# Role of Cytochrome P450 Isoforms in the Metabolism of Abamectin and Ivermectin in Rats

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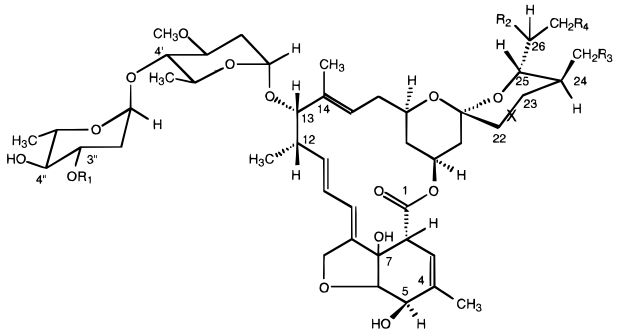
Abamectin (AVM) and ivermectin (IVM) are each metabolized by rat liver microsomes to 3″-*O*-desmethyl (3″-ODMe), 24-hydroxymethyl (24-OHMe), and 26-hydroxymethyl (26-OHMe) derivatives. Microsomes from rats pretreated with dexamethasone (Dex), but not 3-methylcholanthrene (3MC), increased the formation of 3″-ODMe metabolites of both AVM and IVM. Troleandomycin inhibited formation of 3″-ODMe metabolites by >80% by microsomes from Dex-induced rats. Therefore, cytochrome P450 3A plays a major role in this metabolic pathway. Formation of the 26-OHMe metabolites was markedly increased by microsomes from 3MC-treated but not Dex-treated rats. Formation of 24-OHMe from AVM, but not IVM, was slightly increased by microsomes from 3MC-treated rats. Consistent with this observation, anti-rat cytochrome P450 1A1 inhibited formation of 26-OHMe metabolites of AVM and IVM by 90 and 40%, respectively. This antibody also inhibited formation of the 24-OHMe metabolite from AVM by 60% but not from IVM. Thus, cytochrome P450 1A1 is involved in the hydroxylation of the 26-methyl group of both AVM and IVM as well as the 24-methyl group of AVM but not the 24-methyl group of IVM.

**Keywords:** *Avermectin; ivermectin; metabolism; rat; cytochrome P450; isoform*

## INTRODUCTION

The avermectins are a family of macrocyclic lactones produced by *Streptomyces avermitilis* (Fisher and Mrozik, 1989; Burg and Stapley, 1989). Abamectin (avermectin $B_1$, AVM) is a mixture of two components, with the major component avermectin $B_{1a}$ ($B_{1a}$), ≥80% of the mixture, and the minor component avermectin $B_{1b}$ ($B_{1b}$), ≤20% of the mixture, differing by a single methylene group (Figure 1). Ivermectin (IVM) is synthesized from AVM by reducing the 22,23-double bond, producing dihydroavermectin $B_{1a}$ ($H_2B_{1a}$) and dihydroavermectin $B_{1b}$ ($H_2B_{1b}$). These compounds are distinguished by potent anthelmintic and insecticidal activities (Shoop et al., 1995). Both AVM and IVM are registered as antiparasitic drugs for animal health, while AVM has also been developed as an acaricide/insecticide. IVM is also used in humans for the treatment of onchocerciasis or river blindness.

The *in vivo* and *in vitro* metabolic fates of AVM and IVM have been previously studied in different animal species (Halley et al., 1992). AVM and IVM are metabolized in a qualitatively similar way by cattle, sheep, swine, and rat, and the metabolic profiles for each compound are also qualitatively similar among the species. The major metabolites of AVM and IVM in cattle, sheep, swine, and rats are either 24-hydroxymethyl (24-OHMe) or 3″-*O*-desmethyl (3″-ODMe) derivatives. One minor unidentified metabolite of AVM or IVM was also observed in rats and possibly in some of the other species (Halley et al., 1992; Maynard et al., 1989). However, the enzymes responsible for the metabolism have not been identified in any species. This information will be useful to establish the relevance of the involved enzymes to other animals, including humans. Rats have been used widely for toxicology studies, and the rat was also proven to be an appropriate laboratory animal toxicity model for cattle, sheep, and swine (Halley et al., 1992). Thus, we determined which cytochrome P450 isoforms are responsible for the metabolism of AVM and IVM in the rat. Structures of the unknown metabolites were also determined.



| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $C_{22}$-X-$C_{23}$ |
|---|---|---|---|---|---|
| $B_{1a}$ | $CH_3$ | $CH_2CH_3$ | H | H | - $CH_2$ = $CH_2$ - |
| $B_{1b}$ | $CH_3$ | $CH_3$ | H | H | - $CH_2$ = $CH_2$ - |
| $H_2B_{1a}$ | $CH_3$ | $CH_2CH_3$ | H | H | - $CH_2$ - $CH_2$ - |
| $H_2B_{1b}$ | $CH_3$ | $CH_3$ | H | H | - $CH_2$ - $CH_2$ - |
| 24-Hydroxymethyl $B_{1a}$ | $CH_3$ | $CH_2CH_3$ | OH | H | - $CH_2$ = $CH_2$ - |
| 24-Hydroxymethyl $H_2B_{1a}$ | $CH_3$ | $CH_2CH_3$ | OH | H | - $CH_2$ - $CH_2$ - |
| 26-Hydroxymethyl $B_{1a}$ | $CH_3$ | $CH_2CH_3$ | H | OH | - $CH_2$ = $CH_2$ - |
| 26-Hydroxymethyl $H_2B_{1a}$ | $CH_3$ | $CH_2CH_3$ | H | OH | - $CH_2$ - $CH_2$ - |
| 3″-O-Desmethyl $B_{1a}$ | H | $CH_2CH_3$ | H | H | - $CH_2$ = $CH_2$ - |
| 3″-O-Desmethyl $H_2B_{1a}$ | H | $CH_2CH_3$ | H | H | - $CH_2$ - $CH_2$ - |

**Figure 1.** Structures of abamectin, ivermectin, and metabolites.

## MATERIALS AND METHODS

**Chemicals.** [5-$^3$H]$B_{1a}$ (17.79 mCi/mg) and [22,23-$^3$H]$H_2B_{1a}$ (81.00 mCi/mg) were synthesized by the Labeled Compound Synthesis Group, Drug Metabolism II, Merck Research Laboratories (MRL). Unlabeled AVM and IVM were obtained from the Chemical Data Department, MRL. Substrates were prepared by mixing [5-$^3$H]$B_{1a}$ and [22,23-$^3$H]$H_2B_{1a}$ with unlabeled AVM and IVM, respectively; thus only $B_{1a}$ components were radiolabeled. Glucose 6-phosphate, β-nicotinamide ad-

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enine dinucleotide phosphate ($NADP^+$), glucose 6-phosphate dehydrogenase, ethylenediaminetetraacetic acid (EDTA), troleandomycin (TAO), and SKF525A were purchased from Sigma (St. Louis, MO). Rabbit anti-rat P450 3A and preimmune rabbit immunoglobulin G (IgG) were purchased from Oxygene (Dallas, TX). Monoclonal anti-rat 1A1 and control antibody were kindly provided by Dr. Paul E. Thomas (Rutgers University, Piscataway, NJ). All solvents used were of HPLC grade or equivalent. Insta-Gel XF scintillation cocktail was obtained from Packard, Downers Grove, IL.

**Preparation of Liver Microsomes.** Livers pooled from male Sprague−Dawley rats treated with dexamethasone (Dex), 3-methylcholanthrene (3MC), or phenobarbital (PB) were excised and homogenized with 2 vol of 50 mM Tris buffer (pH 7.5) containing 1.15% KCl. Homogenates were centrifuged at 10000*g* for 20 min, and the resulting supernatants were centrifuged at 105000*g* for 60 min. The microsomal pellets were washed with 10 mM EDTA containing 1.15% KCl and recentrifuged at 105000*g* for 60 min. The washed microsomes were resuspended in 10 mM potassium phosphate buffer (pH 7.4) containing 250 mM sucrose and stored at −80 °C until use. The microsomes from untreated male and female rats were prepared separately in a similar manner. The livers were homogenized with 4 vol of 0.1 M phosphate buffer containing 1.15% KCl and 1 mM EDTA (pH 7.4) and centrifuged at 10000*g* for 40 min. A 0.1 M phosphate buffer containing 1 mM EDTA (pH 7.4) was used for subsequent washing and storage of microsomes. The protein concentration and cytochrome P450 content were measured by the method of Smith et al. (1985) and Omura and Sato (1964), respectively.

**Liver Microsomal Incubation.** In the time course studies with microsomes from untreated and Dex-, PB-, and 3MC-pretreated rats, the incubations contained 0.5 mg/mL microsomal protein, 10 $\mu$M substrate (AVM or IVM), 0.1 M potassium phosphate (pH 7.4), 1 mM EDTA, and an NADPH-generating system (10 mM glucose 6-phosphate, 1 mM $NADP^+$, and 1.4 units of glucose-6-phosphate dehydrogenase/mL) in a total volume of 200 $\mu$L. The incubations were initiated by addition of the NADPH-generating system at 37 °C. The reactions were stopped by the addition of an equal volume of methanol. The incubation mixtures were centrifuged, and the supernatants were analyzed by HPLC.

In the inhibition studies with chemical inhibitors, liver microsomes were preincubated with TAO for 15 min or SKF525A for 30 min at 37 °C with an NADPH-generating system before the addition of AVM or IVM (10 $\mu$M). The reactions were stopped after 30 min of incubation.

Immunoinhibition experiments were performed by incubating the liver microsomes with control IgG or the antibody against rat CYP enzymes for 30 min at room temperature before incubation with the substrate in 0.1 M potassium phosphate buffer (pH 7.4), 6 mM $MgCl_2$, 1 mM EDTA, and an NADPH-generating system (10 mM glucose 6-phosphate, 1 mM $NADP^+$, and 0.7 unit of glucose-6-phosphate dehydrogenase/mL).

Metabolites of AVM and IVM for structure identification were generated from incubations with microsomes from 3MC-induced rat liver.

**High-Performance Liquid Chromatography (HPLC).** Metabolite profiles were determined by reverse-phase HPLC analysis on a Zorbax SB-C18 4.6 mm × 250 mm column using a Shimadzu dual pump system with a mobile phase of $CH_3OH/H_2O$ (AVM, 0−50 min, 80/20; 52−55 min, 100/0; 57 min, 80/20; IVM, 0−50 min, 86/14; 52−55 min, 100/0; 57 min, 86/14). The flow rate was 1.0 mL/min, and the eluate was monitored at 245 nm using a flow-through UV detector. One-minute fractions of the column eluate were collected into miniscintillation vials and mixed with scintillation cocktail. The radioactivity in these samples was determined by scintillation spectrometry.

**Mass Spectral Analysis.** Mass spectral analysis was performed on a SCIEX API III mass spectrometer (Ontario, Canada). Samples were analyzed by MS/MS using the ionspray interface and positive ion detection. Samples were analyzed by flow injection in a mobile phase that consisted of 70% acetonitrile:30% 10 mM ammonium acetate containing 0.1% trifluoroacetic acid.

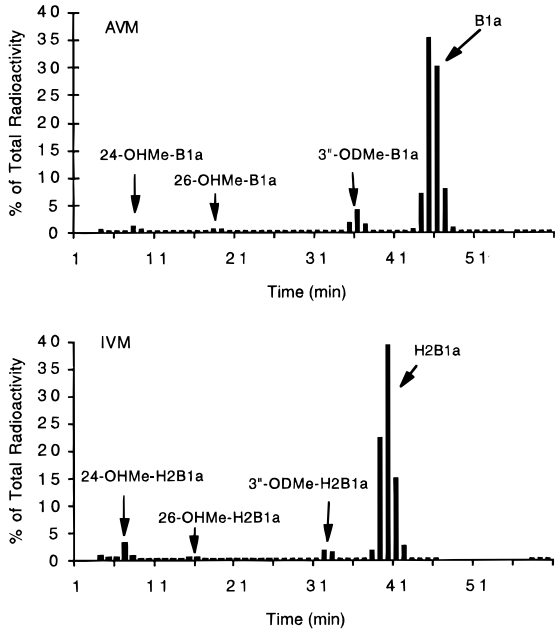


**Figure 2.** HPLC radiochromatograms of extracts from incubations of AVM (top) and IVM (bottom) with untreated rat liver microsomes.

**NMR Analysis.** $^1H$ NMR spectra were acquired at 25 °C on a Varian Unity Plus 400 MHz spectrometer. Standard $B_{1a}$ and its unknown metabolite were dissolved in deuterated chloroform, while standard $H_2B_{1a}$ and its unknown metabolite were dissolved in deuterated benzene.

## RESULTS AND DISCUSSION

**Metabolism of AVM and IVM in Rat Liver Microsomes.** AVM and IVM were metabolized very slowly in liver microsomes from untreated male and female rats. Since the extent of metabolism was very low, differences in metabolism between male and female rats were not detectable. Thus, only male rats were used in the later studies. Rates of total metabolism of AVM and IVM (10 $\mu$M) in male rats were ~22 and 30 pmol/mg of protein/min, respectively.

For both AVM and IVM, at least three metabolites were generated (Figure 2), two of which have been previously identified as 24-OHMe and 3″-ODMe derivatives (Halley et al., 1992). The third metabolite, which may have been produced in other animal species, remained to be identified (Halley et al., 1992; Maynard et al., 1989). Since the extent of metabolism of AVM and IVM in microsomes from untreated rats was too low, microsomes from 3MC-treated rats were used for producing and isolating the unknown metabolites from AVM and IVM for identification.

The positive ion mode mass spectrum of the metabolite from AVM showed the molecular ion peak at *m/z* 907 ($[M + NH_4]^+$) with a characteristic fragment at *m/z* 321 in its daughter-ion spectrum. Both peaks were 16 mass units higher than the corresponding peaks in the spectra of the parent compound (Albers-Schönberg et al., 1981) indicating that hydroxylation had occurred. The $^1H$-NMR analysis of this metabolite, compared to $B_{1a}$, showed the loss of one methyl doublet at 0.95 ppm (i.e., either $C_{24}$-$CH_3$ or $C_{26}$-$CH_3$) consistent with derivatization of one of the methyl groups. Irradiation of the proton on C-24 caused collapse of the 0.95 ppm doublet suggesting that the C-24 methyl group remained intact.

Thus, the C-26 methyl group must have been modified. These results led to identification of the unknown metabolite as 26-OHMe-$B_{1a}$.

Similarly, the identification of the unknown metabolite of IVM was based on the results from positive ion mode mass spectrometry and NMR analysis. The mass spectrum showed the molecular peak at *m/z* 909 ($[M + NH_4]^+$) with a characteristic fragment at *m/z* 323 in its daughter-ion spectrum. These peaks are 16 mass units higher than those from $H_2B_{1a}$. NMR analysis of this compound could not readily discriminate between the 24-hydroxymethyl and 26-hydroxymethyl compounds by a double-irradiation experiment since, unlike the spectrum of $B_{1a}$, the chemical shifts of the protons on C-24 and C-26 are very similar. However, in the $^1$H-NMR spectrum of $H_2B_{1a}$ in deuterated benzene, the chemical shifts of C-24 and C-26 methyl groups are well distinguished. Compared to $H_2B_{1a}$, the spectrum of the unknown metabolite showed a loss of the C-26 methyl doublet indicating that the C-26 methyl group was hydroxylated. Thus, the unknown metabolite of IVM was identified as 26-OHMe-$H_2B_{1a}$.

The 24-OHMe and 3″-ODMe metabolites were also isolated from the incubation of AVM and IVM with microsomes from 3MC-treated rats. The structures were confirmed by comparing their retention times with those obtained from previous studies (Halley et al., 1992) and by mass spectral analyses. The positive ion mode spectra of the 24-OHMe metabolites of AVM and IVM showed the ion peak ($[M + NH_4]^+$) at *m/z* 907 and 909, respectively. For the 3″-ODMe compounds, the most predominant peaks ($[M + NH_4]^+$) were at *m/z* 877 and 879, respectively.

**Effect of Cytochrome P450 Inducers, Inhibitors, and Antibodies.** The metabolism of AVM and IVM by rat liver microsomes was inhibited in the presence of SKF525A (~90%), suggesting the involvement of cytochrome P450s in the biotransformation. To determine the participation of the specific cytochrome P450 in metabolism, AVM and IVM were each incubated with liver microsomes from 3MC-, Dex-, and PB-treated rats, respectively (Soucek and Gut, 1992). Pretreatment of male rats with Dex, but not 3MC, greatly increased the rate of formation of 3″-ODMe-$B_{1a}$ and 3″-ODMe-$H_2B_{1a}$ from AVM and IVM *in vitro*, respectively (Figure 3). The amount of 26-OHMe derivatives generated from both AVM and IVM in microsomal incubations was markedly increased by 3MC treatment but not by Dex administration. Formation of the 24-OHMe metabolite *in vitro* from AVM, but not IVM, was slightly increased following 3MC treatment. Pretreatment of rats with PB had no significant effect on the metabolism of AVM and IVM.

Since the elevation of 3″-ODMe metabolite formation by liver microsomes from Dex-treated rats as well as TAO-treated rats (data not shown) suggested the involvement of the cytochrome P450 3A subfamily in this metabolic pathway, the metabolism of AVM and IVM (10 $\mu$M) in liver microsomes from Dex-induced rats was studied in the presence of TAO, a specific suicide inhibitor of CYP3A (Watkins et al., 1985; Waxman et al., 1988) (Figure 4). Due to the limited amount of metabolites produced by liver microsomes from untreated rats, the inhibition studies were performed only with liver microsomes from induced rats. The inhibition of 3″-ODMe metabolite formation from both compounds approached 80% at concentrations as low as 50 $\mu$M TAO. Formation of the 3″-ODMe metabolite from AVM was

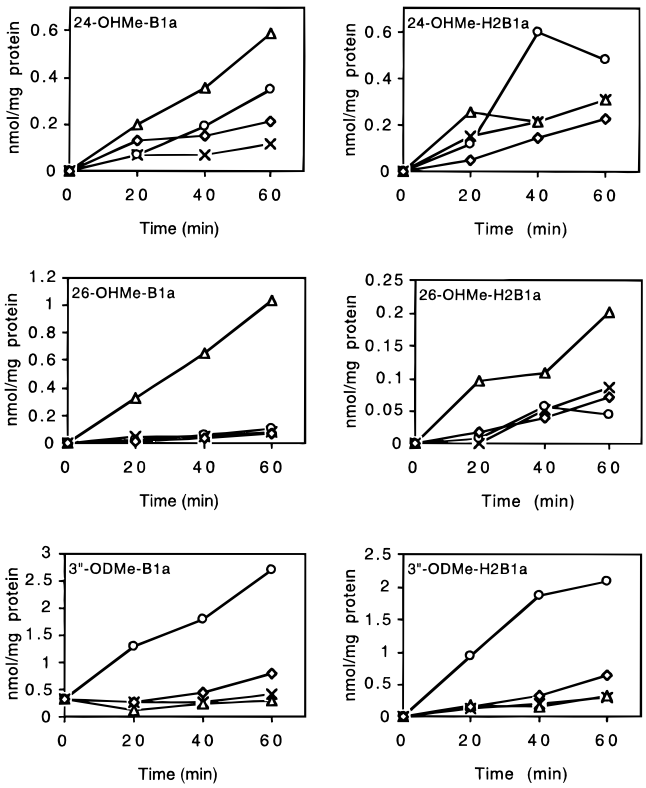


**Figure 3.** Metabolite formation from incubations of AVM (left panels) and IVM (right panels) with liver microsomes from untreated and Dex-, 3MC-, and PB-treated male Sprague–Dawley rats: (○) Dex, (△) 3MC, (◇) PB, and (×) untreated.

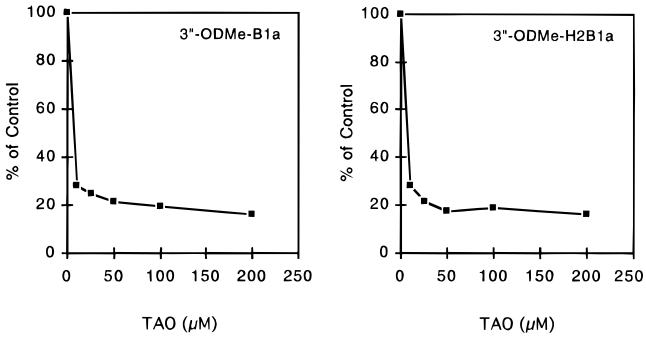


**Figure 4.** Inhibition by TAO of 3″-ODMe metabolite formation from AVM and IVM in incubations with liver microsomes from Dex-treated rats. The metabolite was measured and calculated as the percentage relative to the control sample which had no TAO added.

also inhibited by greater than 80% with antibodies against 3A (Figure 5). However, significant inhibition was also noted by the preimmune (control) IgG. The cause of this nonspecific inhibition is unknown. The combined chemical and immunological inhibition studies clearly establish that the cytochrome P450 3A subfamily is primarily responsible for the 3″-ODMe metabolite formation in Dex-treated microsomes.

To determine the role of the cytochrome P450 1A1 isoform in the metabolism of AVM and IVM, anti-rat P450 1A1 was incubated with substrate and liver microsomes from 3MC-induced rats (Figure 6). In the incubations with AVM, the formation of 24-OHMe-$B_{1a}$ and 26-OHMe-$B_{1a}$ was inhibited by the antibodies by 60 and 90%, respectively. Hence, cytochrome P450 1A1 is the predominant enzyme responsible for the formation of 26-OHMe-$B_{1a}$. Cytochrome P450 1A1 is also a major enzyme for the formation of 24-OHMe-$B_{1a}$, although other cytochrome P450 forms may play a minor

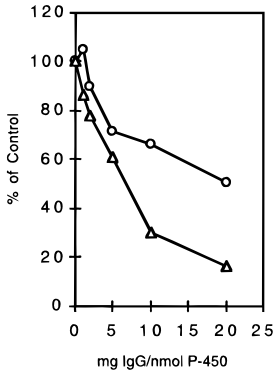


**Figure 5.** Inhibition of formation of the 3″-ODMe metabolite from AVM by anti-P450 3A in incubations with microsomes from Dex-treated rats: (○) preimmune rabbit IgG (control) and (△) polyclonal anti-rat 3A. The metabolite was measured and calculated as the percentage relative to the control sample which had no IgG added.

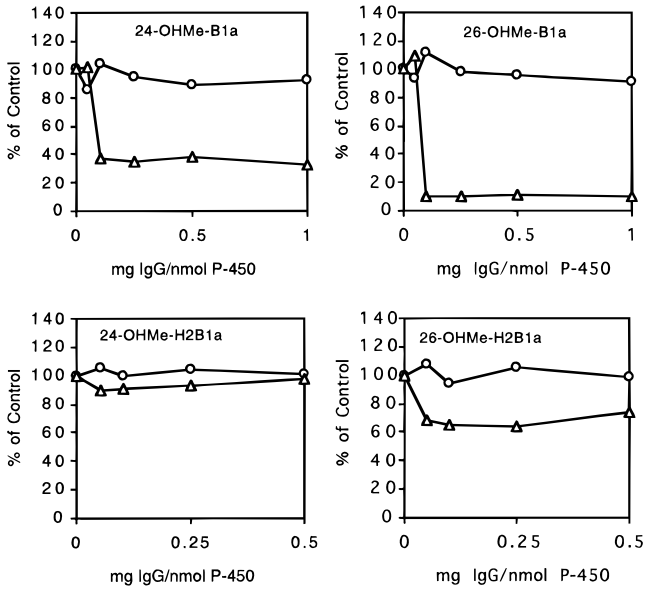


**Figure 6.** Inhibition of metabolism of AVM (top) and IVM (bottom) by anti-P450 1A1 in incubation with microsomes from 3MC-treated rats: (○) control monoclonal antibodies and (△) monoclonal anti-rat 1A1. The metabolite was measured and calculated as the percentage relative to the control sample which had no antibody added.

role. For IVM, antibodies against cytochrome P450 1A1 inhibited 26-OHMe-$H_2B_{1a}$ formation by 40% but had no effect on the formation of 24-OHMe-$H_2B_{1a}$, suggesting that P450 1A1 plays only a partial role in the formation of 26-OHMe-$H_2B_{1a}$ and is not involved in the formation of 24-OHMe-$H_2B_{1a}$. These results are consistent with the observations that the formation of metabolites 26-OHMe-$B_{1a}$ and 24-OHMe-$B_{1a}$ of AVM and 26-OHMe-$H_2B_{1a}$, but not 24-OHMe-$H_2B_{1a}$, of IVM was increased *in vitro* by microsomes from 3MC-treated rats (Figure 3). Anti-rat P450 1A1, on the other hand, had no effect on the formation of 3″-ODMe compounds from either AVM or IVM, thus excluding the involvement of cytochrome P450 1A1 in this O-demethylation pathway.

## CONCLUSION

Previously unidentified metabolites of AVM and IVM from rat liver microsomal studies were determined by MS and NMR to be 26-OHMe-$B_{1a}$ and 26-OHMe-$H_2B_{1a}$, respectively. The cytochrome P450 isoform responsible for each metabolic pathway is listed in Table 1. Cytochrome P450 3A is the major enzyme responsible for the 3″-*O*-desmethylation of both AVM and IVM. Cytochrome P450 1A1 is the predominant enzyme responsible for the hydroxylation of the C-26 methyl group of AVM but contributes only partially to the formation 26-OHMe-$H_2B_{1a}$ from IVM. In addition, cytochrome P450 1A1 plays a major role in hydroxylation of the C-24 methyl of AVM but not of IVM. Formation of 3″-ODMe derivatives of both AVM and IVM was not inhibited by anti-rat P450 1A1. The involvement of P450 2B in the metabolism of AVM and IVM could be excluded since PB-induced microsomes had no effect on the rate of formation of any of the metabolites. These conclusions are based on the results from incubations with liver microsomes from induced rats because of limited quantities of metabolites produced by liver microsomes from untreated rats.

**Table 1. Cytochrome P450 Isoforms Responsible for the Formation of Each Metabolite of AVM and IVM in Induced Liver Microsomes**

| | metabolite | | |
|---|---|---|---|
| parent compd | 24-OHMe | 26-OHMe | 3″-ODMe |
| AVM | 1A1 (major)[a] | 1A1 (predominant)[a] | 3A (major)[b] |
| IVM | N/D[a,c] | 1A1 (partial)[a] | 3A (major)[b] |

[a] Incubation was performed with liver microsomes from 3MC-treated rats. [b] Incubation was performed with liver microsomes from Dex-treated rats. [c] Not determined.

## ABBREVIATIONS USED

AVM, abamectin; IVM, ivermectin; Dex, dexamethasone; 3MC, 3-methylcholanthrene; TAO, troleandomycin; PB, phenobarbital; $NADP^+$, $\beta$-nicotinamide adenine dinucleotide phosphate; EDTA, ethylenediaminetetraacetic acid; IgG, immunoglobulin G.

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