VERAPAMIL METABOLISM IN DISTINCT REGIONS OF THE HEART AND IN CULTURES OF CARDIOMYOCYTES OF ADULT RATS

MARKUS WALLES, THOMAS THUM, KARSTEN LEVSEN, AND JÜRGEN BORLAK

Fraunhofer Institute of Toxicology and Aerosol Research, Center of Drug Research and Medical Biotechnology, Hannover, Germany

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ABSTRACT:

A substantial number of drugs act either directly or indirectly on the heart, but surprisingly, little is known about drug oxidation in the heart. We therefore investigated the metabolism of the calcium antagonist verapamil in microsomal fractions isolated from the left and right ventricle of heart muscle and in primary cultures of cardiomyocytes of adult rats. Metabolism of verapamil proceeded predominantly with microsomal fractions isolated from the right ventricle of rat heart, and in liquid chromatographic-tandem mass spectrometry (LC-MS/MS) and LC-MS3 experiments four metabolites (M1–M4) could be identified. Furthermore, the intermediate biotransformation products M5 to M8 could additionally be identified in cultures of primary cardiomyocytes, thus providing new insight into the mechanisms of the N-dealkylation and O-demethylation pathway of verapamil. We show metabolism of verapamil to be predominant in the right ventricle of the heart, and the data reported herein may explain metabolic inactivation and/or adverse drug reactions of certain cardiovascular drugs on the basis of tissue specific metabolism.

The main site of drug oxidation is the liver, but lung, various sections of the gut, skin, placenta, endometrium, kidney, and brain are secondary sites of metabolism (Henderson and Wolf, 1991; Schuetz et al., 1993; Kivisto et al., 1996; Keeney et al., 1998; McFayden et al., 1998). Surprisingly, little is known about drug oxidation in the heart despite the large number of different drugs used to treat heart diseases and the range of remedies including β-blockers, Calcium antagonists, ACE inhibitors, and ACE receptor antagonists. All of these drugs are subject to cytochrome P450-dependent oxidations (Jurima-Romet and Huang, 1993; Stearns et al., 1995; Oldham and Clarke, 1997; Tracy et al., 1999), but there is very limited information on the expression of P450 genes in heart tissue of laboratory animals, despite their importance in common pharmacotherapy.

The extensive metabolism of the calcium antagonist verapamil in liver tissue is well documented, but little is known about its metabolism in heart tissue. Verapamil suffers from extensive first pass metabolism, which results in low drug bioavailability and considerable variability in therapeutic plasma levels (Woosley and Roden, 1983; Padrini et al., 1985; Piovano et al., 1995). In general, metabolism of verapamil leads to pharmacological inactivation, and thus patients require frequent dosage of this particular drug. There are several cytochrome P450 proteins involved in the metabolism of verapamil in human liver, including CYP3A4, CYP3A5, CYP2C8, CYP2C18, CYP2D6, and CYP2E1 (Tracy et al., 1999). Verapamil is metabolized into two initial breakdown products, namely norverapamil and D-617. These metabolites are subject to further metabolism by the cytochrome P450 system to form additional secondary metabolites.

Recently, we showed that human and rat heart tissue express several genes that code for drug-metabolizing enzymes (Thum and Borlak, 2000a,b). As verapamil acts primarily on cardiomyocytes, we wanted to investigate its metabolism in microsomes of ventricular tissue and in cultures of cardiomyocytes of adult rats.

Materials and Methods

Animals All animal procedures described in this report were approved by the ethical committee of the local government of Hannover, Germany, and the investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication 85-23, revised 1996).

Male Sprague-Dawley rats weighing about 200 g were obtained from Charles River (Sulzfeld, Germany). Food and water were given ad libitum.

Anesthesia. Rats were anesthetized with ketamine (anesthetic) and xylazine-hydrochloride (muscle relaxant) with 0.1 ml of ketamine per 100 g of body weight and 0.05 ml of xylazine-hydrochloride per 100 g of body weight. In addition, 2,000 international units of heparin were given intraperitoneally before surgery. All experimental results are obtained from at least n = 3 individual animals.

Isolation and Cultivation of Adult Cardiomyocytes. The isolation was performed as described previously (Thum and Borlak, 2000b,c). In brief, the thorax was opened by surgical procedures, and the aorta ascendens was anatomically prepared. The heart was perfused in situ with the washing solution for 1 min. After the initial wash, the heart was swiftly removed and mounted onto the perfusion apparatus. Then, different solutions were pumped through the rat heart (for details see Thum and Borlak, 2000b) to remove the cells out of their cellular community. The isolated cells were counted with the Neubauer’s counting chamber (Hecht, Germany) and were cultured at 37°C at 5% CO2 for 1 day. Purity of culture was 95% and further improved by preplating cell suspension on plastic flasks for 2 h as previously reported (Chajeck et al., 1977).

Chemicals. Acetonitrile (Malinkrodt-Baker, Deerfield, Holland), MeOH (Malinkrodt Baker), NH4Ac (Merck, Darmstadt, Germany), acetic acid...
(Fluka, Buchs, Switzerland), norverapamil [5-N(3',4'-dimethoxyphenethyl)amino-2-(3',4'-dimethoxyphenyl)-2-isopropyl-valeronitrile] (RBI/Sigma, Natick, MA), and verapamil [5-N(3',4'-dimethoxyphenethyl)methylamino-2-(3',4'-dimethoxyphenyl)-2-isopropyl-valeronitrile] (lot 56H0925/Sigma-Aldrich, Steinheim, Germany) were used in this study. PR23 was a kind gift of W. L. Nelson (University of Washington, Seattle, WA).

**Tissue Preparation and Preparation of Microsomes.** After explantation hearts were removed immediately using standard surgical procedures, and tissue pieces from rat left and right ventricles were cut into small pieces and homogenized with an ultraturrax (IKA, Staufen, Germany) in KCl buffer (0.15 M, pH 7.4). After centrifugation for 30 min at 11,000 g and 4°C, the supernatant was centrifuged at 170,000 g and 4°C for 60 min. The pellet was resuspended in KCl buffer (0.15 M, pH 7.4) and recentrifuged for 40 min at 200,000 g and 4°C, and the microsomal fraction was transferred into Tris-sucrose buffer (0.25 M sucrose, 20 mM Tris buffer, 5 mM EDTA). Microsomal solutions were shock frozen in liquid nitrogen and stored at −80°C until further use.

**Measurement of Protein Concentration.** Microsomal protein concentrations were determined according to the method of Smith et al. (1985). Protein content was adjusted to approximately 5 mg of protein/ml.

**Incubation Experiments with Verapamil.** One hundred micrograms of microsomal protein was incubated with 1 μM verapamil, 21.4 μM glucose 6-phosphate (Sigma), 4.6 μM NADP (Sigma), and 5 units of glucose-6-phosphate dehydrogenase (Sigma) in a final volume of 2 ml of Tris buffer (pH 7.4, 20 mM, Sigma) for 4 h at 37°C in a shaking water bath. Samples were shock frozen in liquid nitrogen and were stored at −80°C until further analysis. In the case of cardiomyocytes, culture medium containing 1 μM verapamil was added to the culture, and incubations were done for 0.5, 2, 4, and 8 h at 37°C. At the end of the incubation, cardiomyocytes were lysed using an ultrasonic waterbath for 10 min, centrifuged at 1000 RPM for 10 min, and the supernatant was collected and frozen at −20°C until further analysis.

**Measurements of Verapamil and Its Metabolites.** Verapamil and its basic metabolites were analyzed by solid phase extraction followed by high-performance liquid chromatography-mass spectrometry (HPLC-MS).

For solid phase extraction a lipophilic cartridge (RP18, Merck, Germany) was used, which was conditioned with methanol followed by equilibration with water. The sample was loaded onto the cartridge without any organic solvent and washed with 3% methanol to separate any sample matrix. Verapamil and its basic metabolites eluted with 65% methanol containing 3% acetic acid. Eluents were evaporated to dryness and reconstituted in 200 μl of acetonitrile/ammonium acetate (0.01 M, pH 6.0, 50:50, v/v). Aliquots of 20 μl were injected onto the HPLC-MS system.

**HPLC-MS.** HPLC-MS analyses were done on a Waters HPLC instrument (pump 590) coupled to an ion trap mass spectrometer (Esquire from Bruker Daltonik, Germany) operated under positive ion electrospray conditions in the full scan, MS², and in some instances in the MS³ mode. The nebulizer pressure was set to 40 psi and the drying gas temperature to 300°C, while +3 kV were applied to the nebulizing capillary. Full mass spectra were acquired by scanning the mass range of m/z 100 to 500. Collision-induced dissociation (CID) spectra were obtained from the protonated molecules (M + H)⁺. HPLC analysis was carried out with an isocratic elution of 1:1 ammonium acetate buffer (0.01 M, pH 6.0)/acetonitrile. The total run time was 60 min and the flow rate 0.2 ml/min. Separation of verapamil and its metabolites was achieved on a 250- × 2-mm RP select B column with a particle size of 4 μm (Merck).
**Recovery.** Recovery experiments were done in quadruplicates. For this purpose, ventricular microsomal suspensions were spiked with 50 ng/ml of verapamil and norverapamil. Solid phase extraction was done as described above, and the resultant eluent was reduced in volume to 200 μl. Measurement of these extracts was done as described above, and recoveries of 85 ± 4 and 81 ± 5% for verapamil and norverapamil, respectively, were determined.

<table>
<thead>
<tr>
<th>Number</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
<th>R6</th>
<th>R7</th>
<th>M + H</th>
<th>α-Cleavageα</th>
<th>N-C Cleavage</th>
<th>C</th>
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<tr>
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<td>OCH₃</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>CH₃</td>
<td>H</td>
<td>C₅H₇</td>
<td></td>
<td>455.4</td>
<td>151.1</td>
<td>303.3</td>
<td>165.1</td>
<td>289.3</td>
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<td>OCH₃</td>
<td>OCH₃</td>
<td>CH₃</td>
<td>H</td>
<td>C₅H₇</td>
<td></td>
<td>291.3</td>
<td>151.1</td>
<td>289.0</td>
<td>165.1</td>
<td>248.1</td>
</tr>
<tr>
<td>M2 (Norverapamil)</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>H</td>
<td>CH₃</td>
<td>C₅H₇</td>
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</tr>
<tr>
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<td>OCH₃</td>
<td>OH</td>
<td>CH₃</td>
<td>H</td>
<td>C₅H₇</td>
<td></td>
<td>441.4</td>
<td>151.1</td>
<td>289.0</td>
<td>165.1</td>
<td>246.1</td>
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<tr>
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<td>OCH₃</td>
<td>CH₁</td>
<td>H</td>
<td>C₅H₇</td>
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<td>303.3</td>
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<td>CH₁</td>
<td>H</td>
<td>C₅H₇</td>
<td></td>
<td>471.2</td>
<td>291.0</td>
<td>260.0 (−OCH₃)</td>
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<td>OCH₃</td>
<td>CH₁</td>
<td>H</td>
<td>C₅H₇</td>
<td></td>
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<td>CH₁OH</td>
<td>H</td>
<td>C₅H₇</td>
<td></td>
<td>471.4</td>
<td>151.1</td>
<td>165.1</td>
<td>441.3 (−CO)</td>
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<tr>
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<td>CH₂O</td>
<td>H</td>
<td>C₅H₇</td>
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<td>165.1</td>
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<tr>
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<td>OCH₃</td>
<td>CH₁</td>
<td>OH</td>
<td>C₅H₇</td>
<td></td>
<td>471.1</td>
<td>303.1</td>
<td>181.1</td>
<td>291.1</td>
<td>234, 260.0 (−OCH₃)</td>
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</tbody>
</table>

α-α-Cleavage and N-C-cleavage may be accompanied by proton transfer (H-rearrangement).

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**TABLE 1**

Fragmentation scheme and main fragment ions of nine metabolites of verapamil

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**FIG. 1.** (Continued)
Metabolites from rat heart tissue and culture experiments were identified by CID experiments in multiple LC-MS/MS (MS²) and LC-MS³ investigations. The obtained MS/MS spectra of metabolites are depicted in Fig. 1, A to I. Metabolism of verapamil proceeded predominantly with the production of M2 with microsomes isolated from the right ventricle of the rat heart. In strong contrast, no detectable production of dealkyl- and O-demethylverapamil products were observed in left ventricular microsomal incubation experiments.

Microsomal Metabolism Studies with Left and Right Ventricular Tissue. Metabolite M1 (D 617). The MS spectrum shows a protonated molecule (MH⁺) of m/z = 291 and points to oxidative dealkylation of the lower substituted phenylalkylamine moiety (M = 164 Da less than verapamil). Fragmentation in the MS² mode (see Table 1) gives rise to two abundant ions with m/z = 248 (loss of the isopropyl group) and m/z = 260 (loss of a methoxy group), which supports the structure M1 given in the metabolic pathway of Fig. 2.

Metabolite M2 (norverapamil). The full scan MS spectrum displays a protonated molecule (MH⁺) of m/z = 441, 14 Da lower than that of the protonated molecule of verapamil, and this points to oxidative dealkylation of a methyl group. Fragmentation in the MS² mode (see Table 1) produces an abundant ion of m/z = 165 and further ions of m/z = 398, 289, and 151, which are indicative for oxidative N-demethylation. The prominent peak at m/z = 165 can be explained by N-alkyl-cleavage with charge transfer and at m/z = 289 by C-C-cleavage in a-position to the nitrogen with subsequent proton transfer. The fragment of m/z 398 is formed by loss of isopropyl, m/z = 151 by α-cleavage with charge transfer. The structure of metabolite 2 was confirmed with a synthetic reference compound.

Metabolite M3 (PR 23). The full scan MS spectrum shows a protonated molecule (MH⁺) of m/z = 441, 14 Da lower than that of verapamil. This is again highly suggestive of oxidative dealkylation of a methyl group. Fragmentation in the MS² mode (see Table 1) leads to abundant ions of m/z = 289, 165, and 151, which suggests oxidative demethylation in position C-31 or C-33. The prominent peaks at m/z = 289 and 151 point to C-C-cleavage in a-position to the nitrogen (the former accompanied by proton transfer). The ions of m/z = 165 and 151 support the view of oxidative demethylation at position 31 or 33 (see Figs. 1C and 2). It was shown previously that O-demethylation occurs predominantly in position 31 (Nelson et al., 1988). The fragment of m/z = 303 stems from metabolite 4, and both isomers have similar retention times in the chromatogram; an extracted ion chromatogram of M3 and M4 is given in Fig. 5. The structure of metabolite 3 was confirmed with a synthetic reference compound (see Fig. 1J).

Metabolite M4 (PR 24). The full scan MS spectrum shows a protonated molecule (MH⁺) of m/z = 291. This indicates oxidative dealkylation of a methyl group. Fragmentation in the MS² mode (see Table 1) produces an abundant ion of m/z = 165. This is highly suggestive of demethylation at C-atoms 21 or 23. The prominent ion of m/z = 291 stems from a N-alkyl-cleavage with proton transfer. The other fragmentation ions of m/z = 303, (C-C-cleavage in a-position to the nitrogen), 248, and 260 (loss of the isopropyl group or a methoxy group from m/z = 291) confirm an O-demethylation in position 21 or 23. It was shown previously that O-demethylation occurs predominantly in position 21 (Nelson et al., 1988). The peak at m/z = 165 stems from metabolite M2.

Metabolism Experiments with Cultured Primary Cardiomyocyte Cultures of Adult Rats. LC-MS/MS experiments provided clear evidence for the production of M1 to M4 as detailed above. Noticeably, cardiomyocytes produced a number of intermediate metabolites, which were not identified or were only present in trace amounts in the aforementioned microsomal incubation experiments. A discussion of the intermediate biotransformation identified in cultures of cardiomyocytes is given below.

Metabolite M5. The full scan MS spectrum shows a protonated molecule (MH⁺) of m/z = 471, 16 Da higher than that of verapamil. This is highly suggestive of direct hydroxylation of verapamil itself. Fragmentation in the MS² mode gives rise to an abundant ion of...
m/z = 291 and additional peaks at m/z = 248 and 260. The prominent ion of m/z = 291 can be explained by N-alkyl-cleavage with proton transfer, and this points to hydroxylation at the phenylalkylamine moiety. Corroborative evidence is the loss of the isopropyl group (m/z = 248) and methoxy group (m/z = 260) from m/z 291 shown in Fig. 1E. Consequently, the hydroxylation reaction could only occur at C-atoms 2, 3, 6, or 7. When comparing the fragmentation pattern of the hydroxylated metabolite with that of the O-demethylverapamil, no loss of water is observed from the [MH+]o of both metabolites. In contrast, collision-induced dissociation of the hydroxyverapamil M9 metabolite (hydroxyl group in the benzylic position) shows loss of water, which suggests M5 is formed as the oxidative precursor of O-demethylverapamil (M4).

**Metabolite M6.** The full scan MS spectrum shows a protonated molecule (MH+) of m/z = 469, 14 Da higher than that of verapamil. This is highly suggestive of direct oxidation of verapamil. Fragmentation in the MS2 mode (see Fig. 1F and Table 1) leads to an abundant ion of m/z = 291 and further subsequent fragments of m/z = 248 and 260. The prominent ion of m/z = 291 results from a N-alkyl-cleavage with proton transfer, demonstrating that oxidation occurred in the phenylalkylamine moiety. The fragment ions of m/z = 248 and 260 originate from m/z = 291 and indicate loss of the isopropyl and methoxy group, respectively. With the exception of the molecular ion, only small differences in the fragmentation pattern of M6 and O-demethylverapamil are obvious upon CID. The MS data are consistent with the aldehyde structure shown in Fig. 2.

**Metabolite M7.** The full scan MS exhibits a protonated molecule (MH+) of m/z = 471, 16 Da higher than that of verapamil. This indicates oxidative hydroxylation. Fragmentation in the MS2 mode (see Fig. 1G and Table 1) leads to an abundant ion of m/z = 165 and further ions of m/z = 260 and 151. The prominent ion of m/z = 165 is consistent with N-C-cleavage with charge migration, and thus oxidation of the phenylalkylamine moiety can be ruled out. The observation of a peak at m/z = 260 excludes hydroxylation of the higher substituted moiety. Thus, only hydroxylation of the methyl group bound to nitrogen is conceivable. When the spectral data of M7 are compared with those of norverapamil, only slight differences are obvious. Indeed, M7 must be the precursor of norverapamil.

**Metabolite M8.** The full scan MS spectrum shows a protonated molecule (MH+) of m/z = 469, 14 Da higher than that of verapamil. Fragmentation in the MS2 mode (see Fig. 1H and Table 1) produces an abundant ion of m/z = 165 and further peaks at m/z = 441 and 151. The ion of m/z = 441 can be explained by loss of CO and provides strong evidence for the presence of a carboxyl group. The prominent ion of m/z = 165 results from a N-C-cleavage. This indicates oxidation of the higher substituted moiety. Further fragmentation of the ion of m/z = 441 (as shown by MS3 experiments) leads to no other fragments apart from m/z = 165. When the MS data of M8 are compared with that of norverapamil, only slight differences are obvious. We propose oxidation of the methyl group bound to the nitrogen atom. M8 is probably an intermediate of M2 (norverapamil) and an oxidation product of M7.

**Metabolite M9.** The full scan MS spectrum shows a protonated molecule (MH+) of m/z = 471, 16 Da higher than that of verapamil. Fragmentation in the MS2 mode (see Fig. 1I and Table 1) produced an abundant ion of m/z = 291 and further ions with m/z = 453, 303, 260, 248, and 181. Loss of water yields an ion of m/z = 453. The prominent ion of m/z = 291 can be explained by N-C-cleavage and is highly suggestive for hydroxylation of the phenylalkylamine moiety. This is confirmed by the presence of further fragment ions of m/z = 248 and 260. The peak at m/z = 303 is formed by C-C-cleavage in a-position to the nitrogen. The ion of m/z = 181 is formed by N-alkyl-cleavage. Consequently, the hydroxylation occurred at the benzylic position (C-7) or the aromatic ring (C-2, -3, or -6), but aromatic hydroxylation is less likely, as comparison of the MS data of the hydroxylated metabolite (loss of water) with that of O-demethylverapamil (no loss of water) highly suggests the hydroxylation at the benzylic position.

**Kinetics of Verapamil Metabolism in Cultures of Cardiomyocytes and Ventricular Microsomes.** Figure 3 depicts the rate of metabolism in cardiomyocyte cultures after 0.5, 2, 4, and 8 h of incubation. Data from 2 × 10^6 cells/flask are given as a percentage of the peak area observed for verapamil. Essentially, production of metabolites increased with time, but a nonlinear and potentially saturation kinetics is observed when the signal abundancies of metabolites are plotted as log-data. This suggests that the rate of metabolism changes with time in cultures of cardiomyocytes.

Data on the signal abundancies of metabolites M1 to M9 from microsomal experiments are reported in Table 2. With ventricular microsomes, M2 accounted for 3.2%, whereas M3 and M4 were 0.3% of verapamil. For all other metabolites, the levels were very low indeed, and they ranged between 0.02 and 0.1% of verapamill’s peak area. Metabolism studies with cultured cardiomyocytes resulted in essentially similar values, but the production of metabolites differed among the two experimental systems. Indeed, 30-, 10-, 25-, 30-, 170-, and 110-fold increases in the production of M1, M3/M4, M5, M6, M7, and M8 were observed when the signal abundancies in extracts from cultured cardiomyocytes and ventricular microsomes were compared. In both experimental systems, M2 was prominent, accounting for 3.2 and 5.6%, but with cultured cardiomyocytes essentially all metabolites were produced in higher amounts. We speculate with reason that the full spectrum of metabolite production depends on the cellular integrity of cardiomyocytes.

**Discussion**

The extensive biotransformation of verapamil is well documented from both in vivo and in vitro studies, but surprisingly its metabolism in the target tissue is basically unknown. To the best of our knowledge, this is the first report on verapamil metabolism in microsomes of rat heart tissue and rat heart muscle cell cultures. We identified a total of nine metabolites, of which M2 to M4 were produced in sufficient amounts with microsomal fractions, whereas in primary cultures of cardiomyocytes intermediate biotransformation products could additionally be identified (M5–M9). We show M5 to M8 to be key intermediates in the N-dealkylation and O-demethylation pathway of verapamil and provide new insight into the mechanisms of drug oxidation in rat heart tissue. M9 is a further, yet unknown metabolite of verapamil with uncertain pharmacological activity. The identification of M9 necessitates further pharmacological and toxicological investigations, as in vivo metabolism studies do not reflect tissue-
specific metabolism. Thus, earlier reports on verapamil’s disposition in human volunteers should be viewed with caution, particularly if the tissue-specific metabolism is being considered (Eichelbaum et al., 1979; Nelson and Olsen, 1988).

Investigations into the tissue-specific expression of xenobiotic-metabolizing enzymes are ongoing, and we recently reported the expression of cytochrome P450 isozymes in distinct regions of the human heart and in cultures of cardiomycocytes of adult rats (Thum and Borlak, 2000a). In the case of the rat, semiquantitative gene expression patterns suggest that CYP1A1 and CYP2B1/2 are key players in cardiomycocytes, and treatment with the P450 monooxygenase-inducing agent Aroclor 1254 resulted in an approximate 4-fold induction of members of the here named gene families. The constitutive expressed genes CYP2C11 and CYP2E1 are expressed throughout the entire culture period (5 days) but did not respond to Aroclor 1254 treatment. There was good agreement between gene expression and translated protein activity using 7-ethoxyresorufin and testosterone as a substrate (Thum and Borlak, 2000b).

In the case of the human heart, transcripts for CYP1A1, CYP2B6/7, CYP2C8–19, CYP2D6, and CYP4B1 were predominantly expressed in the right ventricle, but mRNA copies of the CYP3A gene family were not detected in any of the human heart tissue examined (n = 7 donors) (Thum and Borlak, 2000a). Also Price and colleagues (1992) reported expression of aromatase mRNA in heart tissue of the fetus, and the cloning and expression of cytochrome P450 2J2 arachidonic acid epoxygenase was reported by Wu and colleagues (1997). These studies show the functional importance of cytochrome P450 monooxygenase isozymes in cardiac physiology. Furthermore, treatment of guinea pigs with β-naphthoflavone resulted in 4-fold increases in 7-ethoxyresorufin-O-deethylase and 7-methoxyresorufin-O-demethylation activity in heart microsomal tissue (McCallum et al., 1993). These findings suggest that cytochrome P450 enzymes expressed in heart tissue can be induced by xenobiotics. There is speculation about the physiological importance of the selective P450 monooxygenase gene and protein expression in distinct regions of the human heart, and this was commented in a recent editorial by Kevin Park (2000).

The precise molecular mechanisms of cytochrome P450-mediated metabolism are still unresolved, but it is generally accepted that P450-mediated oxidations occur via iron-oxo/porphyrin cation radical [Fe(III)-Por] (Dawson and Sono, 1987; Dawson, 1988; Blake and Coon, 1989; Larroque et al., 1990). The catalytic cycle for P450 starts with the binding of the substrate to a pentacoordinate high-spin Fe(III) state that initiates the transfer of an electron from NADPH to give an Fe(II) complex. The reduced enzyme-substrate complex then binds molecular oxygen. Transfer of a second electron to this complex is followed by the uptake of two protons and cleavage of O-O-bond to give rise to the activated enzyme O = Fe(IV)-Por + and water. The oxygen of the activated enzyme is then inserted into the substrate.

The P450-catalyzed oxidations of amines (which would lead to the formation of metabolite M7) have been controversially discussed (see Karki and Dinnocenzo, 1995 and references therein). These authors provide new evidence for the metabolism of substituted N,N-dimethylaminals to proceed via a hydrogen abstraction to yield a neutral α-amino radical. Hydroxy transfer from the enzyme gives rise to carbinolamines. The unstable carbinolamine usually collapses to the corresponding secondary amine and a formaldehyde (see Fig. 4).

Some investigators report the isolation of stable carbinolamines, and thus the metabolism of N-methylcarbazole leads to the stable carbino- lamine 3-OH-N-hydroxy-methyl-carbazole (Ebner et al., 1991) and that of medazepam proceeds via a stable carbinolamine as well (Schwartz and Kolis, 1972).

We propose a carbinolamine as an intermediate in the N-demethylation of verapamil, even though N-formyl derivatives are usually chemically more stable. It has been demonstrated that some carbinolamines can be dehydrogenated (instead of hydrolyzed) yielding stable N-formyl derivatives (Testa and Jenner, 1978); thus, N-formyl derivatives of prenylamine were reported by Remberg et al. (1977), and similarly a N-formyl derivative for bepridil (a class III antiarrhythmic drug) was also investigated (Wu et al., 1987).

We suggest that N-demethylation of verapamil to form norverapamil (M2) proceeds via a carbinolamine (M7) and a N-formyl intermediate (M8), which are sufficiently stable in cultures of cardiomycocytes to be identified by mass spectrometry.

A similar mechanism has been proposed by Watanabe et al. (1982) for the O-demethylation of anisole derivatives, and in the case of verapamil the P450-mediated O-demethylation most likely starts with a H- abstraction and hydroxy transfer from the activated enzyme to form a hemiacetate (M5) followed by dehydrogenation to generate a formate derivative (M6).

The hydrogen atom transfer described in Fig. 3 has been generally accepted as a mechanism for the hydroxylation of hydrocarbons and other substrates that are difficult to oxidize (McMurray and Groves, 1986), and we consider this mechanism as the basis for the formation of hydroxiverapamil (M9).

In Fig. 2 we show verapamil metabolism to proceed via M5 and M6 to yield O-demethylverapamil (M4; Fig. 5); similarly, norverapamil production proceeds via the intermediates M7 and M8 to yield the N-alkylverapamil product (M2). As already stated, the hydroxylation product M9 is a further novel metabolite, as hydroxylation occurred, most certainly, at the benzylic position of C-7 (see Fig. 1), although aromatic ring hydroxylation at atom C-2, -3, or -6 can not be ruled out. Some of these metabolites produced in heart tissue are linked to pharmacological inactivation, and further investigations are required to elucidate their role in causing adverse drug reactions. It is tempting to speculate that adverse drug reactions are, at least in part, the result of tissue-specific and idiosyncratic metabolism in view of the findings that saturable tissue uptake may occur (Padini et al., 1985).

Nelson and coworkers (1988) studied the regiochemistry and substrate stereoselectivity of verapamil O-demethylation using microsomal fractions of rat and human liver as an enzyme source. The authors showed significant substrate stereoselectivity in the formation of one particular O-demethylverapamil metabolite, the S/R ratio being 2.3, whereas marginal substrate stereoselectivity was observed in all of the other O-demethylated metabolites. They also show similarity in rat and

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**TABLE 2**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>M1</th>
<th>M2</th>
<th>M3 + M4</th>
<th>M5</th>
<th>M6</th>
<th>M7</th>
<th>M8</th>
<th>M9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomes</td>
<td>0.04</td>
<td>3.2</td>
<td>0.3</td>
<td>0.02</td>
<td>0.02</td>
<td>0.03</td>
<td>0.02</td>
<td>0.1</td>
</tr>
<tr>
<td>Cardiomyocyte cultures</td>
<td>1.1</td>
<td>5.6</td>
<td>3.1</td>
<td>0.5</td>
<td>0.6</td>
<td>5.0</td>
<td>2.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Fig. 4. Proposed scheme for the metabolic activation of amines via carbinolamine formation.
human liver microsomal verapamil metabolism, although greater substrate enantioselectivity for the N-dealkylation process in rat liver microsomes than in human liver microsomes (Nelson and Olsen, 1988) was observed. With rat liver microsomes, two aliphatic aldehydes were successfully trapped as their O-methylximes; in addition, the alcohols formed from reduction of these aldehydes were observed. In a further study, Cashman (1989) reported enantioselective N-oxygenation of verapamil by flavin-containing monooxygenases (FMOs) of rat and hog liver microsomes. With microsomal preparations of both species, significant stereoselectivity in N-oxygenation of verapamil is observed with S/R ratios of 10.1 and 6.6 for purified hog and rat FMO, respectively. This suggests an important role for FMOs in the stereoselective first pass metabolism of verapamil, although the contribution of individual FMO isozymes still must be determined. Because the major route of biotransformation of verapamil in humans involves the unusual C-N-C-cleavage, it is possible that the stereoselective C-N-C cleavage observed for hog and rat hepatic preparations can account for the stereoselective clearance of verapamil in humans. As discussed above, verapamil undergoes stereoselective first pass metabolism (Vogelgesang et al., 1984), and with regard to its antiarrhythmic activity, (S)-verapamil is 8 to 10 times more potent than (R)-verapamil, although the clearance of the (S)-enantiomer is approximately 3-fold higher than that of the (R)-enantiomer (Eichelbaum et al., 1984).

In a study by Lankford et al. (1994), enantioselective metabolites were determined by using a combination of enantioselective chromatographic separation techniques and fast atomic bombardment MS/MS techniques. Six of the urinary verapamil metabolites were predominantly of the (R)-configuration, and this included N-demethyl, O-demethylverapamil; O,O-demethylverapamil; and O-demethylverapamil and norverapamil, whereas one of the metabolites was predominantly in the (S)-form (N-demethyl, N-dealkylverapamil). The remaining isolated metabolite N-dealkylverapamil comprised approximately equal amounts of the two forms.

In conclusion, we show heart tissue to successfully metabolize verapamil. The biotransformation in heart tissue is potentially linked to pharmacological inactivation and adverse drug reaction. It is tempting to speculate that metabolic breakdown of verapamil in heart tissue is linked to therapeutic inefficacy in some patient populations, such as right ventricular hypertrophy, and to the adverse drug reactions seen with this particular drug (Psaty et al., 1995; Waters, 1997; Pahor et al., 1996; Idle JR, 2000).

In this report, we provide new insight into the mechanisms of drug oxidation in heart tissue and identified novel and key intermediates in the N-dealkylation and O-demethylation pathway of the calcium antagonist verapamil.

References

Chajek T, Stein O and Stein Y (1977) Rat heart in culture as a tool to elucidate the cellular origin of lipoprotein lipase. Biochim Biophys Acta 485:140–144.


