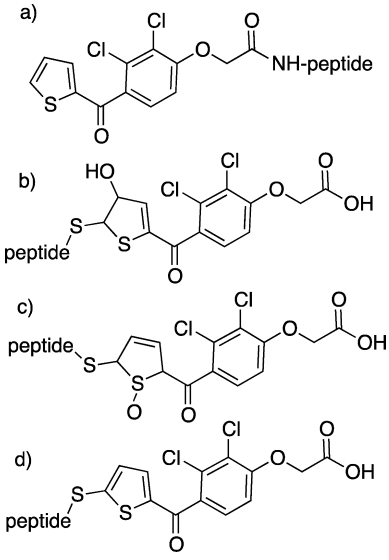
The cell pellet from the incubation of hepatocytes with unlabeled TA (193 mg wet weight) was resuspended by pipetting with 1 mL of KPBS (25 mM potassium phosphate and 150 mM sodium chloride, pH 7.4), to give a total volume of 1.2 mL. An aliquot of this suspension (ca. 15%) was reserved for lipid analysis (see below), and the remainder was centrifuged (100*g* × 5 min). The resulting pellet (ca. 162 mg wet weight) was mixed with 1215 μL of RHS and solubilized exactly as the ^{14}C-containing sample, above. The resulting clear solubilizate was split into 6 equal aliquots, each corresponding to ca. 2 × 10^6 incubated cells. One of the aliquots was reserved for 1D gel electrophoresis, and the remaining five aliquots were immediately submitted to 2DGE separation followed by proteomics analysis as described below.

Preparation of Lipid Extracts and MS/MS Analysis of Phospholipids. A phospholipid fraction from the incubated cells was obtained by Folch extraction, and the extract was analyzed by MS/MS as described earlier.[44] A synthetic preparation of the tienilic acid amide conjugate of DPPE was used as a standard.

Electrophoresis, Phosphorimaging and In-Gel Digestion. These standard procedures were carried out essentially as described earlier.[45−47]

Mass-Spectrometry of Tryptic Digests and Protein Identification. Digested protein samples were submitted to a capillary LC-MS/MS using a LTQ-FT hybrid linear quadrupole ion trap Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (ThermoFinnigan, Bremen, Germany) as described.[48] Raw data files were processed using BioWorks 3.2 software followed by peptide/protein identification using Sequest (ThermoFinnigan), Mascot (Matrix Science, version 2.2), and X!Tandem (www.thegpm.org) database-searching programs with the SwissProt 2011 database with a fragment ion mass tolerance of 0.20 Da and a parent ion tolerance of 20 ppm. The *S*-pyridylethyl (+105) derivative of cysteine residues and methionine *S*-oxidation (+16) were specified as variable modifications. Possible mass shifts for putative adducts of TA to lysine (+312), as well as to cysteine (+328 and +346) were also considered (Scheme 2). The lysine adduct could conceivably form via activation of the carboxyl group of TA as either an acyl glucuronide or an acyl coenzyme A thioester. Alternatively, either epoxidation or *S*-oxidation of the thiophene ring could activate the molecule toward protein sulfhydryl groups to give a +346 adduct, and dehydration of either of these adducts would lead to a +328 adduct.

Scheme 2. Structures of Potential Peptide Adducts of Reactive TA Metabolites



Scaffold software (Proteome Software Inc., version 3.3.1) was used to combine and validate MS/MS based peptide and protein identifications. Peptide identifications with greater than 50% probability as specified by the Peptide Prophet algorithm[49] were accepted for reporting protein coverage. Taking into account the high accuracy of peptide mass determination by LTQ-FTMS (usually within 10 ppm), we performed, for select samples, an additional search of SwissProt database using parent peptide−ion masses (using Decon software; http://omics.pnl.gov/software/DeconTools) and MS-Fit (http://prospector.ucsf.edu) or Mascot searching software. For several identified proteins, we performed visual examination of raw MS and MS/MS spectra for the presence of ion-clusters showing chlorine natural abundance isotopic signature and, independently, for the presence of peptide−ions with calculated mass shifts for putative TA-adducts (see above).

RESULTS AND DISCUSSION

After incubation of hepatocytes with [^{14}C]-TA, the cells were harvested by centrifugation, dissolved in lysis buffer, and

recentrifuged. The soluble supernatant (470 μL) contained 1.06 μCi of radioactivity, corresponding to 0.453 nmol-equiv of TA per mg cell wet weight, while the insoluble material contained less than 4% of total ^{14}C. SDS−PAGE analysis of the solubilizate showed numerous bands of protein with a majority of the density in the region of 25−75 kDa (Figure 1, left).

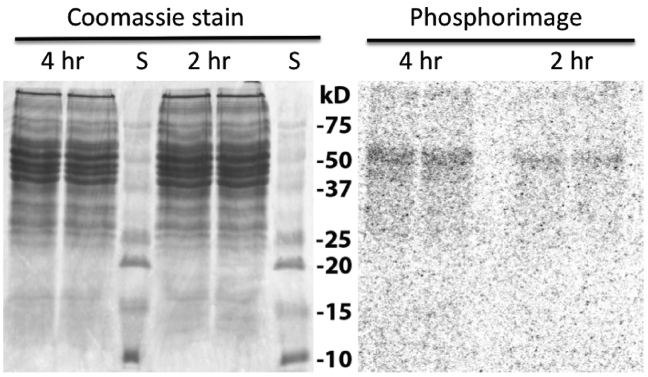


Figure 1. SDS−PAGE gel (left) and phosphorimaging analysis (right) of cellular proteins from human hepatocytes incubated with [^{14}C]-tienilic acid for 2 or 4 h. S = marker protein standards.

Phosphorimaging of a blot of this gel showed a prominent band of radioactivity that increased with incubation time around 50−60 kDa, along with smaller amounts of radioactivity at lower molecular mass. A similar 1D electrophoresis/phosphorimaging result was reported by Bonierbale et al.,[20] who incubated [^{14}C]-TA with human liver microsomes. Their protein band reacted with anti-TA-protein adduct antibodies and with anti-LKM_2 antibodies, and was therefore presumed to represent CYP2C9. The appearance of a band of radioactivity around 50−60 kDa in both studies is consistent with the fact that TA is an efficient suicide substrate for CYP2C9 and labels that protein with essentially 1:1 stoichiometry.[16,19] This result from 1-dimensional SDS−PAGE is significant because many membrane proteins, including P450 enzymes, do not behave well under conditions of 2D gel electrophoresis and are generally not observable in this way.[50]

The remainder of the ^{14}C cell extract was submitted to 2D gel electrophoresis in parallel with the corresponding soluble fraction from cells incubated with nonradioactive TA. A representative blot image is shown in Figure 2. Thirty-six of the spots contained radioactivity that was low but detectable by phosphorimaging (data not shown). Interestingly, essentially all of the radioactive protein spots were associated with low-abundance proteins, while numerous spots for abundant proteins contained no detectable radioactivity (see below).

In addition to the numerous small protein spots distributed across the gel, a large diffuse nonprotein staining spot containing a considerable amount of radioactivity appeared near the low pI/low MW corner of the blot. We observed a similar phenomenon previously in the 2DGE of microsomal proteins from the livers of rats treated with [^{14}C]-bromobenzene[46] or [^{14}C]-thiobenzamide.[48] Because this material came from the microsomal fraction but did not stain as protein and was not observed in the corresponding cytosol fractions, we suspected that it might be lipid-derived. In previous work, we demonstrated that *S*-oxidative bioactivation of thiobenzamide in rat liver in vivo leads to extensive acylation of the amine group in phosphatidylethanolamine (PE) lipids.[44] Conceivably, a TA-amide adduct of PE lipids such as structure **8** (Scheme 3) could account for this radioactive nonprotein material. Such an adduct could potentially be formed via a reactive acyl glucuronide or acyl coenzyme A thioester conjugate of TA, although we know of no specific precedent for such a process. To test this hypothesis, we prepared a synthetic standard of the TA amide of dipalmitoyl phosphatidylethanolamine (TA-DPPE, **8**), characterized its behavior in MS and MS/MS, and used this information to search for analogous TA-PE amides in Folch extracts of the low pI/low MW area of the actual 2D gels from the TA-hepatocyte incubations.

TA-DPPE is readily detectable by electrospray ionization in both positive and negative ion modes, the molar response for

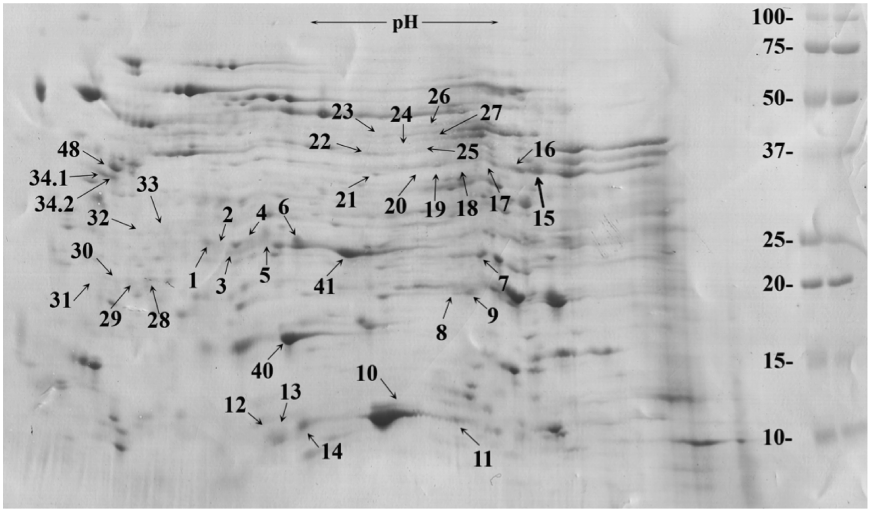
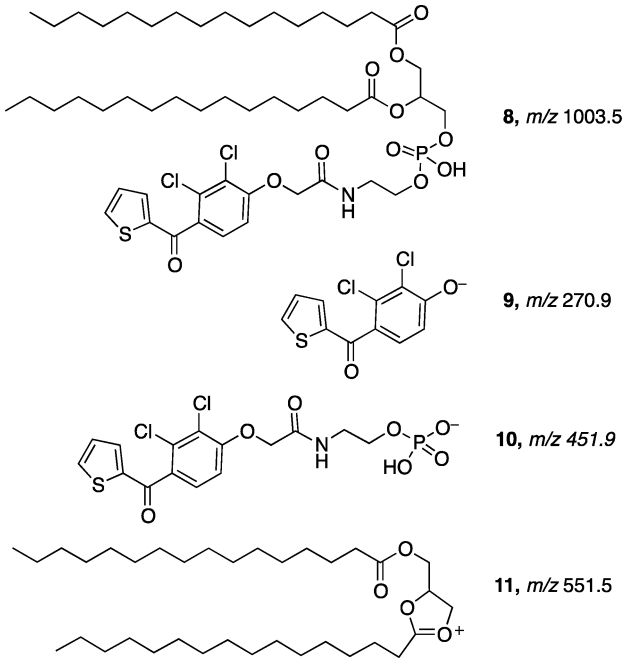


Figure 2. Image of Coomassie stained 2D gel analysis of cellular proteins from human hepatocytes incubated with [^{14}C]-tienilic acid for 2 h. The pH range is from ca. 4 at the far left to 10.0 at the right (but excluding the numbered molecular mass standards at the far right). Proteins found in the numbered spots are listed in Table 1, and all spots other than 40 and 41 were radioactive. Note that some of the spots were faint and do not reproduce well in the photograph, but they are clearly visible to an experienced operator, and the mass spectrometer clearly identified protein(s) in their tryptic digests.

Scheme 3. Structure and Mass Spectral Fragmentation of the Model TA-Phospholipid Adduct



MH^+ being 2.4 times that of $M - H^-$, but when spiked into Folch extract of liver lipids at a mass ratio of 7% that of total lipids, the $M - H^-$ ion gives a 20-fold better s/n ratio due to the high background of other lipids detected above 900 u in positive mode. To detect TA-modified PE lipids in the lipid pool extracted from hepatocytes incubated with TA, we first explored headgroup-specific tandem MS scans using synthetic TA-DPPE. In negative ion mode, the most abundant headgroup-specific product ions from $M - H^-$ were m/z 271 (structure **9**) and m/z 452 (structure **10**). In positive ion mode, the most abundant headgroup-specific product ion from MH+ is m/z 551 representing a neutral loss of 453 (i.e., **10** + H^+), in analogy to the "classic" characteristic neutral loss of 141 from unmodified PEs. The neutral loss of 453 in positive ion mode detects the molecular ion of TA-DPPE with 330 times the sensitivity of detection by precursors m/z 270.9 in negative ion mode. When TA-DPPE is spiked into the Folch extract of control liver tissue to the extent of 7% of total lipids, the scan for neutral loss of 453 detects the TA-DPPE with a s/n ratio of better than 180, which gives a detection limit of 0.04% of total lipid. Nevertheless, we observed no lipids, modified or unmodified, in the Folch extracts from the 2D gels. In corresponding Folch extracts from whole hepatocytes incubated with TA, we observed all of the usual major classes of phospholipids, but no acylated PE lipids were detected. Thus, for all practical purposes, TA-PE lipid adducts are not formed when hepatocytes are incubated with TA. This is very different from the situation with thiobenzamide, which modifies (i.e., acylates) up to 23% of total microsomal PE lipids in rat liver.[44] The nature of the radioactive material in the low MM/low pI area of the gel thus remains to be determined.

Protein spots corresponding to radioactive spots on the phosphorimaged blot were excised from the five nonradioactive gels, pooled, and digested with trypsin, and the digests were analyzed by LC-MS/MS. Thirty-one of the spots contained at least one identifiable protein, and 43 proteins were identified overall (Table 1). Automated searching did not reveal the presence of TA-adducted peptides such as those indicated in Scheme 2 in any of the protein digests examined. This was not surprising in vew of the small amount of sample we had to work with and the generally low level of overall protein adduction. Therefore, we also performed thorough visual examination of mass spectra for the presence of ion clusters with a chlorine isotopic signature and for the presence of peptide ions with calculated mass shifts corresponding to putative TA-derived adducts, but neither approach resulted in detection of any adducts.

Sixteen of the 31 protein spots analyzed (Group A) contained only a single detectable protein; hence, the proteins in these radioactive spots are taken to be target proteins for TA metabolites. However, three of these 16 spots each contained the same single protein, so the total number of nonredundant target proteins identified is 14. Rat or mouse orthologues of 6 of these 14 human proteins are already known as targets for reactive metabolites of 4 different chemicals, whereas 8 of the target proteins in Group A are identified here for the first time.

The other 15 spots each contained from 2 to 4 identifiable proteins (Groups B and C). Since we did not observe any adducted peptides in the digests of these samples, the identity of the target protein(s) in these spots is necessarily ambiguous. Interestingly, however, Group B of Table 1 indicates that 6 of the 15 multiprotein spots (i.e., spots # 1, 10, 12, 13, 22, and 34.1) each contained one target protein from Group A, while one spot (# 27) contained two target proteins from Group A. When analyzing cellular proteins by 2DGE, it is not uncommon to observe the same protein appearing in several spots of the same MW but different pI.[45,46,51,52] Thus, it is possible, perhaps likely, that the radioactivity observed in these seven multiprotein spots is associated with the same target proteins that were also observed in single protein spots (Group A), but since we cannot rule out the possibility that other proteins in these spots may also be adducted, they are not excluded from Table 1. Altogether, the 15 multiprotein spots in Table 1 contain 29 nonredundant proteins (Group C) whose target status is ambiguous. As indicated in Table 1, however, the Reactive Metabolite Target Protein Database (TPDB) (http://tpdb.medchem.ku.edu:8080/protein_database/) indicates that rat or mouse orthologues of 14 of these 29 human proteins are already known as targets for one or more of 12 different chemicals.

Because many membrane proteins do not behave well on 2D gel electrophoresis, we also performed mass spectral analysis of peptides produced by tryptic digestion of proteins in 18 bands cut from a 1D gel separation of the same solubilized proteins used for our 2D gel analysis. Using the same protein identification protocols, we identified more than 400 proteins, with the most populated region being in the 45−60 kDa range as expected (Figure 1). Among the identified proteins, we observed cytochromes P450 2C9, 3A4, 2A6, and 2E1 with sequence coverages from 7 to 38%. No peptides with calculated mass shifts for putative adducts were detected. Possible reasons for this include the low level of adduction, low sequence coverage, and possible instability of adducts in the mass spectrometer. More than 100 proteins were identified with sequence coverages in excess of 70%, but even among these, no adducts were observed. Interestingly, no adducted peptides from CYP2C9 were observed by Koenigs et al. even when purified and reconstituted 2C9 was incubated with tienilic acid in vitro, although in this case whole molecule mass

dx.doi.org/10.1021/tx300103j | Chem. Res. Toxicol. 2012, 25, 1145−1154

Table 1. Proteins Identified in Radioactive 2DGE Spots from Human Hepatocytes Incubated with [^{14}C]-Tienilic Acid

spot #	protein name[a]	SwissProt acc. #	# peptides matched by MS/MS[b]	percent sequence covered[c]	also targeted by[d]
		Group A			
23	2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial	P12694	2	7	
20	acetyl-CoA acetyltransferase, cytosolic (cDNA FLJ53975)	Q9BWD1	3	8	
28	cathepsin B, heavy chain (129−333)	P07858	2	12	MYCO
29	cathepsin B, heavy chain (129−333)	P07858	2	12	MYCO
30	cathepsin B, heavy chain (129−333)	P07858	1	5	MYCO
11	D-dopachrome decarboxylase	P30046	6	46	BB, TB
3	enoyl-CoA hydratase, mitochondrial	P30084	16	52	BHT
14	fatty acid-binding protein, liver	P07148	3	23	BB, TB
25	fumarylacetoacetase	P16930	2	5	BB, TB
21	galactokinase (cDNA FLJ56840)	P51570	14	36	
16	isovaleryl-CoA dehydrogenase, mitochondrial	P26440	7	18	
24	medium-chain specific acyl-CoA dehydrogenase, mitochondrial	P11310	11	27	
34.2	nucleophosmin Isoform 1	P06748-1	9	26	
9	phosphatidylethanolamine-binding protein 1	P30086	6	40	BB, TB
2	prohibitin	P35232	4	15	
19	short-chain specific acyl-CoA dehydrogenase, mitochondrial	P16219	12	34	
		Group B			
27	2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial	P12694	2	7	
10	fatty acid-binding protein, liver	P07148	4	31	BB, TB
12	fatty acid-binding protein, liver	P07148	4	31	BB, TB
13	fatty acid-binding protein, liver	P07148	5	36	BB, TB
22	medium-chain specific acyl-CoA dehydrogenase, mitochondrial	P11310	5	16	
34.1	nucleophosmin Isoform 1	P06748-1	9	26	
1	prohibitin	P35232	12	40	
27	short-chain specific acyl-CoA dehydrogenase, mitochondrial	P16219	4	12	
		Group C			
5	3-hydroxyisobutyrate dehydrogenase, mitochondrial	P31937	3	14	
6	3,2-*trans*-enoyl-CoA isomerase, mitochondrial, Isoform 1	P42126-1	2	9	MYCO
48	α-1 acid glycoprotein	P02763	4	22	
34.1	α-1-acid glycoprotein 1	P02763	3	17	
22	β-ureidopropionase (UPB1 protein fragment)	Q9UBR1	2	6	BB, TB
1	cathepsin D (heavy chain, 169−412)	P07339	8	32	
5	cathepsin D (heavy chain, 169−412)	P07339	8	31	
7	dihydropteridine reductase	P09417	2	11	
22	DnaJ homologue subfamily B member 11	Q9UBS4	2	7	TB
4	endoplasmic reticulum protein ERp29	P30040	5	24	BB, TB
5	endoplasmic reticulum protein ERp29	P30040	7	34	BB, TB
6	endoplasmic reticulum protein ERp29	P30040	16	50	BB, TB
48	endoplasmin?	P14625	18	18	HAL, APAP, AMAP
6	enoyl-CoA hydratase domain-containing protein 2, mitochondrial, Isoform 2	Q86YB7-2	3	14	
15	fructose-bisphosphate aldolase B	P05062	10	28	TB, MYCO, TEU
7	glutathione *S*-transferase Mu 1 (isoform 1)	P09488	9	35	BB, TB, VCN, TEU
4	glutathione *S*-transferase omega-1	P78417	7	30	
6	glutathione *S*-transferase omega-1	P78417	18	49	
15	hydroxyacid oxidase 1	Q9UJM8	6	19	
26	hydroxymethylglutaryl-CoA synthase, mitochondrial	P54868	7	14	TEU
27	isocitrate dehydrogenase [NADP] cytoplasmic	O75874	10	23	BB, TB
26	keratin, type II cytoskeletal 1	P04264	6	11	
10	keratin, type II cytoskeletal 2 epidermal	P35908	6	11	
34.1	keratin, type II cytoskeletal 8	P05787	9	17	
48	keratin, type II cytoskeletal 8	P05787	8	15	
26	lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondrial	P11182	11	25	
5	peroxiredoxin-4	Q13162	4	17	TB
8	peroxiredoxin-6	P30041	5	22	MYCO, TB, ATRZ, BHT, NAPH
22	*S*-adenosylmethionine synthetase isoform type-1	Q00266	5	13	APAP, AMAP
10	serum amyloid A protein	P02735	2	30	

dx.doi.org/10.1021/tx300103j | *Chem. Res. Toxicol.* 2012, 25, 1145−1154

Table 1. continued

spot #	protein name[a]	SwissProt acc. #	# peptides matched by MS/MS[b]	percent sequence covered[c]	also targeted by[d]
		Group C			
12	serum amyloid A2 isoform a	NP_110381	2	11	
13	serum amyloid A2 isoform a	NP_110381	2	11	
8	superoxide dismutase [Mn], mitochondrial	P04179	7	31	
7	triosephosphate isomerase isoform 1(fragm)	Q53HE2	12	52	MYCO, BB, DACT, BHT
27	Tu translation elongation factor, mitochondrial	P49411	8	15	BHT, BENZ
15	voltage-dependent anion-selective channel protein 2, isoform 2	P45880-2	2	9	

[a]Many proteins can appear in more than one spot on a 2D gel (see text). Group A comprises spots in which only one protein was found. Group B lists spots containing two or more proteins, one of which was also identified in a single-protein spot in Group A. Group C spots contain two or more proteins, none of which was observed in Group A. [b]Number of peptides sequenced and matched to the identfied protein. [c]Percent of entire protein sequence covered by peptides observed. [d]Other chemicals whose reactive metabolites are also known[42] to target the identified TA target protein. Abbreviations: AMAP, *m*-hydroxyacetanilide; APAP, *p*-hydroxyacetanilide; ATRZ, atrazine; BB, bromobenzene; BENZ, benzene; BHT, butylated hydroxytoluene; DACT, diaminochlorotriazine; HAL, halothane; MCTP, monocrotaline pyrrole; MYCO, mycophenolic acid; NAPH, naphthalene; TB, thiobenzamide; TEU, teucrin A; VNC, acrylonitrile.

spectrometry clearly showed adduction of the 2C9 protein by tienilic acid.[17]

Among the 14 TA target proteins listed in Group A of Table 1, eight are involved in fatty acid uptake or metabolism. The mitochondrial enzymes short-chain specific acyl-CoA dehydrogenase, medium-chain specific acyl-CoA dehydrogenase, isovaleryl-CoA dehydrogenase, and 2-oxoisovalerate dehydrogenase subunit α, and the cytosolic enzyme fumarylacetoacetase, all play key roles in the metabolism of fatty acids or branched chain keto acids derived from amino acid metabolism, and all are associated with genetically inherited deficiency diseases that have severe consequences at the cellular and organismal level. Another mitochondrial enzyme targeted by TA, enoyl-CoA hydratase, has been implicated in nonalcoholic fatty liver disease, and siRNA knockdown of this enzyme leads to increased accumulation of lipid droplets in both a cell line and in vivo.[53] Liver fatty acid binding protein (LFABP), a major target protein for metabolites of bromobenzene[45] and thiobenzamide,[48] has been suggested to be an early marker for kidney disease and acute kidney failure,[54] and transgenic mice expressing human LFABP show moderate protection against kidney disease in several models of nephropathy.[55,56]

Three of the Group A proteins are very well known and of special interest from the point of view of their potential role in cytotoxicity, namely, nucleophosmin (NPM), prohibitin (PHB), and Raf kinase inhibitory protein (RKIP, also known as phosphatidylethanolamine binding protein or PEBP-1). Nucleophosmin is a ubiquitously expressed phosphoprotein that moves between the cytoplasm, nucleus, and nucleolus and is associated with lipid rafts.[57,58] It binds to DNA, RNA, histones, unfolded proteins, and NF-kappaB, and is involved in a multitude of cellular processes including protein chaperoning, cell proliferation, chromatin assembly and disassembly, transcription coactivation, stress response, and apoptosis. It forms a dimer of pentamers having a ring-like structure that is no doubt related to its chaperone function. Multiple post-translational modifications of NPM are known, including acetylation, phosphorylation ($\geq$6 sites), SUMOylation, ubiquitination, and poly-(ADP-ribosyl)-ation, and are variously associated with specific functions and/or subcellular localizations of NPM. Cellular levels of NPM increase 3-fold upon exposure of human lung epithelial cells to chlorobenzene and 1,2-dichlorobenzene (both of which are significantly hepatotoxic as well as pneumotoxic).[59] NPM is one of several proteins that become glutathionylated in 3T3 cells upon treatment with a nitric-oxide releasing prodrug, and this covalent modification was linked to the activation of kinases associated with stress response and cell death pathways including p38, JNK, and c-jun.[60] Interestingly, NPM (−/−) null mice are embryonically nonviable.

RKIP and its orthologues constitute a large family of proteins conserved throughout evolution and widely expressed in eukaryotic organisms.[61−63] It is a PE binding protein having a discrete ligand binding site that binds cacodylate and could accommodate the headgroup of a phospholipid. Depending on its phosphorylation state, it can inhibit MAP kinases (Raf-MEK-ERK), G-protein coupled receptor kinases, and NF-kappaB kinases. In doing so, it integrates cross-talk in pathways initiated by diverse environmental stimuli that affect cellular proliferation, differentiation, survival, angiogenesis, metastasis, apoptosis, and resistance to apoptosis. In the male mouse, deletion of the RKIP gene leads to decreased rates of reproductive success with wild type females, but knockout females have normal rates of reproductive success with wild type males. Because of its central position overlapping with several major signaling cascades, there is much interest in RKIP as a potential therapeutic target,[63] and one inhibitor (locostatin, (4*S*)-3-[(*E*)-but-2-enoyl]-4-benzyl-2-oxazolidinone) is commercially available.

Members of the prohibitin family of proteins are highly conserved evolutionarily.[64−66] They are subdivided into two groups, PHB1 and PHB2, and at least one from each group is found in every known eukaryotic genome, suggesting that the two types are not functionally redundant. They function in both the cytosol and mitochondria and are required for embryonic development in *C. elegans*, mice, and plants. Rat, mouse, and human PHBs are nearly identical, but they are known to undergo a wide range of post-translational modifications. For example, they are phosphorylated by insulin-, IGF-1-, and EGF-receptor tyrosine kinases. PHBs localize to the cell membrane and inner mitochondrial membrane as well as to the cytoplasm and nucleus depending on cell type and status. PHB1 and PHB2 function together as a 1 MDa complex of 12−16 1:1 heterodimers associated into a ring-like structure anchored to the membrane by transmembrane helices. The complex is speculated to be a hydrolase/unfoldase chaperone that protects mitochondrial membranes from unfolded or improperly associated proteins. PHBs are also thought to act as protein scaffolds to facilitate

cross-talk among signaling cascades, they are essential in metabolism, cell proliferation, and immune regulation, and they play important roles in PI3K/Akt, Raf/ERK, and TGF-β signaling pathways. Knockdown of PHB expression is deleterious in a variety of cell types, and gene deletion is embryonically lethal in mice and flies, whereas overexpression protects cardiomyocytes from hypoxia. Given the pleiotropic roles of PHB, RKIP, and NMP in overall cell function, and the multiplicity of post-translational modifications involved in modulating these roles, it would not be surprising that their post-translational modification by chemically reactive xenobiotic metabolites could contribute to cell injury or death.

In an earlier study, Methogo et al.[43] identified 15 proteins likely to be modified by reactive metabolites of TA in rat liver. However, four of these proteins including PDI-A1, adenosylhomocysteinase, arginase, and hsc 73 were only observed in multiprotein spots, so their adduction by TA is ambiguous. According to the TPDB, hsc 73 is a known target for butylated hydroxy toluene and naphthalene, arginase is a known target for bromobenzene and thiobenzamide, adenosylhomocysteinase is not listed as a target, and PDI-A1 is a target for 10 other chemicals. However, five proteins identified by Methogo et al. as appearing in single-protein spots were not listed in their summary list of likely targets. These proteins (and other chemicals known to target them) include albumin (bromobenzene and thiobenzamide), β-actin (butylated hydroxytoluene and mycophenolic acid), 4-hydroxyphenylpuruvate dioxygenase (mycophenolic acid), and UDP-glucose pyrophosphorylase and keratin Kb1. Among all of the above rat liver proteins, only two, fumarylacetoacetase and triosephosphate isomerase, are in common with the human hepatocyte target proteins listed in Table 1. There could be several reasons for the differences between the two lists of TA targets. One could be methodological, in that Methogo used Western blotting with antiadduct antibodies to detect adducted proteins, whereas we used radioactivity and phosphorimaging. Another is that species differences between rat and human may be larger than expected. A third potential reason is purely statistical, i.e., given the very large number of potential target proteins vs the small size of each target protein list, it is not surprising that the overlap between the two lists from two different laboratories might be small.

Currently, the TPDB contains information on 540 individual adduction events involving 46 different chemicals and 356 nonredundant proteins, but only 42 of these proteins are human proteins, only 21 of these 42 are targets for more than one chemical, and only 11 of these 21 have rat or mouse orthologues that are also known to be targets for reactive metabolites. Viewed another way, the 42 human proteins in the TPDB include albumin, hemoglobin, 8 isoforms of tubulin that were adducted relatively selectively by exposing A549 lung cancer cells to chemically reactive isothiocyanates that may not require metabolic activation, and 8 members of the serine hydrolase family that are adducted very selectively by nerve gas analogues. While the numbers of target proteins known for several "paradigm" protoxins are relatively good (e.g., 62 for thiobenzamide, 46 for teucrin, 45 for bromobenzene, 40 for naphthalene, 34 for butylated hydroxytoluene, and 33 for acetaminophen), the data on protein targets of the other 40 chemicals in the TPDB are much more sparse, and the overall data for human proteins are particularly sparse. It is thus difficult to draw mechanistic conclusions or even to formulate specific hypotheses, from comparisons of small sets of targets for a few chemical agents. It is a stark realization that the 356 known proteins in the TPDB represent not an abundance but rather a paucity of information within the vast space defined by the dimensions of chemical agent, animal species, target tissue, and individual protein. This in turn raises an important question about the degree of concordance between human targets vs animal targets across the other variables (i.e., chemical agent and tissue). Unfortunately, this question cannot be answered with currently available data, but given the demonstrated feasibility of identifing reactive metabolite target proteins from isolated human hepatocytes, it should be possible to address this important question more directly in the future.

AUTHOR INFORMATION

Corresponding Author

*Address correspondence to: Dr. Robert P. Hanzlik University of Kansas, Department of Medicinal Chemistry, Room 4048, Malott Hall, 1251 Wescoe Hall Drive, Lawrence, KS 66045. Tel: 785-864-3750. Fax: 785-864-5326. E-mail: rhanzlik@ku.edu.

Funding

The research described herein was supported in part by NIH grant GM-21784 (to R.P.H.).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. Patrick Dansette, Université Paris-Descartes, for helpful discussions.

ABBREVIATIONS

2DGE, 2-dimensional gel electrophoresis; AST, aspartate aminotransferase; BSP, bromosulfophthalein; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CYP2C9, cytochrome P450 2C9; DILI, drug-induced liver injury; DPPE, dipalmitoyl phosphatidylenthanolamine; DTT, dithiothreitol ((2*S*,3*S*)-1,4-bis(sulfanyl)butane-2,3-diol); EDC, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride; IDILI, idiosyncratic DILI; HOBt, hydroxybenzotriazole; MM, molecular mass; PBS, phosphate-buffered isotonic saline (pH 7.4); PE, phosphatidylethanolamine; TA, tienilic acid ([2,3-dichloro-4-(2-thienylcarbonyl)phenoxy]acetic acid); TAI, tienilic acid isomer ([2,3-dichloro-4-(3-thienylcarbonyl)phenoxy]-acetic acid); TPDB, reactive metabolite target protein database

REFERENCES

(1) Meanwell, N. (2011) Improving drug candidates by design: A focus on physicochemical properties as a means of improving compound disposition and safety. *Chem. Res. Toxicol. 24*, 1420−1456.

(2) Johnson, W. W. (2008) Many drugs and phytochemicals can be activated to biological reactive intermediates. *Curr. Drug Metab. 9*, 344−351.

(3) Kaplowitz, N. (2005) Idiosyncratic hepatotoxicity. *Nat. Rev. Drug Discovery 4*, 489−499.

(4) Walgren, J. L., Mitchell, M. D., and Thompson, D. C. (2005) Role of metabolism in drug-induced idiosyncratic hepatotoxicity. *Crit. Rev. Toxicol. 35*, 325−361.

(5) Nakayama, S., Atsumi, R., Takakusa, H., Kobayashi, Y., Kurihara, A., Nagai, Y., Nakai, D., and Okazaki, O. (2009) A zone classification system for risk assessment of idiosyncratic drug toxicity using daily dose and covalent binding. *Drug Metab. Dispos. 37*, 1970−1977.

(6) Obach, R. S., Kalgutkar, A. S., Soglia, J. R., and Zhao, S. X. (2008) Can in vitro metabolism-dependent covalent binding data in liver

microsomes distinguish hepatotoxic from nonhepatotoxic drugs? An analysis of 18 drugs with consideration of intrinsic clearance and daily dose. *CRC Crit. Rev. Toxicol. 21*, 1814−1822.
(7) Takakusa, H., Masumoto, H., Yukinaga, H., Makino, C., Nakayama, S., Okazaki, O., and Sudo, K. (2008) Covalent binding and tissue distribution/retention assessment of drugs associated with idiosyncratic drug toxicity. *Drug Metab. Dispos. 36*, 1770−1779.
(8) Usui, T., Mise, M., Hashizume, T., Yabuki, M., and Komuro, S. (2009) Evaluation of the potential for drug-induced liver injury based on in vitro covalent binding to human liver proteins. *Drug Metab. Dispos. 37*, 2383−2392.
(9) Roth, R. A., and Ganey, P. E. (2011) Animal models of idiosyncratic drug-induced liver injury: current status. *Crit. Rev. Toxicol.*, 1−17.
(10) Uetrecht, J. (2009) Immune-mediated adverse drug reactions. *Chem. Res. Toxicol. 22*, 24−34.
(11) Zimmerman, H. J., Lewis, J. H., Ishak, K. G., and Maddrey, W. C. (1984) Ticrynafen-associated hepatic injury: Analysis of 340 cases. *Hepatology 4*, 315−323.
(12) Park, B. K., Laverty., H., Srivastava, A., Antoine, D. J., Naisbitt, D., and Williams, D. P. (2011) Drug bioactivation and protein adduct formation in the pathogenesis of drug-induced toxicity. *Chem.-Biol. Interact. 192*, 30−36.
(13) Homberg, J. C., Andre, C., and Abauf, N. (1984) A new anti-liver-kidney microsome antibody (anti-LKM_2) in tienilic acid-induced hepatitis. *Clin. Exp. Imunol. 55*, 561−570.
(14) Mansuy, D. (1997) Molecular structure and hepatotoxicity: Compared data about two closely related thiophene compounds. *J. Hepatol. 26* (Suppl. 2), 22−25.
(15) Neuberger, J., and Williams, R. (1989) Immune mechanisms in tienilic acid associated hepatotoxicity. *Gut 30*, 515−519.
(16) Lopez-Garcia, M. P., Dansette, P. M., and Mansuy, D. (1994) Thiophene derivatives as new mechanism-based inhibitors of cytochromes P-450: Inactivation of yeast-expressed human liver cytochrome P-450 2C9 by tienilic acid. *Biochemistry 33*, 166−175.
(17) Koenigs, L. L., Peter, R. M., Hunter, A. P., Haining, R. L., Rettie, A. E., Friedberg, T., Pritchard, M. P., Shou, M., Rushmore, T. H., and Trager, W. F. (1999) Electrospray ionization mass spectrometric analysis of intact cytochrome P450: Identification of tienilic acid adducts to P450 2C9. *Biochemistry 38*, 2312.
(18) Lecoeur, S., Bonierbale, E., Challine, D., Gautier, J.-C., Valadon, P., Dansette, P. M., Catinot, R., Ballet, F., Mansuy, D., and Beaune, P. H. (1994) Specificity of *in vitro* covalent binding of tienilic acid metabolites to human liver microsomes in relationship to the type of hepatotoxicity: Comparison with two directly hepatotoxic drugs. *Chem. Res. Toxicol. 7*, 434−442.
(19) Lopez-Garcia, M. P., Dansette, P. M., and Coloma, J. (2005) Kinetics of tienilic acid bioactivation and functional generation of drug-protein adducts in intact rat hepatocytes. *Biochem. Pharmacol. 70*, 1870−1882.
(20) Bonierbale, E., Valadon, P., Pons, C., Desfosses, B., Dansette, P. M., and Mansuy, D. (1999) Opposite behaviors of reactive metabolites of tienilic acid and its isomer toward liver proteins: Use of specific anti-tienilic acid—protein adduct antibodies and the possible relationship with different hepatotoxic effects of the two compounds. *Chem. Res. Toxicol. 12*, 286−296.
(21) Zimmerman, H. J., Abernathy, C. O., Lukacs, L., and Ezekiel, E. (1982) Effects of ticrynafen on hepatic excretory function in the isolated perfused rat liver. *Hepatology 2*, 255−257.
(22) Nishiya, T., Kato, M., Suzuki, T., Maru, C., Kataoka, H., Hattori, C., Mori, K., Jindo, T., Tanaka, Y., and Manabe, S. (2008) Involvement of cytochrome P450-mediated metabolism in tienilic acid hepatotoxicity in rats. *Toxicol. Lett. 183*, 81−19.
(23) Belghazi, M., Jean, P., Poli, S., Schmitter, J.-M., Mansuy, D., and Dansette, P. M. (2001) Use of isotopes and LC-MS-ESI-TOF for mechanistic studies of tienilic acid metabolic activation. *Adv. Exp. Med. Biol 500*, 139−144.
(24) Masubuchi, N., Makino, C., and Murayama, N. (2007) Prediction of *in vivo* potential for metabolic activation of drugs into chemically reactive intermediates: Correlation of *in vitro* and *in vivo* generation of reactive intermediates and *in vitro* glutathione conjugate formation in rats and humans. *Chem. Res. Toxicol. 20*, 455−464.
(25) Mansuy, D., Valadon, P., Erdelmeier, I., Lopez-Garcia, M. P., Amar, C., Girault, J.-P., and Dansette, P. M. (1991) Thiophene *S*-oxides as new reactive metabolites: Formation by cytochrome P450 dependent oxidation and reaction with nucleophiles. *J. Am. Chem. Soc. 113*, 7825−7826.
(26) Dansette, P. M., Amar, C., Smith, C., Pons, C., and Mansuy, D. (1990) Oxidative bioactivation of the thiophene ring by hepatic enzymes. *Biochem. Pharmacol. 39*, 911−918.
(27) Valadon, P., Dansette, P. M., Girault, J.-P., Amar, C., and Mansuy, D. (1996) Thiophene sulfoxides as reactive metabolites: Formation upon microsomal oxidation of a 3-aroylthiophene and fate in the presence of nucleophiles *in vitro* and *in vivo*. *Chem. Res. Toxicol. 9*, 1402−1413.
(28) Dansette, P. M., Amar, C., Valadon, P., Pons, C., Beaune, P. H., and Mansuy, D. (1991) Hydroxylation and formation of electrophilic metabolites of tienilic acid and its isomer by human liver microsomes. *Biochem. Pharmacol. 41*, 553−560.
(29) Lopez-Garcia, M. P., Dansette, P. M., Valadon, P., Amar, C., Beaune, P. H., Guengerich, F. P., and Mansuy, D. (1993) Human-liver cytochromes P-450 expressed in yeast as tools for reactive-metabolite formation studies. Oxidative activation of tienilic acid by cyrochromes P-450 2C9 and 2C10. *Eur. J. Biochem. 213*, 223−232.
(30) Beaune, P. H., Dansette, P. M., Mansuy, D., Kiffel, L., Finck, M., Leroux, J. P., and Homberg, J. P. (1987) Human anti-endoplasmic reticulum autoantibodies appearing in a drug-induced hepatitis are directed against a human liver cytochrome P450 that hydroxylates the drug. *Proc. Natl. Acad. Sci. U.S.A. 84*, 551−555.
(31) Jean, P., Lopez-Garcia, M. P., Dansette, P. M., Mansuy, D., and Goldstein, J. L. (1996) Oxidation of tienilic acid by human yeast-expressed cytochromes P-450 2C8, 2C9, 2C18 and 2C19. *Eur. J. Biochem. 241*, 797−804.
(32) Hutzler, J. M., Balogh, L. M., Zientek., M., Kumar, V., and Tracy, T. S. (2009) Mechanism-based inactivation of cytochrome P450 2C9 by tienilic acid and (±)-suprofen: A comparison of kinetics and probe substrate selection. *Drug Metab. Dispos. 37*, 59−65.
(33) O'Donnell, J. P., Dalvie, D. K., Kalgutkar, A. S., and Obach, R. S. (2003) Mechanism-based inactivation of human recombinant P450 2C9 by the nonsteroidal anti-inflamatory drug suprofen. *Drug Metab. Dispos. 31*, 1369−1367.
(34) Kazui, M., Nishiya, Y., Ishizuka, T., Hagihara, K., Farid, N., Okazaki, O., Ikeda, T., and Kurihara, A. (2010) Identification of the human cytochrome P450 enzymes involved in the two oxidative steps in the bioactivation of clopidogrel to its pharmacologically active metabolite. *Drug Metab. Dispos. 38*, 92−99.
(35) Pereillo, J.-M., Maftouh, M., Adrieu, A., Uzabiaga, M.-F., Fedeli, O., Savi, P., Pascal, M., Herbert, J.-M., Maffrand, J.-P., and Picard, C. (2002) Structure and stereochemistry of the active metabolite of clopidogrel. *Drug Metab. Dispos. 30*, 1288−1295.
(36) Dansette, P. M., Rosi, J., Bertho, G., and Mansuy, D. (2011) Cytochromes P450 catalyze both steps of the major pathway of clopidogrel bioactivation, whereas paraoxonase catalyzes the formation of a minor thiol metabolite iisomer. *Chem. Res. Toxicol. 25*, 348−356.
(37) Alvarez, F. J., and Slade, R. T. (1992) Kinetics and mechanism of degradation of zileuton, a potent 5-lipoxygenase inhibitor. *Pharm. Res. 9*, 1465−1473.
(38) Li, F., Chordia, M. D., Woodling, K. A., and Macdonald, T. L. (2007) Irreversible alkylation of human serum albumin by zileuton metabolite 2-acetylbenzothiophene *S*-oxide: A potential model for hepatotoxicity. *Chem. Res. Toxicol. 20*, 1854−1861.
(39) Joshi, E. M., Heasley, B. H., and Macdonald, T. L. (2009) 2-Abt-*S*-oxide detoxification by glutathione *S*-transferases A1−1, M1−1 and P1−1: Implications for toxicity associated with zileuton. *Xenobiotica 39*, 197−204.
(40) Joshi, E. M., Heasley, B. H., Chordia, M. D., and Macdonald, T. L. (2004) In vitro metabolism of 2-acetylbenzothiophene: Relevance to zileuton hepatotoxicity. *Chem. Res. Toxicol. 17*, 137−143.

(41) Evans, D. C., Watt, A. P., Nicoll-Griffith, D. A., and Baillie, T. A. (2004) Drug—protein covalent adducts: An industry perspective on minimizing the potential for drug bioactivation in drug discovery and development. *Chem. Res. Toxicol. 17*, 3−16.
(42) Reactive Metabolite Target Protein Database, http://tpdb.medchem.ku.edu:8080/protein_database/.
(43) Methogo, R., Dansette, P., and Klarskov, K. (2007) Identification of liver protein targets modified by tienilic acid metabolites using a two-dimensional western blot-mass spectrometry approach. *Int. J. Mass Spectrom. 268*, 284−295.
(44) Ji, T., Ikehata, K., Koen, Y. M., Esch, S. W., Williams, T. D., and Hanzlik, R. P. (2007) Covalent modification of microsomal lipids by thiobenzamide metabolites *in vivo*. *Chem. Res. Toxicol. 20*, 701−708.
(45) Koen, Y. M., Gogichaeva, N. V., Alterman, M. A., and Hanzlik, R. P. (2007) A proteomic analysis of bromobenzene reactive metabolite targets in rat liver cytosol *in vivo*. *Chem. Res. Toxicol. 20*, 511−519.
(46) Koen, Y. M., and Hanzlik, R. P. (2002) Identification of seven proteins in the endoplasmic reticulum as targets for reactive metabolites of bromobenzene. *Chem. Res. Toxicol. 15*, 699−706.
(47) Koen, Y. M., Williams, T. D., and Hanzlik, R. P. (2000) Identification of three protein targets for reactive metabolites of bromobenzene in rat liver cytosol. *Chem. Res. Toxicol. 13*, 1326−1335.
(48) Ikehata, K., Duzhak, T., Galeva, N. A., Ji, T., Koen, Y. M., and Hanzlik, R. P. (2008) Protein targets of reactive metabolites of thiobenzamide in rat liver in vivo. *Chem. Res. Toxicol. 21*, 1432−1442.
(49) Keller, A., Nesvizhskii, A. I., Kolker, E., and Aebersold, R. H. (2002) Empirical statistical model to estimate the accuracy of peptide identifications made by ms/ms and database search. *Anal. Chem. 74*, 5383−5392.
(50) Galeva, N. A., and Alterman, M. A. (2002) Comparison of one-dimensional and two-dimensional gel elecrophoresis as a separation tool for proteomic analysis of rat liver microsomes: Cytochromes P450 and other membrane proteins. *Proteomics 2*, 713−722.
(51) Meier, B. W., Gomez, J. D., Kirichenko, O. V., and Thompson, J. A. (2007) Mechanistic basis for inflammation and tumor promotion in lungs of 2,6-di-*tert*-butyl-4-methylphenol-treated mice: Electrophilic metabolites alkylate and inactivate antioxidant enzymes. *Chem. Res. Toxicol. 20*, 199−207.
(52) Qiu, Y., Burlingame, A. L., and Benet, L. Z. (1998) Identification of the hepatic protein targets of reactive metabolites of acetaminophen *in vivo* in mice using two-dimensional gel electrophoresis and mass spectrometry. *J. Biol. Chem. 273*, 17940−17953.
(53) Zhang, X., Yang, J., Guo, Y., Ye, H., Yu, C., Xu, C., Wu, S., Sun, W., Wei, H., Gao, X., Zhu, Y., Qian, Z., Jiang, Y., Li, Y., and He, F. (2010) Functional proteomic analysis of nonalcoholic fatty liver disease in rat models: Enoyl-coenzyme a hydratase down-regulation exacerbates hepatic steatosis. *Hepatology 51*, 1190−1199.
(54) McMahon, B., and Murray, P. T. (2010) Urinary liver fatty acid-binding protein: Another novel biomarker of acute kidney disease. *Kidney Internat. 77*, 657−659.
(55) Kamiho-Ikemori, A., Sugaya, T., Matsui, K., Yokoyama, T., and Kimura, K. (2011) Roles of human liver type fatty acid binding protein in kidney disease clarified using hL-FABP chromosamal transgenic mice. *Nephrology 16*, 539−544.
(56) Kunzendorf, U., Haase, M., Rölver, L., and Haase-Fielitz, A. (2010) Novel aspects of pharmacological therapies for acute renal failure. *Drugs 70*, 1099−1114.
(57) Colombo, E., Alcalay, M., and Pelicci, P. G. (2011) Nucleophosmin and its complex network: A possible therapeutic target in hematological diseases. *Oncogene 30*, 2595−2609.
(58) Okuwaki, M. (2008) The structure and functions of NPM/nucleophosmin/B23, a multifunctional nucleolar acidic protein. *J. Biochem. (Tokyo) 143*, 441−448.
(59) Mörbt, N., Tomm, J., Feltens, R., Mögel, I., Kalkhof, S., Murugesan, K., Wirth, H., Vogt, C., Binder, H., Lehman, I., and von Bergen, M. (2011) Chlorinated benzenes cause concomitantly oxidative stress and induction of apoptotic markers in lung epithelian cells (A549) at nonacute toxic concentrations. *J. Proteome Res. 10*, 363−378.
(60) Townsend, D. M., Findlay, V. J., Fazilev, F., Ogle, M., Fraser, J., Saavedra, J. E., Ji, X., Keefer, L. K., and Tew, K. D. (2006) A glutathione S-transferase π-activated prodrug causes kinase activation concurrent with *S*-glutathionylatioin of proteins. *Mol. Pharmacol. 69*, 501−508.
(61) Granovsky, A., and Rosner, M. R. (2008) Raf kinase inhibitory protein: A signal transduction modulator and metastasis suppressor. *Cell Res. 18*, 452−457.
(62) Klysik, J., Theroux., S. J., Sedivy, J. M., Moffit, J. S., and Boekelheide, K. (2008) Signaling crossroads: The function of raf kinase inhibitory protein in cancer, the central nervous system and reproduction. *Cell. Signalling 20*, 1−9.
(63) Zeng, L., Imamoto, A., and Rosner, M. R. (2008) Raf kinase inhibitory protein (RKIP): A physiological regulator and future therapeutic target. *Expert Opin. Ther. Targets 12*, 1275−1287.
(64) Artal-Sanz, M., and Tavernarakis, N. (2009) Prohibitin and mitochondrial biology. *Trends Endocrinol. Metab. 20*, 394−401.
(65) Mishra, S., Ande, S. R., and Nyomba, B. L. G. (2010) The role of prohibitin in cell signaling. *FEBS J. 277*, 3937−3946.
(66) Theiss, A. L., and Sitaraman, S. V. (2011) The role and therapeutic potential of prohibitin in disease. *Biochim. Biophys. Acta 1813*, 1137−1143.