Capture Compound Mass Spectrometry Sheds Light on the Molecular Mechanisms of Liver Toxicity of Two Parkinson Drugs

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Capture compound mass spectrometry (CCMS) is a novel technology that helps understand the molecular mechanism of the mode of action of small molecules. The Capture Compounds are trifunctional probes: A selectivity function (the drug) interacts with the proteins in a biological sample, a reactivity function (phenylazide) irreversibly forms a covalent bond, and a sorting function (biotin) allows the captured protein(s) to be isolated for mass spectrometric analysis. Tolcapone and entacapone are potent inhibitors of catechol-O-methyltransferase (COMT) for the treatment of Parkinson’s disease. We aimed to understand the molecular basis of the difference of both drugs with respect to side effects. Using Capture Compounds with these drugs as selectivity functions, we were able to unambiguously and reproducibly isolate and identify their known target COMT. Tolcapone Capture Compounds captured five times more proteins than entacapone Capture Compounds. Moreover, tolcapone Capture Compounds isolated mitochondrial and peroxisomal proteins. The major tolcapone-protein interactions occurred with components of the respiratory chain and of the fatty acid β-oxidation. Previously reported symptoms in tolcapone-treated rats suggested that tolcapone might act as decoupling reagent of the respiratory chain (Haasio et al., 2002b). Our results demonstrate that CCMS is an effective tool for the identification of a drug’s potential off targets. It fills a gap in currently used in vitro screens for drug profiling that do not contain all the toxicologically relevant proteins. Thereby, CCMS has the potential to fill a technological need in drug safety assessment and helps reengineer or to reject drugs at an early preclinical stage.

Key Words: Capture compound mass spectrometry; tolcapone; entacapone; hepatotoxicity; drug safety; Parkinson’s disease.

The main challenge for the pharmaceutical industry is the selection of drug candidates that possess not only high efficacy but also low toxicity. Even in the past decade, hepatotoxicity and cardiovascular toxicity were the most prominent reasons accounting for two-thirds of market withdrawals (Schuster et al., 2005). More than 70% of toxic reactions were identified during the compulsory regulatory animal toxicity tests (Olson et al., 2000). Only half of the new pharmaceuticals that produced hepatotoxicity in the clinical stage had already shown concordant reactions in the obligatory animal toxicity studies. Therefore, the development of additional new tools for the investigation of potential human hepatotoxicity is a necessity for the pharmaceutical industry (Schuster et al., 2005).

Traditional drug safety assessment is based either on selectivity screens against a panel of target-related recombinant proteins or on phenotypical cell-based assays. However, there is a shortage of technologies that can detect unforeseen drug off-target interactions to native endogenous proteins. We show here that capture compound mass spectrometry (CCMS) (Koster et al., 2007) can be used to identify the sets of proteins that interact with two drugs for Parkinson’s disease, tolcapone and entacapone. Tolcapone and entacapone are potent inhibitors of catechol-O-methyltransferase (COMT), a key enzyme in the metabolism of dopamine (Axelrod and Tomchick, 1958). Inhibition of COMT gives rise to elevated levodopa levels and thereby exerts a therapeutic effect on patients suffering from Parkinson’s disease. Tolcapone has a much higher efficacy; however, it showed hepatotoxic effects in humans and rats (Assal et al., 1998), while this has not been reported for entacapone. With respect to animal toxicity, the literature appears contradictory: While preclinical trials on tolcapone in animal species, among them rats, appear to have not shown toxic effects (Schläppi et al., 1996, cited in Haasio, 2003), severe toxic effects were reproducibly observed in more recent studies in rats (Haasio et al., 2002a,b). This effect must be attributed to the structural features of the drug molecules different from the moiety required for COMT binding because the latter is identical in tolcapone and entacapone.

CCMS can identify the set of proteins that interact with the drug directly from biological samples containing native endogenous proteins. From this set of interactions, conclusions can be drawn with respect to which molecular pathways may be affected by the drug. This information can be used to guide follow-up experiments to assess drug safety. This makes CCMS a platform technology that helps foresee adverse events.
in humans. The mechanism by which a drug is potentially hepatotoxic can be better understood and compared to a related drug exhibiting lower hepatotoxicity, e.g., entacapone.

A Capture Compound consists of three main functionalities: (1) the drug molecule to be investigated, (2) an adjacent photoreactive functionality for photo cross-linking, and (3) the more distant sorting function, e.g., biotin for the isolation of captured proteins (Fig. 1). A sorting function permits the isolation of the proteins that are covalently linked to the Capture Compounds. In our case study, streptavidin magnetic beads were used to isolate all biotinylated proteins. The technology exclusively probes those proteins that interact with the drug molecule that is part of the Capture Compound. The complexity of a biological sample is reduced to the interaction partners of the drug. The identification of the captured proteins is accomplished by high-resolution mass spectrometry.

The capture process is simple and easy to perform. After incubation of the Capture Compound with a cell lysate, photolysis leads to the generation of a nitrene within the reactivity group. The nitrene then forms a covalent cross-link between the Capture Compound and the proteins that show an affinity-based interaction with the drug molecule in the Capture Compound. Non–cross-linked proteins can be washed away employing the sorting function biotin, streptavidin magnetic beads, and stringent washing conditions. The captured biotinylated proteins are then digested by trypsin, and the resulting peptides are analyzed by high-resolution mass spectrometric analysis.

We show that CCMS discriminates between tolcapone and entacapone. Both are of the same type and have the same mode of action but distinguish themselves by their therapeutic efficacy and the strength of the hepatotoxic effects. Capture Compounds containing these two drugs allow the capturing of the target protein COMT; however, tolcapone additionally binds to essential proteins in critical pathways such as fatty acid β-oxidation and oxidative phosphorylation. Our results demonstrate that CCMS is an effective tool for the generic and swift identification of both a drug’s mode of action and its potential off targets responsible for adverse side effects.

**MATERIALS AND METHODS**

**Chemical Synthesis of Entacapone and Tolcapone Capture Compounds**

The detailed description of the Capture Compound synthesis will be published elsewhere. Structures of final compounds are shown in Figure 2. Analysis of the Capture Compounds by mass spectrometry and nuclear magnetic resonance (NMR) confirmed the identity and structure of the final reaction products. Purity of the compounds was determined by 1H-NMR and was found to be greater than 95%.

**Preparation of molecular structures.** Atomic coordinates for the ternary complex between COMT, the cosubstrate S-adenosyl-methionine, and the
Bradford (1976). Protein concentrations of the supernatants were determined according to their absorbance. The suspensions were cleared by centrifugation at 10,000 rpm for 15 min. The supernatants were collected, and the protein concentration was determined using Bradford reagent (Bio-Rad, Hercules, CA), which is based on the binding of Coomassie blue to protein. The absorbance at 595 nm was measured using a spectrophotometer, and the concentration of protein was calculated using a standard curve generated with bovine serum albumin (BSA).

Molecular docking. In order to reduce the rotational degrees of freedom and to focus on the interactions of the selectivity and the reactivity functions with the protein, the Capture Compounds were modeled without the polyethylene glycole linker and the biotin moiety. Unrestricted flexible docking between COMT and Tep-Bz-CC was performed with Surfex-Dock (Jain, 2003) included in the SYBYL package using the default settings. The cocrytalized ligand was extracted, and the proteins were generated based on the ligand. Twenty poses were sampled. The results were investigated for the correct binding of the catechol moiety in the binding pocket and the positioning of the cross-linking unit in vicinity of the protein surface, especially close to polar side chains. The polyethylene glycol linker and the biotin moiety (based on the PDB file 1H1D) were added manually and minimized using standard procedures.

COMT Affinity Assay

Bioanalytical services for the determination of 50% inhibitory concentration (IC50) values were provided by MDS Pharma Services Inc. (Pitou, Taipei, Taiwan). IC50 equals the molar concentration of an antagonist, which produces 50% of the maximum possible inhibitory response for that antagonist.

Preparation of Cell Lysates and Rat Liver Subcellular Fractions

HepG2 cell lysate was purchased from InVivo Biotech (Berlin, Germany), and frozen rat liver was purchased from BLS Preclinical Services (Berlin, Germany). Fractionation of rat liver was carried out essentially as described (Emig et al., 1995). Briefly, rat liver was homogenized using a motor-driven glass-Teflon homogenizer (Sartorius Stedim Biotech GmbH, Aubagne Cedex, France) in 10 volumes of homogenization buffer per gram of tissue wet weight (0.32M sucrose, 5mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid [HEPES]/NaOH, pH 7.4, complete protease inhibitor cocktail [Roche, Mannheim, Germany]) using 12 strokes at 900 rpm at 4°C. The homogenate was centrifuged at 1000 × g for 20 min. The supernatant was collected using the caproMag, a device for handling of magnetic beads (Caprotec Bioanalytics GmbH). The characteristics of the caproBox are as follows: capture temperature 0.5°C–4°C, λ = 275–375 nm with λmax = 312 nm, irradiance Ie 10–12 mW/cm², and irradiation energy for each sample (15 mm² irradiation area of the closed tube and 10 mm height of the reaction mixture) –1.4 J. Irradiation with the caproBox was carried out for 20 min, with mixing at intervals of 2.5 min. After ultraviolet (UV) light exposure, the beads were collected using the caproMag, a device for handling of magnetic beads (Caprotec Bioanalytics GmbH), and washed first six times with 200 µl of ultrapure water and then twice with ultrapure water. Until further analysis, beads were stored at 4°C in ultrapure water.

For capture experiments using fractions of rat liver, the initial protein amounts in the capture reactions were 0.4 mg for mitochondrial or microsomal fraction and 1.4 mg in case of the cytosolic fraction, respectively. Capture buffer and WB were supplemented with 0.1% n-dodecyl-β-maltoside (Glycon, Luckenwalde, Germany).

SDS-Polyacrylamide Gel Electrophoresis

Subsequent to the capture experiments, the captured proteins with the covalently attached Capture Compounds are bound to the streptavidin beads via biotin-streptavidin interactions. To analyze the captured proteins by SDS-polyacrylamide gel electrophoresis (PAGE), the beads were resuspended in 7 µl Laemmli buffer (Laemmli, 1970) and heated to 95°C for 5 min. Subsequently, the beads were separated from the released proteins using a magnet. For comparability, sets of different Capture Compounds must be used in parallel with the same amount of protein starting material. The entire amount from one capture experiment was loaded in one lane (if not indicated otherwise). Consequently, Capture Compounds interacting with few proteins will show fewer bands on a gel than those interacting with many proteins. Gels were stained using the FireSilver Kit (Proteome Factory, Berlin, Germany) according to the manufacturer’s instructions.

Western Blot

After separation by SDS-PAGE, captured proteins were transferred to a nitrocellulose membrane (Whatman, Kent, UK). Proteins were stained with Ponceau red (Sigma, Steinheim, Germany) to control the blotting efficiency (data not shown). The membrane was blocked for 1 h at room temperature with a solution of 5% (wt/vol) skimmed milk powder in Tris-buffered saline (20mM Tris-HCl, pH 7.5, 150mM NaCl [TBS], supplemented with 0.1% [vol/vol] Tween 20 [TBS-T]). Incubation with the primary antibody was performed for 1 h at room temperature or overnight at 4°C, followed by three wash steps in TBS-T and incubation with the secondary antibody for 1 h at room temperature. Antibodies were diluted in 5% skimmed milk powder in TBS-T as follows: anti-COMT 1:2500, secondary anti-goat antibody conjugated to horseradish peroxidase 1:2000. After three washes in TBS-T and one wash in TBS, membranes were treated with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) according to the manufacturer’s instructions. Hyperfilm ECL films (GE Healthcare, Muenchen, Germany) were used to detect the chemiluminescence. In case of blots for the detection of biotinylated proteins, streptavidin-horseradish peroxidase was used instead of a first antibody at a dilution of 1:1000 in 5% skimmed milk powder in TBS-T and blots were developed directly after washing three times with TBS-T and once with TBS.
In-Solution Digest

For analysis of the complex protein mixture obtained after capture experiments, the washed beads were resuspended in 10 μl 50 mM ammonium bicarbonate and 1 μl trypsin (0.5 μg/μl) (sequencing grade; Roche) for 16 h at 37°C on a thermoshaker (Eppendorf, Hamburg, Germany). Subsequently, tryptic peptides were desalted using Stage Tips (Proxeon Biosystems A/S, Odense, Denmark) and eluted according to the manufacturer’s instructions. The eluate was evaporated to dryness in a miVac DNA vacuum centrifuge (Genevac, Ipswich, UK) and stored at −20°C until mass spectrometric analysis.

In-Gel Digestion

Silver-stained gels were washed twice for 10 min with LC-MS grade water. Gel bands were excised, cut into small pieces, and washed twice with each 100 μl water and 100 μl 50% ethanol (vol/vol). Gel bands were shrunk with 50 μl 100% ethanol for ~5 min. Subsequently, the washing and shrinking steps were repeated. Protein digestion was carried out by rehydration of bands for 20 min on ice with 12.5 ng/μl of trypsin solution in 50 mM NH₄HCO₃ and subsequent incubation for 16 h at 37°C. The extraction of peptides was carried out in two consecutive steps by incubating the gel pieces with 50% acetonitrile (ACN) with 2.5% formic acid (FA) for 15 min. The pooled supernatants were then dried in a miVac DNA vacuum centrifuge (Genevac). Desalting, elution, evaporation, and storage of tryptic peptides were performed as described for in-solution digested samples.

Nano LC-MS/MS

The protein digest was redissolved in 5 μl of 5% FA. Subsequently, peptides were loaded onto a nanoflow Biosphere C18 precolumn (5 μm, 120 Å, 20 × 0.1 mm; Nanoseporation, Nieuwkoop, the Netherlands) coupled to a nanoflow Biosphere C18 analytical column (5 μm, 120 Å, 105 × 0.075 mm). The experiments were performed on an Easy-nLC liquid chromatography system (Proxeon Biosystems A/S) connected to an LTQ Orbitrap XL Mass Spectrometer (Thermo Electron, Bremen, Germany) utilizing a nanoelectrospray ion source (Proxeon Biosystems A/S). For the analysis of in-solution digest samples, peptides were eluted during an 80-min linear gradient from 5% ACN/0.1% FA to 40% ACN/0.1% FA, followed by an additional 2 min increase to 100% ACN/0.1% FA and remaining at 100% for another 8 min with a controlled flow rate of 300 nl/min.

For the analysis of extracted gel bands, a linear 40-min gradient increasing from 5% ACN/0.1% FA to 40% ACN/0.1% FA, followed by an additional 2 min increase to 100% ACN/0.1% FA and remaining at 100% for another 8 min with a controlled flow rate of 300 nl/min was used.

The mass spectrometric analysis was performed in the data-dependent mode to automatically switch between orbitrap-MS and LTQ-MS/MS (MS²) acquisition. The mass spectrometer duty cycle was controlled by setting the injection time automatic gain control. Survey full-scan MS spectra (from m/z 400–2000) were acquired in the orbitrap with a resolution of r = 60,000 at m/z 400 (after accumulation to a target value of 500,000 charges in the linear ion trap). The most intense ions (up to five, depending on signal intensity) were sequentially isolated for fragmentation in the linear ion trap using collision-induced dissociation (CID) at a target value of 10,000 charges. The resulting fragment ions were recorded in the LTQ.

For accurate mass measurements in the MS mode, the singly charged polydimethylcyclosiloxane background ion (Si(CH₃)₂O)₆H⁺ (m/z 445.120025) generated during the electrospray process from ambient air was used as lock mass for real-time internal recalibration. Target ions already mass selected for CID were dynamically excluded for the duration of 60 s. Charge-state screening and rejection of ions for CID with unassigned charge state were set. Further mass spectrometric settings were as follows: Spray voltage was set to 1.7 kV, normalized collision energy was 35% for MS2. The minimal signal required for MS² was 500 counts. An activation q = 0.25 and an activation time of 30 ms were applied for MS² acquisitions. After each analysis of an in-solution digest sample, the system was washed by performing at least one linear gradient that was used for the respective peptide separation.

Peptide Identification via Database Search

Proteins were identified by automated database searching against the UniProtKB/Swiss-Prot database (release 56.5) by using SEQUEST implemented in BioWorks 3.3.1 SP1 (Thermo Fisher Scientific). Specific search parameters were used in the SEQUEST analyses were 5-ppm precursor tolerance, 1-amu fragment ions tolerance, and full trypsin specificity allowing for up to two missed cleavages. Phosphorylation at serine, threonine, and tyrosine; oxidation of methionines; deamidation of asparagines and glutamine; acetylation at lysine and serine; formylation at lysine; and methylation at arginine, lysine, serine, threonine, and asparagine were allowed as variable modifications. No fixed modifications were used in the database search.

The SEQUEST peptide identifications were required to satisfy minimum XCorr values of 2, 2.5, and 3 for singly, doubly, and triply charged peptides, respectively; a minimum ΔCn of 0.1; and a peptide probability ≥ 0.001. Peptides fulfilling these criteria were accepted for analysis without further validation. The estimated percentage of false discovery peptide identifications was determined using the reversed protein database approach and was < 1%.

RESULTS

Design of Capture Compounds

Tolcapone and entacapone are potent inhibitors of COMT for the treatment of Parkinson’s disease. The catechol groups of these drugs compete with dopamine for coordination of the catechol moiety with the magnesium ion in the COMT-binding pocket (Bonifacio et al., 2007). While tolcapone is much more efficacious than entacapone in raising dopamine levels in the brain, tolcapone is well known for serious side effects, limiting its therapeutic utility. In particular, tolcapone was temporarily withdrawn due to the drug’s implication in fulminant liver failure and the consequent death of three patients; now monitoring of liver enzymes is mandatory during drug treatment (Unger et al., 2008).

In order to investigate the molecular mechanisms underlying the cause of tolcapone’s hepatotoxicity, we designed Capture Compounds containing tolcapone and entacapone as selectivity functions, respectively (Fig. 2). With these Capture Compounds, we aimed to identify the proteins interacting with tolcapone and entacapone, respectively, thereby identifying interactions that are potentially related to tolcapone’s underlying hepatotoxicity. To ensure optimal interaction of the selectivity and the photoreactivity groups with the target protein, molecular docking studies were carried out and the molecular design was adjusted accordingly. Figure 3 shows a molecular model visualizing the interaction between COMT and the tolcapone Capture Compound with accessible catechol moiety (Tcp-Bz-CC). The model showed that the catechol moieties were expected to target the binding pocket, whereas the amide residue of entacapone and the benzyl side of tolcapone protruded outside the protein and pointed in opposite direction of the catechol group. Due to the preferred mechanisms of the cross-linking reaction, the linker between the selectivity and the reactivity groups had to ensure a position of the photoreactivity group close to polar side chains on the protein surface. We observed a 100-fold reduction of the affinity of the entacapone Capture Compound Ecp-Am-CC
and the biotin-sorting function with linker (orange) protrudes from the protein. The catechol group (magenta) is positioned close to polar side chains on the protein surface. Tolcapone is nestled into the lipophilic COMT crevice, while the photoreactivity of COMT is colored according to the lipophilic potential. The catechol group of moiety (Tcp-Bz-CC) into the dopamine-binding pocket of COMT. The surface (Fig. 2, with cellular proteins inducing different responses. Thus, we perfectly.

An important role and due to its flexibility cannot be modeled compared to entacapone and concluded that the model may not show the entire picture. It is possible that the linker might play an important role and due to its flexibility cannot be modeled perfectly.

Different parts of a drug may produce different interactions with cellular proteins inducing different responses. Thus, we chose two points of attachment for each drug to the scaffold (Fig. 2, 1). In one set, we used the catechol moiety responsible for the interaction with COMT as attachment point (2, Tcp-Ct-CC, and 6, Ecp-Ct-CC, Fig. 2). In addition, we linked the drugs at the opposite end via the benzylic or the amino group, respectively (5, Tcp-Bz-CC, and 7, Ecp-Am-CC, Fig. 2). As a result, different pharmacophoric elements of entacapone and tolcapone were presented to the complex protein mixtures tested. As a control, the Capture Compound without selectivity group (scaffold) was used.

The design of the Capture Compounds was carried out with the aim that for one attachment position, the Capture Compound functionalities should not interfere with the interaction between the drug and its known target COMT. In case of entacapone, the Capture Compound with attachment via the amino group should be able to bind COMT as the original drug. To verify the quality of our molecular design, we commissioned standard affinity measurements between three of the Capture Compounds and the purified target protein COMT. Published K_D values for the affinity of the drug entacapone to COMT vary by one order of magnitude between 0.3nM (Bonifacio et al., 2007) and 10nM (Tervo et al., 2003). We determined the IC_50 of the entacapone drug to be 430nM for the entacapone Capture Compound Ecp-Am-CC (Supplementary data). As outlined above, the reduction of affinity is probably due to the linker properties and its lack of affinity to the proteins. As expected, Capture Compounds with the reversed architecture in which the drugs were linked by the catechol had no affinity to COMT. Their IC_50 were larger than 10,000nM. We performed the experiments at concentrations that were about 10-fold to 20-fold higher than the IC_50. This is a C_max aimed for in drug development. Our results demonstrate that although the attachment of the Capture Compound scaffold may lead to a reduction of affinity, entacapone is still able to effectively bind COMT—the pharmacophoric properties of the drug are retained.

### Validation of the CCMS Approach with Soluble Rat Liver Proteins

In order to demonstrate the validity of the CCMS approach, we first tested our Capture Compounds in the cytosolic fraction of rat liver, which shows a reduced protein complexity compared to whole-cell lysates. We used rat liver lysates due to the availability of healthy rat organs and used the cytosolic fraction to demonstrate the effectiveness of our methodology. Other fractions of the lysates were used as well to explore which proteins interact with the drug. UV irradiation induces a covalent bond between the reactivity function of the Capture Compound and the protein interacting with the drug. As the Capture Compound contains a biotin moiety (sorting function), interacting proteins become biotinylated by irreversible cross-link to the Capture Compound during the capture process and can be not only isolated by streptavidin beads but also detected in an anti-biotin (i.e., streptavidin) Western blot (Fig. 4A), demonstrating the covalent bond to the Capture Compound. By SDS-PAGE and silver staining (Fig. 4B), all isolated proteins were visualized. In classic pull-down experiments, frequently background proteins are isolated that do not interact with the selectivity group, but stick to the beads, or are only interaction partners of proteins that directly bind the selectivity group. In capture experiments, direct binders of the selectivity group will form covalent bonds with the Capture Compounds and can be visualized in the anti-biotin Western blot. Unspecific background proteins should be visible in the silver-stained gel however; they should not be visible in the anti-biotin Western blot. Comparison of Figures 4A and 4B shows that all protein bands visualized in the silver-stained SDS-PAGE are also visible in the anti-biotin Western blot. Consequently, the comparison of silver-stained SDS-PAGE and anti-biotin Western blot clearly shows that the isolated proteins were indeed covalently bound to the biotin-containing Capture Compounds. Western blots directed against the COMT protein (Fig. 4C) demonstrated that the target is indeed captured and—comparing Figures 4A and 4C—covalently bound. Analysis of the respective samples by mass spectrometry in addition to Western blots revealed the unambiguous and reproducible isolation and identification of the known drug target COMT with the Capture Compounds containing the free
catechol moiety (Tcp-Bz-CC and Ecp-Am-CC) (Table 1), while compounds with attachment at the opposite end (Tcp-Ct-CC and Ecp-Ct-CC) did not bind COMT, as expected. These, however, presented different pharmacophoric elements of the drugs to the protein mixture and allowed capturing of proteins that interacted with these elements of the drugs.

Notably, the tolcapone Capture Compounds interacted with bile salt sulfotransferase (ST2A1) and alcohol sulfotransferase A (ST2A2), independently of the attachment point (Tcp-Bz-CC and Tcp-Ct-CC, Figs. 4A and 4B, lanes 1 and 2). Due to the very similar molecular weight, the two sulfotransferases comigrated in SDS-PAGE but were unambiguously identified by mass spectrometry. An interaction with these proteins was also observed with the entacapone Capture Compound linked via the catechol moiety (Ecp-Ct-CC, Figs. 4A and 4B, lane 3) but not or to a much lower extent with the amide-linked entacapone Capture Compound (Ecp-Am-CC, Figs. 4A and 4B, lane 4). ST2A1 and ST2A2 belong to the group of cytosolic sulfotransferases, phase II detoxification enzymes involved in the biotransformation of a wide variety of structurally diverse endo- and xenobiotics, including many therapeutic agents and endogenous steroids (Nowell and Falany, 2006). Binding of both drugs by these enzymes is in accordance with the rather unspecific role of the sulfotransferases in detoxification processes (Nowell and Falany, 2006). As the scaffold control did not show this interaction, we suggest that the binding of sulfotransferases with both Capture Compounds is likely to reflect the physiological detoxification process.

TABLE 1
Proteins Captured by Tolcapone and Entacapone in the Soluble Protein Fraction of Rat Liver. The Corresponding Silver-Stained SDS-PAGE is Shown in Figure 4. Processes are Given as Retrieved from SwissProt Annotation via http://www.expasy.org

<table>
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<th>Gel band</th>
<th>Abbreviation</th>
<th>Name</th>
<th>Molecular weight (kDa)</th>
<th>Accession</th>
<th>Cellular process</th>
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</thead>
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<td>Clathrin heavy chain 1</td>
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<td>P11442</td>
<td>Endocytosis</td>
</tr>
<tr>
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<td>DHB4</td>
<td>Peroxisomal multifunctional enzyme type 2</td>
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<td>P97852</td>
<td>Fatty acid β-oxidation</td>
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<tr>
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<td>78</td>
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<tr>
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<td>Alcohol sulfotransferase A</td>
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<td>P22789</td>
<td>Detoxification</td>
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<tr>
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pathway of these two compounds. Indeed, it has been shown previously that tolcapone is metabolized by sulfation (Jorga et al., 1999).

The Capture Compounds with free catechol moiety exposed to the cytosol showed, apart from the sulfotransferases, strong and specific interaction only with the target protein COMT. Similarly, the entacapone Capture Compound Ecp-Am-CC1 revealed no interaction except for the sulfotransferases. However, the tolcapone Capture Compounds with attachment via the catechol group (Tcp.Ct-CC1) revealed additional interacting proteins in the cell lysate. Notably, additional peroxisomal proteins were captured. Peroxisomal acyl-coenzyme A oxidase 3 (ACOX3) and peroxisomal multifunctional enzyme type 2 (DBH4, also MFP-2 or MFE-2) play essential roles in fatty acid \( \beta \)-oxidation. The phenotypical data (Baes et al., 2000; Huyghe et al., 2006b; Yu et al., 2003) associated with the two captured peroxisomal enzymes suggest that tolcapone-related side effects may be partly due to the interaction of these enzymes with tolcapone (see “Discussion” section). These off-target proteins could only be identified using the tolcapone Capture Compounds attached via the phenolic group, confirming the importance of several different attachment points. Moreover, these results gave an initial indication that CCMS can indeed identify the molecular basis of drug side effects in general and tolcapone in particular.

**Tolcapone Interacts with Components of the Respiratory Chain**

In order to reveal the mode of hepatotoxic action of both tolcapone and entacapone in the human liver, we performed capture experiments in whole-cell lysates of the human hepatocyte cell line HepG2. As the chemical structure of entacapone and tolcapone at the catechol end is identical and the pilot experiments using cytosolic rat liver fractions suggested that primarily tolcapone attached via the catechol group captures relevant off targets, we decided to focus on compounds with this attachment point. To generate a comprehensive coverage of drug-protein interactions, we designed two additional Capture Compounds differing in the linker length between the drug molecule and the reactivity group (3 and 4, Tcp.Ct-CC, Fig. 2).

Capture experiments in HepG2 whole-cell lysates revealed that tolcapone Capture Compounds, independent of linker length, interacted with a large number of different proteins in the cell, while entacapone Capture Compounds showed relatively few interactions, independent of the attachment point. As expected, even fewer proteins were found when capturing was performed with the scaffold control (Fig. 5A). LC-MS/MS analysis of the respective complex protein mixtures led to the identification of the tolcapone and entacapone interaction partners. Proteins identified in the control experiment with scaffold were excluded from further analysis. We established the overlap between the captured proteins to classify the interaction partners of the respective drugs. The tolcapone compounds captured a total of 124 proteins; with the entacapone Capture Compounds, however, only 20 proteins were identified (full protein lists are given in the Supplementary data: table of LC-MS/MS results). While some proteins were captured exclusively by one Capture Compound, the majority of proteins were captured independent of the linker length and thus considered as interaction partners with highest confidence. For an overall functional classification of the proteins, we performed Gene Ontology (Ashburner et al., 2000) annotation via BioMart (Smedley et al., 2009) of the ENSEMBL Genome Browser (http://www.ensembl.org, build 52 NCBi63) according to cellular component terms.

For the 20 proteins specifically captured with the entacapone Capture Compounds, the cellular distribution could not be linked in a straightforward way to mitochondrial function and toxicity based on literature. However, in case of tolcapone, a considerably large proportion of the 124 captured proteins were assigned to the mitochondria (Fig. 5B) and, in particular, within the mitochondrial membrane. To gain deeper insight into the role of the captured proteins in metabolism, we then carried out a Kyoto Encyclopedia of Genes and Genomes pathway (Aoki and Kanehisa, 2005) analysis via the Database for Annotation, Visualization and Integrated Discovery (DAVID, V6, 2008, http://david.abcc.ncifcrf.gov/) (Dennis et al., 2003). We found that the captured proteins are essential components of the respiratory chain, the bile acid synthesis, and peroxisomal fatty acid \( \beta \)-oxidation. Enzymes functioning in bile acid synthesis and peroxisomal fatty acid \( \beta \)-oxidation are in accordance with the results obtained from the soluble fraction of rat liver. In particular, the human homolog of ACOX3 was again reproducibly captured, implicating a possible impact on fatty acid \( \beta \)-oxidation by tolcapone in humans. In a previous study, focally mild microvesicular steatosis was observed (Assal et al., 1998). Our finding may hint at the mechanism that could underlie steatosis induction. Notably, the captured mitochondrial proteins contained subunits from each of the complex in the respiratory chain (Fig. 5C), e.g., eight subunits of ATP synthase. While nearly all subunits were captured by two or three of the tolcapone Capture Compounds, none were isolated by entacapone Capture Compounds. The comparison to the structurally similar but far less toxic drug entacapone suggests that the observed tolcapone-protein interactions are a possible cause of its toxicity. Further experiments should clarify to what extent this contributes to the overall toxicity of the compound. Publications by Haasio et al. (2002a,b) showed unambiguously that tolcapone in contrast to entacapone produces symptoms in rats that are typical for decoupling agents of the respiratory chain. Our results are in very good agreement with these in vivo observations.

_Detailed Analysis of Proteins Interacting with Tolcapone in Rat Liver Mitochondria and Microsomal Fractions_

To investigate the interaction partners of tolcapone in mitochondria and peroxisomes in detail, we carried out capture experiments in the mitochondria and microsomal fractions of...
rat liver. In both preparations, we again found that tolcapone Capture Compounds capture significantly more proteins than the respective entacapone compounds. Consistent with the results described above, we identified key enzymes of the fatty acid β-oxidation pathway, such as peroxisomal multifunctional enzyme type 2, peroxisomal acyl-coenzyme A oxidase 3, and the long chain fatty acid-CoA ligase 1, which is found both in the mitochondrial and peroxisomal membranes as specific interaction partners of tolcapone with the potential to induce side effects (Fig. 5D, Table 2).

**DISCUSSION**

Tolcapone and entacapone are potent inhibitors of COMT for the treatment of Parkinson’s disease (Schapira et al., 2000). Although these two drugs are similar, and even structurally identical with respect to the COMT-binding moiety, they show a number of pharmacological differences and thus different clinical effectiveness (Deane et al., 2004). A recent Cochrane meta-analysis of 14 studies in 2566 patients (Deane et al., 2004) found both drugs to be statistically superior to placebo in increasing “on” time and decreasing “off” time. Furthermore, tolcapone was found to have an approximately twice as long therapeutic effect compared to entacapone. This difference is reflected by the pharmacological profile, where tolcapone is characterized by greater bioavailability and higher COMT affinity. Tolcapone increases the half-life of the dopamine precursor levodopa by 80 versus 40% for entacapone (Factor, 2008). However, a significant number of patients treated with
Proteins Captured by Tolcapone and Entacapone in Solubilized Mitochondrial and Microsomal Fractions of Rat Liver. Corresponding Protein Bands are Depicted in Figure 5D. Processes are Given as Retrieved from SwissProt Annotation via http://www.expasy.org

<table>
<thead>
<tr>
<th>Gel band</th>
<th>Abbreviation</th>
<th>Name</th>
<th>Molecular weight (kDa)</th>
<th>Accession</th>
<th>Cellular process</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CLH</td>
<td>Clathrin heavy chain 1</td>
<td>193</td>
<td>P49951</td>
<td>Endocytosis</td>
</tr>
<tr>
<td>2</td>
<td>DHB4</td>
<td>Peroxisomal multifunctional enzyme type 2</td>
<td>80</td>
<td>P97852</td>
<td>Fatty acid β-oxidation</td>
</tr>
<tr>
<td>3</td>
<td>ACOSL1</td>
<td>Long chain fatty acid-CoA ligase 1</td>
<td>79</td>
<td>P18163</td>
<td>Fatty acid metabolism</td>
</tr>
<tr>
<td>4</td>
<td>ACOX3</td>
<td>Peroxisomal acyl-coenzyme A oxidase 3</td>
<td>79</td>
<td>Q63448</td>
<td>Fatty acid β-oxidation</td>
</tr>
<tr>
<td>5</td>
<td>DHEX2</td>
<td>Glutamate dehydrogenase 1, mitochondrial</td>
<td>62</td>
<td>P10860</td>
<td>Glutamate catabolism</td>
</tr>
<tr>
<td>6</td>
<td>ARHL1</td>
<td>Protein ADP-ribosylarginine hydrolase-like protein 1</td>
<td>40</td>
<td>Q5XIB3</td>
<td>Protein-amino acid deribolysis</td>
</tr>
<tr>
<td>7</td>
<td>DHB13</td>
<td>17-beta hydroxysteroid dehydrogenase 13</td>
<td>34</td>
<td>Q5M875</td>
<td>Oxidation/reduction</td>
</tr>
<tr>
<td>8</td>
<td>AUHM</td>
<td>Methylglutaconyl-CoA hydratase, mitochondrial</td>
<td>34</td>
<td>Q9JLZ3</td>
<td>Branched amino acid catabolism</td>
</tr>
<tr>
<td>9</td>
<td>ECHM</td>
<td>Enoyl-CoA hydratase, mitochondrial</td>
<td>32</td>
<td>P14604</td>
<td>Fatty acid β-oxidation</td>
</tr>
</tbody>
</table>

Tolcapone shows disturbed levels of liver enzymes, and in 1998, even three patient fatalities attributed to tolcapone-induced hepatotoxicity were reported (Deane et al., 2004).

In this study, we have used the CCMS technology to elucidate the molecular basis of the difference in the protein-binding profiles between Capture Compounds of tolcapone and entacapone. We have used protein fractions obtained from rat liver as well as lysates of the human hepatocyte cell line HepG2 and obtained consistent results. The conditions used in the preparation of the lysates were chosen to ensure that the membrane protein complexes were solubilized but still native, i.e., non-denaturing conditions were chosen. The application of the CCMS technology reproducibly and unambiguously demonstrated that besides binding the actual target (COMT), tolcapone Capture Compounds interact with a large number of proteins carrying out essential functions in the respiratory chain, fatty acid β-oxidation, and bile acid synthesis. In the liver, fatty acids are metabolized by β-oxidation in mitochondria and peroxisomes and by ω-oxidation in microsomes. Peroxisomal β-oxidation is responsible for the metabolism of very long chain fatty acids. Impairment of correct peroxisomal function may lead to the accumulation of long fatty acids or of hydrogen peroxide through the peroxisomal oxidative reactions. Both mechanisms might contribute to the hepatotoxicity of tolcapone and help explain the side effects observed in animals and humans. Further studies including in vivo experiments are necessary to establish the connection between human toxicological events and the results of CCMS experiments.

ACOX3 belongs to the family of fatty acyl-CoA oxidases. It has been shown that mice lacking fatty acyl-CoA oxidases developed steatohepatitis, demonstrating the importance of this class of enzymes for proper liver function (Yu et al., 2003). Peroxisomal multifunctional enzyme type 2 (MFP-2, also D-bifunctional protein, DHB4) has been described previously (Adamski et al., 1995). It plays a central role in peroxisomal β-oxidation as it handles most, if not all, peroxisomal β-oxidation substrates (Huyghe et al., 2006a). In humans, deficiency of this enzyme causes a severe developmental syndrome with abnormalities in several organs, leading to death within the first year of life (Huyghe et al., 2006a). Accumulation of branched long chain fatty acids and very long chain fatty acids as well as a disturbed synthesis of bile acids were documented for these patients. Moreover, lack or mutations of DHB4 are a cause of D-bifunctional protein deficiency. The clinical manifestations of this deficiency are similar to those of disorders of peroxisomal assembly, including X-linked adrenoleukodystrophy, Zellweger cerebro-hepato-renal syndrome, and neonatal adrenoleukodystrophy (Wanders et al., 1990; Watkins et al., 1989). Premature death is observed in one-third of MFP-2 knockout mice, accompanied by more severe aberrations in bile acid metabolism and excessive accumulation of very long chain fatty acids in brain and liver (Baes et al., 2000; Huyghe et al., 2006b).

Besides potential interference with β-oxidation, our results show that tolcapone Capture Compounds interact with proteins of the inner mitochondrial membranes and with components of the respiratory chain. Although insertion of tolcapone into the mitochondrial membrane was not shown directly, the results demonstrate that tolcapone interacts with proteins localized within the membrane. A molecular mechanism in which tolcapone compromises the function of the respiratory chain is in accordance with cell physiological data reporting a decrease in membrane potential in the presence of tolcapone (Haasio et al., 2002a), similar to the bona fide decoupling agent 2,4-dinitrophenol. A toxicological study (Haasio et al., 2002b) in which rats were treated with either entacapone or tolcapone reported that no treatment-related findings were observed in entacapone-treated rats; however, animals treated with tolcapone showed increased respiration, decreased activity and drowsiness, and elevation of the rectal body temperature. Although the publications did not address liver toxicity
directly, our results shed light on the reported clinical and cell physiological observations in the study. Mitochondrial and peroxisomal proteins in the liver were identified as off-target proteins bound by tolcapone Capture Compounds. Indeed, the malfunction of the respiratory chain, fatty acid β-oxidation, or bile acid synthesis alone would likely lead to hepatotoxicity (Jaeschke et al., 2002; Pessayre et al., 1999).

After the first launch of tolcapone in Europe (EU) in 1997, three cases of fatal hepatic toxicity were found during postmarketing surveillance (Assal et al., 1998). For this reason, marketing authorization for tolcapone was suspended in the EU in 1998 and labeling was tightened in the United States. Only in 2002, two studies by Haasio et al. (2002a,b) described in vitro and in vivo experiments in rats that point to a decoupling activity of tolcapone in the respiratory chain as explained above. By the application of CCMS technology, we could now, nearly 10 years after three patient fatalities, elucidate the molecular interactions underlying tolcapone’s hepatotoxic potential and would certainly have triggered guided safety tests at the preclinical stage.

Several other hypotheses exist on the mechanism of tolcapone’s hepatotoxicity. In particular, a correlation of elevated levels of liver transaminase in patients with single nucleotide polymorphisms in the UDP-glucuronidyl transferase 1A gene complex, an enzyme that contributes to the metabolism of tolcapone in the liver, has been reported (Acuna et al., 2002). The authors suggested that impaired activity of UDP-glucuronidyl transferase rendered patients susceptible to hepatotoxic events caused by tolcapone. While this could be of value for the risk prediction for administration of tolcapone to individual patients, this finding does not explain the hepatotoxic potential of tolcapone in the first place. Rather, according to our findings, the primary cause for tolcapone’s hepatotoxic potential may be at the level of off-target binding of the drug to proteins identified in our study. Another hypothesis is that tolcapone elicits idiosyncratic drug reactions through reactive metabolites formed from the original drug. They may involve the formation of reactive metabolites and may involve responses of the immune system (Dieckhaus et al., 2002). This has been relatively well characterized in the case of the antiepileptic drug felbamate. Indeed, the formation of reactive metabolites from tolcapone in vitro under the experimental conditions chosen has been observed (Smith et al., 2003), leading to the formation of covalent metabolite-glutathione adducts, which may cause glutathione depletion. However, in the study by Smith et al., the formation of reactive metabolites was artificially favored. Although other modes of toxicity cannot be ruled out completely, in our study, we observed a direct binding of the drug to off-target proteins, which can be linked to the adverse effects observed in tolcapone-treated rats and in patients.

Proteins involved in drug side effects cannot be a priori predicted. Typical established tests for drug specificity and safety are restricted (1) to profiling the selectivity in vitro on a set of recombinantly expressed members of the protein family to which the desired drug target belongs. Or (2) safety assessments are only conducted with respect to an available range of phenotypic cell-based assays. Both approaches cannot cover all possible drug-protein interactions potentially leading to side effects and in particular fail to reveal the molecular identity of unanticipated off-target binders. CCMS provides a unique solution to fill exactly this vital technological gap in a direct, unbiased, and straightforward way. Although drug-protein interaction data do not directly prove that the physiological function of the proteins is compromised, the data directly define and guide further safety assessments. Furthermore, CCMS can be applied to native endogenous proteins from virtually any biological or clinical sample. The application of CCMS in preclinical drug development will help reducing costs by enabling faster GO/NO-GO decisions and will elucidate drug safety by pinpointing interactions that might lead to unwanted toxicological side effects at early stages.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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