Coupling of Liquid Chromatography/Tandem Mass Spectrometry and Liquid Chromatography/Solid-Phase Extraction/NMR Techniques for the Structural Identification of Metabolites following In Vitro Biotransformation of SUR1-Selective ATP-Sensitive Potassium Channel Openers

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ABSTRACT:

SUR1-selective ATP-sensitive potassium channel openers (PCOs) have been shown to be of clinical value for the treatment of several metabolic disorders, including type I and type II diabetes, obesity, and hyperinsulinemia. Taking into account these promising therapeutic benefits, different series of 3-alkylamino-4H-1,2,4-benzothiadiazine 1,1-dioxides structurally related to diazoxide were developed. In view of the lead optimization process of the series, knowledge of absorption, distribution, metabolism, excretion, and toxicity parameters, and more particularly the metabolic fate of these compounds, is a fundamental requirement. For such a purpose, two selected promising compounds [7-chloro-3-isopropylamino-4H-1,2,4-benzothiadiazine 1,1-dioxide (BPDZ 73) and 7-chloro-3-(3-pentylamino)-4H-1,2,4-benzothiadiazine 1,1-dioxide (BPDZ 157)] were incubated in the presence of phenobarbital-induced rat liver microsomes to produce expected mammal in vivo phase I metabolites. The resulting major metabolites were then analyzed by both mass spectrometry (MS) and NMR to completely elucidate their chemical structures. The two compounds were also further incubated in the presence of nontreated rats and human microsomes to compare the metabolic profiles. In the present study, the combined use of an exact mass liquid chromatography (LC)/tandem MS platform and an LC/solid-phase extraction/NMR system allowed the clarification of some unresolved structural assessments in the accurate chemical structure elucidation process of the selected PCO drugs. These results greatly help the optimization of the lead compounds.

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ABBREVIATIONS: KATP, channel, ATP-sensitive potassium channel; PCO, potassium channel opener; BPDZ 73, 7-chloro-3-isopropylamino-4H-1,2,4-benzothiadiazine 1,1-dioxide; BPDZ 157, 7-chloro-3-(3-pentylamino)-4H-1,2,4-benzothiadiazine 1,1-dioxide; PB, phenobarbital; P450, cytochrome P450; LC, liquid chromatography; SPE, solid-phase extraction; MS/MS, tandem mass spectrometry; ACN, acetonitrile; COSY, correlation spectroscopy; BPDZ 44, 3-(1,2-dimethylpropylamino)-4H-pyrido[4,3-e][1,2,4]thiadiazine 1,1-dioxide; BPDZ 154, 6,7-dichloro-3-isopropylamino-4H-1,2,4-benzothiadiazine 1,1-dioxide; BPDZ 256, 6-chloro-3-cyclobutylamino-7-fluoro-4H-1,2,4-benzothiadiazine 1,1-dioxide.

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of metabolic disorders such as diabetes, obesity, and hyperinsulinemia (Rasmussen et al., 2000; Hansen, 2006). Taking into account these potential therapeutic benefits, a series of new compounds belonging to the 3-alkylamino-4H-1,2,4-pyrido- and 3-alkylamino-4H-1,2,4-benzo-thiadiazine 1,1-dioxides were developed during the past decade by our group. Among these drugs, BPDZ 44 (1), BPDZ 73 (2), BPDZ 154 (3), BPDZ 157 (4), BPDZ 415 (5), and BPDZ 256 (6) (Fig. 1) were identified as potent and selective pancreatic PCOs (Pirotte et al., 1994; de Tullio et al., 2003, 2005). However, despite their excellent in vitro profiles, many of these compounds exhibited unexpected in vivo results in terms of potency and/or side effects. These negative results could be explained by unfavorable pharmacokinetic or metabolic properties. Therefore, within the drug discovery and the lead optimization process of these interesting drugs, the study of their metabolism and, more especially, the determination of the metabolites’ chemical structures seem to be essential. Indeed, the knowledge of the metabolic brittleness zones could be relevant to suppress or to reduce the formation of undesirable metabolites. Early knowledge of in vitro metabolism during the drug design process is also helpful to assist transition from in vitro to in vivo studies.

For these purposes, the BPDZ 73 and BPDZ 157 in vitro metabolic profiles were studied using liver microsomes. In a first step, parent compounds were incubated in the presence of phenobarbital (PB)-induced rat liver endoplasmic reticulum vesicles (microsomes) because this is a very well established method to produce expected in vivo phase I metabolites. Indeed, PB treatment increases the expression level of the majority of drug-metabolizing enzyme cytochromes P450 (P450s) (Waxman and Azaroff, 1992). Therefore, the use of such liver microsomes is expected to produce in vivo phase I metabolites in large amounts. As in vivo screenings are conducted in rats, metabolism was studied, in a second step, using noninduced rat liver microsomes to highlight the putative relationships between the in vivo activity and the drugs’ metabolic fate. Finally, to extrapolate results to the human species, incubation of the parent compounds was also conducted in the presence of pooled human liver microsomes.

Usually the major metabolites generated during incubation are analyzed by liquid chromatography (LC) coupled to a mass spectrometer (Oliveira and Watson, 2000; Clarke et al., 2001). Nevertheless, some specific structural modifications remain very difficult to determine using this approach. Thus, other analytical techniques are required to resolve the complete chemical structures of metabolites. It is well known that for organic compounds, 1H and/or 13C NMR spectroscopy is the most powerful, the most helpful, and the simplest method for structural analysis. Despite its great interest, the relative poor sensitivity of NMR and the technical difficulty to directly combine this instrument with an LC system has not permitted the use of this technical approach for a long time, especially with small amounts of compounds. The classic method used to obtain NMR data for major compounds from a mixture was to use a preparative-scale (or semi-preparative-scale) separation and purification to evaporate the mobile phase and to dissolve the residues in a deuterated solvent before classic NMR tube analysis. Even if this method appears to be simple and has been applied with relative success for a long time, several disadvantages could be noticed: the work-up step could be very time-consuming, and the evaporation step could generate some negative chemical reactions (i.e., oxidation, solvolysis, degradation…) that could deteriorate the nature of the analyte and could concentrate into the solution nondesired compounds (i.e., salts and/or solvent impurities). More recently, the developments of the hyphenated LC/NMR and particularly of LC/solid-phase extraction (SPE)/NMR have enhanced its possible applications to mixture separation and resolution (Corcoran and Spraul, 2003; Silva Elipe, 2003; Simpson et al., 2004). The introduction of an SPE step between LC and NMR was shown to be attractive and efficient especially in terms of time consumption and sensitivity (Clarkson et al., 2005; Sandvoss et al., 2005). The main advantages of LC/SPE/NMR compared with classic LC/NMR and conventional preparative step-tube NMR are as follows: possible complete automation and hyphenation, increased sensitivity (i.e., as a result of the multitrapping process and a concentration effect linked to a highly concentrated re-elution band), elimination of the LC solvents by flushing the cartridges with water to remove acids from the elution buffer, avoidance of contaminations and flushing with deuterated solvent (reduction of salt and/or impurities’ negative effects and use of classic NMR sequences), and minimization of artifacts as a result of possible degradations. In addition, LC separations can be carried out under optimized and typical LC conditions, and the nondeuterated solvents can be evaporated completely. This procedure reduces the need for residual solvent suppression and guarantees an optimal quality of the recorded NMR spectra. However, the SPE step (trapping and re-elution) has to be developed very carefully and could be sometimes the limiting and the time-consuming step of the process, especially with unknown products or very polar compounds (Wilson et al., 2006). Despite these disadvantages, LC/SPE/NMR, coupled with LC/tandem mass spectrometry (MS/MS) techniques, remains a convenient platform for accurate chemical structure elucidation (Barbuchi et al., 2006; Yang, 2006; Tatsis et al., 2007). We decided to use such a platform for metabolism studies of SUR1-selective benzo-thiadiazine 1,1-dioxides. Among those, BPDZ 73 and BPDZ 157 were chosen, according to structural features and pharmacological interest, for an in vitro evaluation of their metabolism. Their major metabolites were then analyzed using both LC/MS/MS (quadrupole-time of flight mass spectrometer) and LC/SPE/NMR.

Materials and Methods

Chemicals and Reagents. The compounds BPDZ 73 and BPDZ 157 have been synthesized at the Drug Research Center (Laboratoire de Chimie Phar-
macuteutique) of the Université de Liège. Acetonitrile (ACN) and methanol of high-performance liquid chromatography grade were obtained from Biosolve B.V. (Valkenswaard, The Netherlands). NAD, NADP, and glucose-6-phosphate dehydrogenase were bought from Roche Diagnostics GmbH (Mannheim, Germany). Glucose 6-phosphate, ammonium formate, EDTA, Tris, succrose, and leucine-enkephalin were obtained from Sigma-Aldrich (St. Louis, MO). Formic acid and phosphoric acid were of an analytical grade from Merck (Darmstadt, Germany). PB was provided by Certa (Braine-l’Alleud, Belgium). Deuterated ACN was purchased from Euriso-Top (Gif sur Yvette, France). PB-induced rat liver microsomes were isolated from male Sprague-Dawley rats (≥300 g) treated with PB (50 mg/kg) administered intraperitoneally daily for 4 days. Twenty-four hours after the final treatment, rats were killed and their livers excised, blotted dry, weighed, then minced, and homogenized in 4 volumes of ice-cold homogenization buffer (Tris, 0.01 M; succrose, 0.25 M; EDTA, 0.1 mM, pH 7.4) using a Potter apparatus. The homogenate was centrifuged (9000g) for 20 min at 4°C. The supernatant was isolated and further centrifuged (106,000g) for 60 min at 4°C. The pellet was suspended in an ice-cold homogenization buffer, and the suspension was recentrifuged (106,000g) for 40 min at 4°C. Microsomal pellets were finally resuspended in 0.1 M Tris buffer, pH 7.4, containing 0.1 mM EDTA to yield a protein concentration of approximately 16 to 24 mg/ml. Noninduced rat liver microsomes came from our library and were produced following the same steps. Pooled human liver microsomes were supplied by BD Biosciences Europe (Erembodegem, Belgium).

Metabolism. The preferred in vitro biological test system selected to metabolize the parent compounds (BPDZ 73 and BPDZ 157) was the PB-induced male rat liver microsome system. The parent compounds were dissolved in methanol and added directly to the incubation medium to reach a final substrate concentration of 200 μM and a final percentage in methanol lower than 1%. The incubations were performed at 37°C in a water-shaking bath with a final protein content of 1 mg/ml in a total volume of 1 ml. The reactions were initiated by addition of a NADPH-generating system. The reactions were stopped after an incubation time of 60 min by addition of 1 ml of methanol and 2 ml of ACN and by a subsequent vortexing step. Samples were further centrifuged at 2000g for 5 min. The supernatant was further decanted into a glass tube, and organic solvents were evaporated under an inert nitrogen flux to concentrate the samples. The residue was finally redissolved in 300 μl of a mixture composed of 10 mM ammonium formate buffer, pH 3.0, and ACN (50/50; v/v). Nonincubated samples (T0) were also prepared for comparison. These samples were prepared by the addition of methanol and ACN before supplementing the incubation medium with the NADPH-generating system. The same experimental procedure was applied to parent compounds in the presence of noninduced rat and pooled human liver microsomes.

LC/MS/MS Conditions. The LC analyses were carried out on a high-performance liquid chromatography Alliance 2695 system obtained from Waters (Milford, MA). The samples (10 μl) were injected by an autosampler, and the analysis was carried out using a Luna C18 analytical column (150 × 4.6 mm, i.d.; particle size = 5 μm) from Phenomenex (Torrance, CA) with a mixture of two mobile phases: 0.2% formic acid/methanol (95:5; v/v) (A) and ACN (B). The following linear elution gradient was applied: 0 min, 0% ACN; 30 min, 95% ACN; 35 min, 95% ACN; 35,10 min, 100% ACN; and 45.10 min, 100% ACN. The column was then re-equilibrated with 100% of the mobile phase A for a 10-min period with initial conditions before the next injection. The flow rate was 0.5 ml/min. The column temperature was set to 40°C. A quadrupole-time of flight II mass spectrometer from Micromass (Manchester, UK) operating in positive electrospray ionization mode was directly coupled to the LC device. The metabolite detection was performed by monitoring the total ionic current within 0.5-s regular intervals and a range m/z extending from 90 to 650. The optimum cone voltage was 31 V. Collision-induced dissociation was performed using a collision energy ramp between 20 and 25 eV with argon as the collision gas. For accurate mass measurements, the mass spectrometer was calibrated with a mixture of 0.1% phosphoric acid and ACN (50.50; v/v) to give a resolution of approximately 9000. Leucine-enkephalin (m/z 556.227) was used for lock-mass correction during accurate mass measurements of detected metabolites and their product ions resulting from collision-induced dissociation. All of the data acquisitions were achieved by using MassLynx version 4.1 software from Waters.

LC/SPE/NMR Conditions. The LC/SPE/NMR platform used is a completely hyphenated and on-line system. The LC separations were carried out on an Agilent 1100 series LC system from Agilent (Waldbraun, Germany) equipped with a quaternary pump, a column thermostat, an autosampler, and a diode array detector. The analytic separations were performed on an Alltech Hypersil BDS C18 column (150 × 4.6 mm, i.d.; particle size = 3 μm) from Alltech (Breda, The Netherlands) using mobile phase A: 10 mM ammonium formate, pH 3.0, and mobile phase B: ACN with a flow rate of 0.8 ml/min and the following linear gradient: 0 min, 10% ACN; 24 min, 40% ACN; 27 min, 60% ACN, and 30 min, 10% ACN. The column temperature was set to 40°C. The eluate followed the flow path to a Prospekt II automated SPE unit from Bruker/Spark Holland (Emmen, The Netherlands) under the control of Bruker Hystar 3.0 software. To ensure that the used SPE conditions were adapted to the tested compounds, it was initially verified that the (multi)trapping and the elution steps with small and defined quantities of the parent compounds (10–30 μg injected) led to 1H NMR spectra with sufficient signal-to-noise ratio to obtain reliable integration of each signal within a reasonable scan accumulation. Indeed, the nature of the SPE cartridge had to be chosen; the flow rate of water added during trapping to the mobile phase had to be adjusted; and the nature of the eluting deuterated solvent had to be defined. Taking into account these data, the minimal amount of metabolites was estimated to obtain useful one- and two-dimensional NMR spectra, and the experimental conditions were defined. From this work, it appears that a minimal quantity of 20 μg injected is required for each metabolite. Then a concentrated solution was prepared by pooling together 20 incubated samples before evaporation under an inert nitrogen flux. The residue was redissolved in 500 μl of a mixture composed of 10 mM ammonium formate, pH 3.0, and ACN (50/50; v/v), vortexed, and centrifuged at 2000g for 5 min. The supernatant was decanted and used as an injection solution. The peaks of interest (detected using the UV response at 254 nm) were trapped on Hyssphere GP cartridges (10 × 2 mm) from Spark Holland (Emmen, The Netherlands) by addition of water at a 2-ml/min flow rate to the postcolumn eluate. Three consecutive injections (80 μl/injection) were trapped on the same cartridge to enhance the metabolite concentration. After the trapping process, the cartridges were dried under a constant nitrogen flow to remove residual nondeuterated solvents. The collected fractions were further directly transferred from the SPE cartridges to the NMR cell probe with 290 μl of ACN-d6. The NMR measurements were carried out using an AV500 MHz spectrometer from Bruker BioSpin (Rheinstetten, Germany) equipped with a 3-mm (60 μl) dual inverse 1H/13C z-gradient flow probe.

1H NMR Experiments. "H NMR sequence was used with a pulse width of 30°, a relaxation delay of 2 s, and 64 K data points processed into 32 K points with exponential line broadening of 0.3 Hz. A double presaturation nuclear Overhauser effect spectroscopy pulse sequence (lc1pcwps) adapted to SPIE with shaped pulses for suppression of ACN and water signals was also used to obtain spectra with enhanced signal-to-noise ratio. 13C decoupling was applied to eliminate 13C satellites of the solvents. The spectral window was 10 KHz with 16 K data points processed into 32 K points using zero filling and exponential line broadening of 1 Hz. The number of scans was adapted to obtain the appropriate signal-to-noise ratio.

Two-dimensional correlation spectroscopy (COSY) spectra were acquired without solvent presaturation using the magnitude mode sequence. The spectra were recorded with a spectral window of 7 KHz in both dimensions and transferred into 1 K data points with 128 increments. Data were zero-filled in f1 to 1024 points and processed using the sine bell function with exponential line broadening of 0.3 Hz in f1 and 1 Hz in f2.

Results

Compounds BPDZ 73 and BPDZ 157 were submitted to biotransformation and subsequent analysis, but to avoid repetition and dispersion, the LC/MS/MS and LC/SPE/NMR results will be mainly focused on BPDZ 157 incubated in the presence of PB-induced rat liver microsomes, which could be considered as the most interesting biotransformation profile in terms of metabolism complexity. Only the final results concerning the metabolic profiling of BPDZ 73 will be presented and further discussed.

In Vitro Metabolism. Biotransformation of BPDZ 157 was performed by incubation of the compound in the presence of PB-induced
male rat liver microsomes and an NADPH-generating system. After the incubation step, the samples were analyzed by LC. Metabolite production was highlighted by comparing chromatograms of samples having undergone (or not; T₀ samples) a biotransformation mediated by rat liver microsomes. T₀ samples were prepared like the other samples, but P450s were inactivated by addition of ACN and methanol before starting the biotransformation reactions. As a result, no biotransformation occurred in these samples.

As shown in Fig. 2, the parent product BPDZ 157 is eluted at a retention time of approximately 25 min (relative retention time = 1). The comparison between the chromatograms of samples that have undergone (or not) P450-mediated biotransformation for an incubation period of 1 h highlights the presence of six major metabolites absorbing at 254 nm (see Fig. 2). These products were named M1 to M6, according to their elution order, and were characterized by relative retention times and estimated percentages of 0.47 (3%), 0.58 (2%), 0.69 (10%), 0.70 (18%), 0.73 (15%), and 0.79 (2%), respectively. As expected in reversed-phase LC, the relative retention times of these peaks were lower than that of the parent product, which indicates that the metabolites are more polar compounds.

Metabolism of BPDZ 157 was also studied using microsomes from noninduced rat and pooled human liver. Metabolites M1 to M6 were also detected in the metabolization profile recorded for samples resulting from BPDZ 157 incubation in the presence of nontreated rat liver microsomes (data not shown). Nevertheless, all the metabolites were produced in smaller amounts, which reinforces the utility of using PB-induced liver microsomes in the metabolite chemical structure elucidation process. Incubation of BPDZ 157 in the presence of pooled human liver microsomes results in the production of the major metabolites M3 to M6. Metabolites M1 and M2 were not detected with the used techniques, but interestingly two new minor metabolites (named M7 and M8) were highlighted in human species (data not shown). Because the latter were produced in small amounts, no further inquiries were conducted here to elucidate their chemical structures.

**LC/MS/MS Analyses of BPDZ 157.** The mass spectrometer was initially used in time of flight scan mode to determine the molecular masses of peaks eluted during chromatographic separations by total ionic current monitoring. The BPDZ 157 metabolites M3, M4, and M5 were characterized by a protonated molecule [M + H]⁺ at m/z = 318 higher than for the parent compound (BPDZ 157 + H)⁺ at m/z = 302) and were equivalent to the mass increment of an oxygen atom. Because the P450-mediated reaction involves the combination of oxygen with the organic substrate to produce a molecule of water and a monooxygenated metabolite, the mass increment of these biotransformation products can be explained by the introduction of one oxygen atom on the chemical structure of the parent compound BPDZ 157 to form a hydroxyl group. The protonated molecule [M + H]⁺ of M2 exhibited an m/z of 334, which corresponds to a mass increment equivalent to two oxygen atoms (32 Da) and introduction of two hydroxyl groups on the parent chemical structure. The metabolite M1 had a mass of 232 Da, which is equivalent to the loss of 70 Da by BPDZ 157 and which could result from the removal of the pentyl side chain. Finally, the M6 metabolite was characterized by a [M + H]⁺ at m/z = 316.

To validate those assumptions and to localize structural modifications induced by the P450s, the product ion spectra in time of flight MS/MS mode of each metabolite and the parent compound were further performed (Table 1 summarizes the MS/MS results obtained with the parent BPDZ 157 and its major metabolites). As illustrated in Fig. 3A, the product ion spectrum of [BPDZ 157 + H]⁺ (m/z = 302) showed a series of signals at m/z = 126, 142, 190, and 232. The product ions at m/z = 232, 190, 142, and 126 were issued from the loss of 70 Da (C₆H₁₀), 112 Da (C₆H₁₁N₂), 160 Da (C₆H₅N₂O₃S), and 176 Da (C₆H₁₀N₂O₃S), respectively. This fragmentation pathway may be explained according to Fig. 3B. The fragmentation of the protonated molecule [M + H]⁺ (m/z = 232) corresponding to metabolite M1 generates a series of peaks at m/z = 126, 142, and 190. These product ions are common to those of the parent compound and thus result from the same fragmentation mechanism. Consequently, it is probable that compound BPDZ 157 is metabolized in a compound.

**TABLE 1**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Protonated Molecule</th>
<th>Product Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>[C₁₂H₁₀ClN₃O₃S]⁺</td>
<td>218.9965 [C₆H₅ClNO₂S]⁺</td>
</tr>
<tr>
<td>M2</td>
<td>[C₁₂H₁₆ClN₃O₃S]⁺</td>
<td>218.9965 [C₆H₅ClNO₂S]⁺</td>
</tr>
<tr>
<td>M3</td>
<td>[C₁₂H₁₄ClN₃O₃S]⁺</td>
<td>218.9965 [C₆H₅ClNO₂S]⁺</td>
</tr>
<tr>
<td>M4</td>
<td>[C₁₂H₁₄ClN₃O₃S]⁺</td>
<td>218.9965 [C₆H₅ClNO₂S]⁺</td>
</tr>
<tr>
<td>M5</td>
<td>[C₁₂H₁₄ClN₃O₃S]⁺</td>
<td>218.9965 [C₆H₅ClNO₂S]⁺</td>
</tr>
<tr>
<td>M6</td>
<td>[C₁₂H₁₄ClN₃O₃S]⁺</td>
<td>218.9965 [C₆H₅ClNO₂S]⁺</td>
</tr>
</tbody>
</table>

![Fig. 2. Metabolic profile of compound BPDZ 157. Column: Alltech Hypersil BDS C18 (150 × 4.6 mm, i.d.; particle size = 3 μm). Other conditions: see under LC/SPE/NMR Conditions. - - - - - - - - BPDZ 157 not metabolized; -----, BPDZ 157 metabolized; - - - - - - gradient in ACN.](image-url)
of mass 232 Da (metabolite M1) characterized by the loss of the penty1 group. The product ion spectrum of metabolite M2 (m/z = 334) was characterized by peaks at m/z = 126, 142, 190, 232, 272, 298, and 316. Because some common signals (at m/z = 126, 142, 190, and 232) were found in the product ion spectra of BPDZ 157 and M1, it can be concluded that the introduction of the two oxygen atoms was carried out neither on the benzene core nor on the 1,2,4-thiadiazine 1,1-dioxide cycle but rather on the penty1 group. However, the MS analyses do not allow precise localization of the position of the two oxygen atoms on the alkyl side chain. The metabolites M3, M4, and M5 (m/z = 318) displayed similar product ion spectra that were characterized by peaks at m/z = 126, 142, 190, 232, and 300. As for the metabolites previously analyzed, some product ions (at m/z = 126, 142, 190, and 232) were common to the entities generated during MS/MS analysis of the parent compound and thus resulted from identical fragmentation pathways. Analysis of the product ion spectra of M3, M4, and M5 led to the same conclusion as that established for metabolite M2. Indeed, the existence of an identical fragmentation pathway to that of BPDZ 157 implies that the metabolites resulted from hydroxylation on carbon atoms located on the side alkyl group of BPDZ 157.

Finally, the product ion spectrum of the sixth metabolite detected (M6, m/z = 316) is also characterized by identical product ions as those of BPDZ 157 (peaks at m/z = 126 and 190) and leads to the same conclusion as that established above: a fragmentation pathway identical to that of the parent compound. By comparison with the metabolites M2, M3, M4, and M5, the mass increment detected (14 Da) for M6 can be explained by the formation of a carbonyl group. Nevertheless, the data obtained by fragmentation were not sufficient to determine the exact position of that modification. However, the presence of product ions at m/z = 126 and 190 allowed us to conclude that such a modification is neither on the benzene core nor on the 1,2,4-thiadiazine 1,1-dioxide cycle. Figure 3C highlights the putative structures of the major BPDZ 157 metabolites.

**LC/SPE/NMR Analyses of BPDZ 157.** As described previously, LC/MS/MS analysis of BPDZ 157 metabolites led to some uncertainties about the exact localization of the hydroxyl groups on the alkylamino side chain. Consequently, in this case, the use of the NMR approach could be very helpful to raise the structural uncertainties.

As the NMR spectrometry technique requires larger sample quantities than MS (more than 10 µg of each metabolite), a concentrated solution (prepared as previously described from the pool of 20 incubated samples) was used for LC/SPE/NMR analysis. The workup used to prepare the concentrated solution changed the proportions of each metabolite but did not alter the chromatographic profile.

After chromatographic separations, each metabolite and the parent peak were collected onto SPE cartridges as a result of three consecutive injections (80 µl/injection) in a multiple trapping sequence. After drying for residual nondeuterated solvent removal, each BPDZ 157 metabolite was transferred into the NMR probe with deuterated ACN. From the UV data obtained after LC separation of the concentrated solution and by comparison with data measured from control solution of BPDZ 157, the amount of each metabolite trapped after three injections was estimated as follows: M1 (~20 µg), M2 to M5 (~18–25 µg), and M6 and BPDZ 157 (~15 µg).

Two one-dimensional proton spectra (one using classic quantitative proton sequence and a second using a double nuclear Overhauser effect spectroscopy presaturation) and a two-dimensional COSY spectrum were measured for each metabolite. The classic proton spectra (N.S. from 128 to 1024 scans, 10 min to 1 h) allowed the integration of each signal (if the signal-to-noise ratio was sufficient), whereas the double presaturation sequence furnished more sensitivity and resolution but could not be used for integration. The COSY spectra (N.S. from 8 to 64 scans, 25 min to 3 h) were important to define the proton-proton correlations obtained from the proton and COSY data, and Fig. 4 shows the classic proton spectra (30° pulse) for the different BPDZ 157 metabolites.

First, the similarity between the 1H proton spectra of the trapped parent peak and BPDZ 157 reference in deuterated ACN was verified. The 1H NMR spectrum of BPDZ 157 is characterized by chemical shifts (δH) of 0.95 (protons of the two terminal methyl groups 5′ and 6′), 3.79 (proton in position 2′), 5.65 (proton in position 1′), and 8.87 ppm (proton in position 4). The chemical shifts of the protons located on the benzene ring were monitored between 7 and 8 ppm (7.14, 7.53, and 7.75). It is interesting to note that the proton couples located in
and 4, expected to be chemically identical, appear to resonate at different \( ^1H \) values (1.64 and 1.52 ppm). This feature is corroborated by the COSY spectrum that suggests the splitting of the CH2 group in position 3 and 4 into two distinct signals. It was not possible to precisely assign correspondence signals from the obtained NMR data, but this observation seems to indicate that these two pairs of protons have different magnetic environments (diastereotopic protons), probably resulting from conformational isomerism.

In concern to M1, the LC/MS/MS analysis indicated a probable loss of the alkyl side chain. This putative structure was confirmed by the NMR data because no signals corresponding to the alkyl side chain were monitored in the \( ^1H \) spectrum, and the \( ^1H \) integration signal indicated the presence of two protons at this position.

The LC/MS/MS data of M2 suggested that this metabolite resulted from the introduction of two oxygen atoms on the BPDZ 157 chemical structure corresponding to a double hydroxylation of the alkyl...
side chain but could not exactly locate modifications. Examination of the proton data (Table 2) shows that, compared with the parent compound, the aromatic peaks are not affected. Such a feature confirms the assumption made by MS analysis that the introduction of the two oxygen atoms was carried out neither on the benzene core nor on the 1,2,4-thiadiazine 1,1-dioxide cycle but rather on the pentyl group. NMR analysis clearly showed that the two OH groups are linked to position 3’ and 4’ of the pentyl group. This assumption is supported by the disappearance of the CH$_2$ signals around 1.5 ppm and the appearance of signals with up-field chemical shifts (resulting from the $-I$ effect of the hydroxyl groups) and which couple with the two CH$_3$ located around 1.2 ppm (shown by COSY analysis). Moreover, a signal that integrates for two protons was detected at 3.4 ppm and could be attributed to the newly introduced OH groups.

After MS analysis, metabolites M3, M4, and M5 were expected to be monohydroxylated derivatives, but the position of the OH group could not be determined using the generated MS/MS data. Again, examination of the NMR spectra gave access to the exact localization of the modification site.

The NMR spectral information generated for M3 showed that the hydroxyl group was attached on one of the terminal CH$_3$: disappearance of one of the CH$_3$ signals around 1 ppm, presence of a CH$_2$ signal at low shield that couples with a CH$_2$ signal at 1.74 ppm, and presence of a putative OH signal at 2.9 ppm. Compared with the parent compound, the aromatic peaks are not affected.

The $^1$H spectral data of metabolites M4 and M5 are similar and indicate the presence of a hydroxyl group on the position 3’ or 4’. Indeed, the aromatic signals of these two compounds remained unchanged and confirmed the absence of modifications on the aromatic cores, although evidence for the modification of the CH$_3$ groups 3’ or 4’ could be clearly noted: disappearance of one CH$_2$ signal around 1.5 ppm, unfolding of the CH$_3$ and of one CH$_2$ signal, and appearance of a deshielded CH (coupling with a CH$_3$ signal) and of a OH signal. It is noteworthy that BPDZ 157 structure analysis shows that introduction of a hydroxyl on the methylene group induces two stereogenic centers (in 2’ and the carbon hosting the hydroxyl group). Such a feature implies the possible existence of four stereoisomers divided into two couples of enantiomers, which are diastereoisomers between them (see Fig. 5A). Moreover, the NMR spectra obtained with M6 are not usable, suggesting the probable presence of more than one compound in the LC peak.

**Metabolism of BPDZ 73.** As for BPDZ 157, BPDZ 73 biotransformation step was followed by sample analysis using LC/MS/MS and LC/SPE/NMR to elucidate the chemical structure of BPDZ 73 metabolites. The retention time of the parent compound was approximately 19.1 min after analysis of the T$_0$ and the incubated samples.

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**Fig. 5.** A, potential stereoisomers of M4 and M5. B, structures of the BPDZ 157 metabolites resulting from LC/MS/MS and NMR analysis.
Two other chromatographic peaks of strong intensity (peaks N1 and N2 corresponding to retention times of approximately 11.7 and 14.3 min with relative percentages of 48 and 4%, respectively) were not present in the T<sub>S</sub> sample, but were also observed (Fig. 6). The retention times of these peaks, lower than that of the parent compound, indicate that these metabolites are more polar.

The parent compound BPDZ 73 was further characterized by the protonated ion [M + H]<sup>+</sup> at m/z = 274 for which the fragmentation led to five main ion products at m/z = 126, 142, 190, 208, and 232. The product ion at m/z = 232 is clearly linked to the parent compound with a loss of 42 Da (C<sub>6</sub>H<sub>12</sub>) corresponding to the departure of an isopropyl group. The ion products at m/z = 126, 142, and 190 resulted from the loss, by the native compound, of 148 Da (C<sub>6</sub>H<sub>9</sub>N<sub>2</sub>O<sub>2</sub>S), 132 Da (C<sub>5</sub>H<sub>8</sub>N<sub>2</sub>O<sub>2</sub>S), 84 Da (C<sub>4</sub>H<sub>7</sub>N<sub>2</sub>), and 66 Da (C<sub>3</sub>H<sub>7</sub>N) respectively. The fragmentation of the protonated molecule [M + H]<sup>+</sup> of the metabolite N1 ([M + H]<sup>+</sup> = 232 Da) generated a series of peaks at m/z = 126, 142, and 190. These product ions are common to those obtained from the parent compound and thus result from the same mechanism of fragmentation. It is consequently probable that compound BPDZ 73 is metabolized into a compound of mass 232 Da (N1 metabolite) as a result of the loss of an isopropyl group. The metabolite N2 is characterized by a protonated molecule [M + H]<sup>+</sup> at m/z = 290. The fragmentation of metabolite N2 generated peaks at m/z = 126, 142, 190, 209, 232, and 273. Three of those are common with the product ions of the parent compound, which indicates that the 16-Da mass increment detected for N2 is localized on the alkyl side chain. As for BPDZ 157, a comparison of NMR spectra of each metabolite and the parent compound allowed us to identify the structural changes undergone by BPDZ 73 during the biotransformation step.

Analysis of <sup>1</sup>H NMR spectrum of the N1 metabolite confirms the results obtained by MS, namely, that this compound is obtained by the loss of an isopropyl group (Fig. 6). The N2 metabolite was generated in lower quantity than N1, and only a small amount close to the detection limit had been harvested by the LC/SPE/NMR method. However, longer acquisition times allowed us to circumvent this technical problem and to obtain results enabling chemical structure elucidation of this metabolite. Because the quantity of metabolite N2 was relatively low, no COSY spectrum could be obtained to precisely allocate the hydroxyl group introduction on the BPDZ 73 chemical structure.

Incubation of BPDZ 73 in the presence of nontreated rat liver microsomes exhibited a similar profile to that obtained with PB-induced liver microsomes, although all the metabolites were produced in smaller amounts. The N1 and N2 metabolites were also highlighted in the samples resulting from incubation of BPDZ 73 with pooled human liver microsomes, but interestingly two other minor metabolites were also detected in these samples. Nevertheless, they were produced in too small amounts, and further chemical structure elucidation was not considered.

**Discussion**

The complete elucidation of the chemical structure of metabolites obtained with two benzo-thiadiazine dioxide-type PCOs (BPDZ 73 and BPDZ 157) after incubation with rat liver microsomes (expected phase I metabolites) was achieved by use of two complementary techniques: LC/MS/MS and LC/SPE/NMR.

LC/MS/MS provided information on the molecular mass of each metabolite and on chemical structure by examining their respective fragmentation pathway. However, in several cases, such information was not sufficient to achieve a complete elucidation of their chemical structure. Concretely, in the present study, the MS analysis indicated that compound BPDZ 157 generated six major metabolites, of which three exhibited the same molecular mass and the same fragmentation pathway. These metabolites were expected to result from the addition of one oxygen atom on a carbon atom of the alkylamino side chain at the 3-position leading to hydroxylation in an undefined position. It was shown that the chromatographic separation of these metabolites and their trapping on individual SPE cartridges followed by elution of the trapped metabolites with an appropriate deuterated solvent before NMR recording provided complementary information for a complete elucidation of the structure of the three hydroxylated metabolites of BPDZ 157. The biotransformation profile of compound BPDZ 157 is shown in Fig. 5B. These data show that an important percentage of the parent compound is rapidly transformed. Although no oxidation occurred on the benzenic ring, the alkylamino side chain at the 3-position appears to be the main metabolic brittleness of 3-alkylamino-4-benzothiadiazine 1,1-dioxides. M1, M2, and M3 structures are completely resolved, and we can conclude that biotransformation products M4 and M5 are probably diastereoisomers. It is unfortunate that the stereochemistry of each of these metabolites could not be solved using the collected data. This study also highlights the importance of NMR and MS data for the complete structural determination of the metabolites.

The study of the metabolism of BPDZ 73 has shown the rapid biotransformation of this lead compound into one major metabolite corresponding to 3-amino-7-chloro-4H-benzo-thiadiazine 1,1-dioxide (N1). Some 3-amino-substituted benzo-thiadiazine 1,1-dioxides have been described in the literature to have a potent hypotensive effect in a rat in vivo model (Raffa and Grana, 1965). These data could be in accordance with the unexpected hypotensive effect of SUR1-selective 3-alkylamino-benzo-thiadiazine 1,1-dioxides. In contrast, the minor metabolite (N2) of BPDZ 73 was shown to be structurally identical to BPDZ164, a potent and selective pancreatic PCO in vitro. Indeed, comparing the <sup>1</sup>H NMR spectrum of N2 with that of a synthetic...
compound of similar structure (BPDZ164) confirmed the structural hypothesis. Consequently, N2 is clearly a compound hydroxyLATED on one of the two methyl groups of the alkyl chain at the 3-position (Fig. 6). Introduction of such a group induces the apparition of one stereogenic center. Nevertheless, the stereochemical structure of N2 could not be determined by using the measured MS and NMR data.

This work also showed that the use of PB-induced liver microsomes allows the production of large amounts of biotransformation products, easily highlighted minor metabolites, and facilitated the chemical structure elucidation process. Observation of similar major metabolites in rat and human species reinforces and confirms the choice of rat species for further in vivo investigations.

We can conclude that the combined use of rat PB-induced microsomes model with LC/MS/MS and LC/SPE/NMR analysis constitutes a convenient and rapid approach for identifying and elucidating the chemical structure of SUR1-selective PCOs metabolites generated in classic in vitro conditions. This study has especially shown the rapid biotransformation of 3-alkylamino-4H-1,2,4-benzothiadiazine 1,1-di oxides through hydroxylation and/or dealkylation of the alkylamino side chain.

Taking into account these data and information from the literature, it appears that an enhancement of the metabolic stability of the side chain at the 3-position could be required to avoid or reduce undesired in vivo side effects. For that purpose, some chemical modifications of the alkylamino chain have been conducted. Compounds bearing a 1-methylcyclopentylamino or a 2-fluoroethylamino group at the 3-position have been synthesized and evaluated in terms of metabolic stability and activity. The presence of such substituents drastically reduced the total amount of generated phase 1 metabolites (using the PB-induced rat microsomes model) and especially the nonwished N-dealkylated derivatives (data not shown). Moreover, the introduction of a 2-fluoroethylamino side chain maintained a very interesting pharmacological profile (data not shown). By pointing the metabolic brittle zones of 3-alkylamino-4H-benzo thiadiazine 1,1-dioxides, this work has greatly helped the lead optimization process of the SUR1-selective PCOs, giving access to new improved drug candidates.

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References