OXIDATIVE METABOLISM OF MONENSIN IN RAT LIVER MICROSOMES AND INTERACTIONS WITH TIAMULIN AND OTHER CHEMOTHERAPEUTIC AGENTS: EVIDENCE FOR THE INVOLVEMENT OF CYTOCHROME P-450 3A SUBFAMILY

CARLO NEBBIA, LUCIANO CEPPA, MAURO DACASTO, MONICA CARLETTI, AND CARLO NACHTMANN

Department of Animal Pathology, Division of Pharmacology and Toxicology, University of Turin (C.Ne., L.C., M.D., M.C.); and Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d’Aosta, Turin, Italy (C.Na.)

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ABSTRACT:

Monensin (MON) is an ionophore antibiotic widely used in veterinary practice as a coccidiostatic or a growth promoter. The aims of this study were to characterize the P-450 isoenzyme(s) involved in the biotransformation of the ionophore and to investigate how this process may be affected by tiamulin and other chemotherapeutic agents known to produce toxic interactions with MON when administered concurrently in vivo. In liver microsomes from untreated rats (UT) or from rats pretreated, respectively, with ethanol (ETOH), phenobarbital (PB), pregnenolone 16α-carbonitrile (PCN), or demethasone (DEX), the rate of MON demethylation was the following: DEX > PCN > PB >= UT = ETOH > β-NIF; similar results were obtained by measuring total MON metabolism. In addition, the extent of triacetyloleandomycin-mediated P-450 complexes was greatly reduced by the prior addition of 100 µM MON. In DEX-treated microsomes, MON O-demethylation was found to fit monophasic Michaelis-Menten kinetics (K_M = 67.6 ± 0.01 µM; V_max = 4.75 ± 0.76 nmol/min/mg protein). Tiamulin markedly inhibited this activity in an apparent competitive manner, with a calculated K_i (Dixon plot) of 8.2 µM and an IC_50 of about 25 µM. At the latter concentration, only ketoconazole or metyrapone, which can bind P-450 3A, inhibited MON O-demethylation to a greater extent than tiamulin, whereas α-naphthoflavone, chloramphenicol, or sulphametasine was less effective. These results suggest that P-450 3A plays an important role in the oxidative metabolism of MON and that compounds capable of binding or inhibiting this isoenzyme could be expected to give rise to toxic interactions with the ionophore.

Monensin (MON)1 (see Fig. 1), an ionophore antibiotic derived from Streptomyces cinnamonensis, is used widely as a coccidiostatic in avian species and as growth promoter in cattle. As with other ionophores, MON is able to form lipophilic complexes with monovalent cations easily crossing cellular and subcellular membranes; the high affinity toward Na⁺ displayed by the drug results in an influx of this ion with a corresponding efflux of H⁺ and K⁺, leading, in turn, to a secondary increase in intracellular Ca²⁺ (Donoho, 1984). These cation imbalances are thought to cause a number of severe biochemical and histological changes, including mitochondrial swelling, decrease in ATP production, lipid peroxidation, and eventual loss of the integrity of cell membranes (Mollenhauer et al., 1990). MON actually is characterized by a narrow safety margin, and several accidental poisonings have been described not only in horses, in which the drug is contraindicated, but also in pigs, cattle, chickens, turkeys, sheep, goats, rabbits (Langston et al., 1985), and even dogs (Wilson, 1980). Interestingly, pigs and broiler chicks exposed to recommended doses of MON or of other ionophores should not be administered concurrently with chemotherapeutic agents such as tiamulin (TIAM), triacetyloleandomycin (TAO), chloramphenicol (CAF), or sulphametasine (SMZ), because of the onset of severe, often lethal toxicoses characterized by clinicopathological findings closely resembling those arising from accidental overdosages of the corresponding ionophore(s) (Pietsch and Ruffle, 1986; Anadon and Martinez-Larrañaga, 1991).

Metabolism studies with ¹⁴C-MON have been performed in several food-producing species and in the rat as well, indicating that the ionophore is biotransformed extensively to a large number of metabolites—more than 50—that are qualitatively but not quantitatively similar among species (Donoho, 1984). Six fecal metabolites have been identified tentatively either in steers and rats or in calves and chickens, which appear to be the result of an O-demethylation and/or hydroxylation at various positions along the carbon backbone of the ionophore molecule (Fig. 1) (Donoho et al., 1978; Davison, 1984). The generation of other minor metabolites has been documented, such as a decarboxylated MON in which the methoxyl group –OCH₃ has been replaced by a ketone, but in no instance did the ionophore appear to undergo conjugative biotransformations (Donoho, 1984).
As reported recently, the formation of a characteristic type I binding spectrum, the increase in the rate of NADPH oxidation, the NADPH-dependent production of formaldehyde, as well as its modulation under various conditions [use of preparations from sodium phenobarbital (PB)-pretreated rats, NADPH omission or replacement with NADH, addition of metyrapone (MET)], indicate that MON appears to be a substrate of P-450-dependent monoxygenases in rat liver microsomes (Ceppa et al., 1997). Nonetheless, there is a lack of information concerning which P-450 subfamily is involved primarily in MON metabolism. A study therefore was undertaken to define further the in vitro microsomal metabolism of the ionophore and to identify the P-450 isoform(s) concerned with its oxidative biotransformation. Investigations also were carried out on the ability in affecting the rate of the in vitro metabolism of MON displayed by TIAM and other drugs known to give rise to toxic interactions when administered concurrently in vivo.

Materials and Methods

Chemicals. Authentic, pure MON was a kind gift from Dr. Giuseppe Pradella (Eli-Lilly Italia, Sesto Fiorentino, Italy). TIAM hydrogen fumarate was kindly provided by Dr. Renger F. Witkamp (University of Utrecht, the Netherlands). Ketoconazole (KCZ) was donated by Dr. Marcel Janssen (Janssen Research Foundation, Beerse, Belgium). Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and BSA were purchased from Boehringer Mannheim (Mannheim, Germany), whereas all other reagents came from Sigma (St. Louis, MO).

Induction Protocols and Preparation of Hepatic Microsomes. Male Wistar rats (Charles River Italia, Calco, Italy) in the weight range of 120 to 150 g were fed standard laboratory chow (MIL-rats feeds; Morini, San Polo d’Enza, Italy) and allowed free access to tap water. After an acclimatization period of 1 week, they were allocated to one of the following treatment groups (n = 6 for each group): control (no treatments), PB (0.05% in tap water for 7 days), ETOH (15% in tap water for 10 days), DEX (50 mg/kg i.p. for 3 days), βNAD (50 mg/kg in corn oil i.p. for 3 days), or pregnenolone 16α-carbonitrile (PCN) (40 mg/kg in corn oil i.p. for 4 days). At the end of the induction protocol, animals were deprived of food overnight and sacrificed by CO2 asphyxia followed by decapitation. Housing conditions and euthanasia were as recommended by the Italian law on animal experimentation (Decreto Legislativo 116/92). Microsomal fractions from each individual were isolated by the method of Ceppa et al. (1997). Nonetheless, there is a lack of information concerning which P-450 subfamily is involved primarily in MON metabolism. A study therefore was undertaken to define further the in vitro microsomal metabolism of the ionophore and to identify the P-450 isoform(s) concerned with its oxidative biotransformation. Investigations also were carried out on the ability in affecting the rate of the in vitro metabolism of MON displayed by TIAM and other drugs known to give rise to toxic interactions when administered concurrently in vivo.

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Microsomal Incubations. Incubations were carried out at 37°C under air in a Dubnoff water bath with constant shaking (60 cycles/min). Typically, the incubates (1-ml volume) consisted of the following components (final concentrations): 0.1 M Tris-HCl, pH 7.4, 5 mM MgCl2, 0.3 mM NADP, 6.4 mM glucose 6-phosphate, 1 U of glucose-6-phosphate dehydrogenase, and 0.7 to 1.2 mg of microsomal protein according to the source of microsomes. After a preincubation of 3 min, reactions were started by adding MON dissolved in ETOH (15 µl) and stopped after 15 min by the addition of 0.25 ml of 10% ice-cold trichloroacetic acid (w/v). The O-demethylation rate was estimated by measuring the amount of released formaldehyde on an aliquot of clear supernatant according to Ceppa et al. (1997), after the subtraction of appropriate blanks run under the same conditions and containing the solvent alone. When studying the effects of TIAM and other drugs on the rate of MON O-demethylation, appropriate amounts of the test compounds were dissolved in ETOH and added to the mixture; after 3 min of preincubation at 37°C, the reactions were started by adding the ionophore.

Kinetic Analysis and IC50 Determination. Kinetic parameters of MON O-demethylation were determined in DEX microsomes with six different concentrations of the substrate and, namely, 25, 50, 75, 100, 200, and 300 µM. The use of higher substrate concentrations was hindered by the partial precipitation of the ionophore. The rate of O-demethylation was estimated as detailed above. Apparent Km and Vmax values were calculated by using the Prism nonlinear regression data analysis program (Graphpad Software, San Diego, CA). The effects of TIAM (0, 5, 10, and 25 µM) on this enzyme activity were investigated in DEX microsomes with three different concentrations of MON (0.25, 0.1, and 0.025 mM). The reciprocal of MON O-demethylation rate was plotted against tiamulin concentrations (Dixon plot), and the apparent K50 was estimated. The IC50 was determined in DEX microsomes at a fixed substrate concentration (0.25 mM) from a plot of remaining MON O-demethylation activity versus logarithm of TIAM concentrations up to 250 µM. The resulting data were analyzed by nonlinear regression, and IC50 was calculated by using Prism software (Graphpad Software).

P-450 Determination. P-450 content was estimated as the carbon monoxide-dithionite reduced-difference spectrum by using an extinction coefficient of 90 mM cm−1 (Rutten et al., 1987).

Thin-Layer Chromatography (TLC). Incubations were performed essentially as described for the assessment of O-demethylation, except for microsomal protein content (1 mg in all cases) and a longer incubation time (30 min). The reaction was quenched with 2 ml of dichloromethane, and samples thereafter were placed in an ultrasonic water bath for 10 min, mixed, and centrifuged at 1,500 g for 10 min. The organic phase was decanted, whereas the aqueous one was subjected to a further extraction with 1 ml of dichloromethane. The extracts were combined and evaporated to dryness, and the residues were dissolved in 100 µl of ETOH. Five microliters of the ethanolic solution was applied on an HF 254 Silica Gel plate (Merck, Darmstadt, FRG). Plates were developed in an equilibrated chamber by means of a solvent system consisting of ethyl acetate/ETOH/water (70:30:3, v/v/v). Spots were visualized by reaction with acetic acid as described by Donoho et al. (1978). In this respect, it should be noted that, although the standards of MON metabolites were not available, both the parent compound and the main biotransformation derivatives can be revealed by acid vanillin (Donoho et al., 1978). Therefore, spots other than those corresponding to MON standards will be referred to generically as “metabolites” in the following sections.

HPLC. The extent of substrate disappearance was taken as a further index of the rate of MON microsomal metabolism. Incubation conditions were as described above (O-demethylation paragraph), except that MON was dissolved in methanol and the incubation time was 30 min. Heat-inactivated microsomes (10 min in boiling water) were used as the enzyme source for blanks. Reactions were carried out by the addition of 1 ml of methanol, and tubes were placed in an ultrasonic water bath for 5 min. Samples thereafter were fortified with the external standard (0.5 mM naringin, final concentration) and filtered (0.8 µm; Schleicher & Shuell, Germany). A sample aliquot then was injected directly
Materials and Methods

Microsomal suspensions (DEX microsomes) were diluted in 0.1 M Tris, pH 7.4, to a protein concentration of 1.5 mg/ml and divided into a sample and a reference cuvette, which were placed in a UVIKON 941 spectrophotometer (Kontron, Milan, Italy) at 35°C. After a baseline was recorded between 500 and 370 nm, a 20 μM TAO (final concentration) was used, which included an RP C18 column Licrospher 100 5 μm (250 × 4 mm), an internal pump to deliver the mobile phase (methanol/water/acetic acid, 94:6:0.1, v/v/v) and an external pump Varian 9001 to deliver the vanillin reagent, both operating at a flow rate of 0.7 ml/min, and a UV detector set at 520 nm.

Influence of MON on TAO-Mediated Complex Formation with P-450. Microsomal metabolism of MON was estimated further into HPLC. Analyses were performed essentially with the method of Rodewald et al. (1994), which is based on the postcolumn derivatization of the ionophore in a reaction chamber at 90°C, by means of a vanillin reagent (methanol/sulfuric acid/vanillin, 95:2:3, v/v/v). A Varian LC 5005 liquid chromatograph was used, which included an RP C18 column Licrospher 100 5 μm (250 × 4 mm), an internal pump to deliver the mobile phase (methanol/water/acetic acid, 94:6:0.1, v/v/v) and an external pump Varian 9001 to deliver the vanillin reagent, both operating at a flow rate of 0.7 ml/min, and a UV detector set at 520 nm.

Results

Rate of O-Demethylation in Microsomes from Untreated or Induced Rats. As depicted in Fig. 2, the highest O-demethylation rate was detected in DEX microsomes (3.27 ± 0.19 nmol formaldehyde/min/mg protein) followed by PCN and PB microsomes, whereas microsomes from untreated (UT) or ETOH- or βNAF-pretreated rats were found far less active in this respect. When expressing data as means ± S.E. of two separate incubations performed in triplicate. Open columns denote the specific activity, and solid columns represent the turnover number.

Assays were performed as indicated in Materials and Methods. Values are means ± S.E. of two separate incubations performed in triplicate. Open columns denote the specific activity, and solid columns represent the turnover number.

three spots from PCN or PB incubates, two spots from UT incubates, and only one spot from βNAF incubates. As judged by eye, the size of the spots pertaining to MON metabolites was clearly greater, at least in lanes related to DEX incubates.

HPLC. The microsomal metabolism of MON was estimated further by evaluating the amount of consumed substrate. UT microsomes metabolized about 4% of the added substrate, whereas the rate of metabolism of microsomes from DEX-pretreated rats was almost 10-fold greater. Intermediate values were obtained from PCN microsomes and PB microsomes, respectively (Table 1).

Influence of MON on TAO-Mediated Complex Formation with P-450. Upon the incubation of TAO (20 μM) with DEX microsomes followed by NADPH addition, a characteristic absorption peak appeared at 456 nm and developed fully after 20 min (Fig. 4A), the addition of MON before TAO and NADPH clearly inhibited the formation of TAO-P-450 complex (Fig. 4B); in particular, the amount of complex averaged 0.47 ± 0.06 nmol/mg protein with TAO only and 0.05 ± 0.01 nmol/mg protein with MON + TAO.

Kinetics of MON O-Demethylation. Because the results mentioned above appeared to indicate that microsomes from untreated rats did not metabolize MON to a significant extent, all the subsequent parameters were determined using DEX microsomes. Preliminary investigations (data not shown) revealed that the formaldehyde formation was linear with respect to protein content (0.5–1.5 mg/ml) and time of incubation (0–15 min). Based on these data, Vmax and apparent Km were estimated using 1 mg/ml protein and an incubation time of 10 min. According to nonlinear regression analysis (Fig. 5), the
apparent $K_{\text{m}}$ and $V_{\text{max}}$ were 4.75 ± 0.6 mmol formaldehyde/min/mg protein and 67.6 ± 0.01 μM, respectively; the reaction was found to fit monophasic Michaelis-Menten kinetics. This was confirmed by the good linearity displayed by the Eadie-Hofstee plot (Fig. 5, inset).

**Influence of Tiamulin and Selected Drugs on MON O-Demethylation.** Taking into account the importance of TIAM in the generation of toxic interactions in veterinary practice, the effects of graded concentrations of this antibiotic (0, 5, 10, 25, 50, 62.5, 125, and 250 μM) on MON O-demethylation were examined using DEX microsomes. TIAM caused a concentration-related inhibition of this reaction (Fig. 6) with a calculated IC$_{50}$ of about 25 μM. The inhibitory mechanisms of TIAM on MON O-demethylation were investigated by means of a Dixon plot (Fig. 7). Plots related to $1/V$ versus the TIAM concentrations showed linear correlations ($r > 0.93$), and, from the common intersection point, an approximate value of $K_{\text{i}}$ (8.2 μM) was calculated. Moreover, because the lines intersected in the second quadrant, a competitive inhibition mechanism could be suggested (Dixon and Webb, 1984).

The effects on the rate of MON O-demethylation of a number of drugs, such as KCZ, MET, α-naphthoflavone (αNAF), CAF, or SMZ, were finally tested at a concentration corresponding to the calculated IC$_{50}$ for TIAM (25 μM). As shown in Fig. 8, KCZ (−95%) and MET (−86%) depressed the rate of reaction to a greater extent than TIAM, αNAF and CAF were far less active in this respect, and no inhibition was observed with SMZ.

**TABLE 1**

Total MON metabolism (% of metabolized substrate/mg protein × 30 min) in liver microsomes from rats untreated or pretreated with PB, PCN, or DEX.

Microsomes, buffer containing an NADPH generating system, and 0.25 mM MON were incubated for 30 min; reactions were stopped with methanol, and the amount of MON was determined by HPLC as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Source of Microsomes</th>
<th>Extent of MON Metabolism (%)</th>
</tr>
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<tbody>
<tr>
<td>UT</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td>PB</td>
<td>11.4 ± 0.2</td>
</tr>
<tr>
<td>PCN</td>
<td>20.8 ± 0.2</td>
</tr>
<tr>
<td>DEX</td>
<td>42.9 ± 1.3</td>
</tr>
</tbody>
</table>

Values are means ± S.E. of four to six incubations.
Discussion

Results from this study provide evidence for the main involvement of P-450 3A in the oxidative metabolism of MON in rat hepatic microsomes.

As a first line of evidence, the in vivo pretreatment with DEX or PCN, which are reported to induce, respectively, P-450 3A1 and 3A2 in rat liver (Debri et al., 1995), resulted in the maximal rate of microsomal MON O-demethylation as assessed by the measurement of the released formaldehyde. By contrast, microsomes from rats pretreated with PB, a relatively weaker inducer of P-450 3A1 and 3A2, metabolized the ionophore at a rate of about one-third of that recorded in DEX microsomes. In line with the prevalent involvement of P-450 3A in the ionophore metabolism, an even more pronounced difference was recorded between DEX or PCN microsomes and PB microsomes when expressing the rate of MON O-demethylation in terms of turnover numbers. Microsomal fractions in which P-450 2E1 or P-450 1A1, 1A2, and 2A1 were overexpressed after ETOH or βNAF in vivo administration (Paine, 1991) did not display significant differences in the ability of carrying out MON O-demethylation with respect to microsomes from untreated rats.

As a second line of evidence, the extent of either metabolite production as detected by TLC, or of total MON metabolism, as measured by HPLC, was most pronounced in microsomes from rats pretreated with selective P-450 3A inducers. In the former case, the number and/or size of the spots that could be visualized by vanillin spraying revealed remarkable qualitative and quantitative differences in metabolite production among the different sources of microsomes. On the whole, the MON-metabolizing capacity was DEX > PCN > PB >> UT > βNAF microsomes, thereby matching the results obtained by measuring the rate of O-demethylation. Accordingly, DEX or PCN microsomes were the most active in total MON metabolism and showed about 10 (DEX) or 5 (PCN) times more activity than UT microsomes, whereas PB microsomes exhibited relatively lower activities. Moreover, a good quantitative correlation was found between these data and those arising from the measurement of the amount of released formaldehyde. It therefore may be concluded that the increase in the total in vitro metabolism of the ionophore achieved by the in vivo pretreatment with P-450 3A inducers most likely reflects the production of O-demethylated metabolites, which represent the most abundant biotransformation derivatives in the rat after the in vivo exposure to MON (Donoho et al., 1978).

The macrolide antibiotic TAO is long known to undergo a P-450 3A-mediated N-demethylation, eventually leading to the formation of stable metabolic complexes absorbing at 456 nm that are best detected with microsomes from rats treated with specific P-450 3A inducers such as DEX or PCN (Bensoussan et al., 1995). In our study, when 100 μM MON was added before 20 μM TAO to a microsomal suspension from DEX-pretreated rats, the amount of 456 nm absorbing complex was reduced drastically (−90%) with respect to that obtained with TAO only. This finding is consistent with results concerning the in vitro metabolism of the ionophore mentioned above and stresses further the role played by P-450 3A in MON oxidative biotransformation.

TIAM, a semisynthetic derivative of the diterpene antibiotic pleu-rotumulin, is used widely in veterinary practice, being active against Mycoplasma spp., Staphylococci, Streptococci, Treponema, and other Spirochetae (Högenhauer, 1979). It finds application mainly in swine dysentery and enzootic pneumonia as well as in several Mycoplasma infections of chickens and turkeys. As mentioned above, the simultaneous administration of MON and TIAM gives rise to toxic interac-
tions in pigs (Umemura et al., 1985; Van Vleet et al., 1987), chickens (Hillbrich and Trautwein, 1980; Frigg et al., 1983), and turkeys (Horrox, 1980; Weisman et al., 1980). It first was hypothe-
sized that the simultaneous administration of both drugs could induce a slower degradation and elimination of the ionophore (Meingassner et al., 1979). Some years later, Laczay et al. (1990) postulated that TIAM administration could result in an enhancement of the oxidative metabolism of MON and, hence, in the generation of toxic and/or reactive metabolites, because the combined administration of MON and TIAM in chickens produced a remarkable increase in hepatic P-450 content, in the activity of several monoxygenases, as well as in the formation of thiobarbituric acid-reactive substances (TBARS). In this respect, it should be noted that the addition of MON to a medium containing microsomes from PB-induced rats and an NADPH-regenerating system failed either to affect P-450 content and its spectral characteristics (Cepa et al., 1997) or to increase the NADPH-mediated microsomal generation of TBARS (unpublished observations). It has been reported recently that, upon the in vitro incubation with pig liver microsomes, TIAM proved to form stable,
metabolic intermediate complexes with P-450 3A, thereby inhibiting a number of P-450 3A-mediated biotransformations, with $K_{i}$ values ranging from 7 (6β-hydroxylation of testosterone) to 9 μM (N-demethylation of ethylmorphine) when using microsomes from rifampicin-induced pigs (Witkamp et al., 1995). In line with those results, we found that TIAM inhibits microsomal MON O-demethylation in DEX microsomes, apparently in a competitive manner, showing a $K_{i}$ (8.2 μM) almost superimposable to those mentioned above but several times lower than the apparent $K_{m}$ of MON O-demethylase (67.6 μM).

These findings suggest that both drugs most likely compete for the same P-450 isoenzymes (3A) but with quite different substrate affinities, so that relatively small TIAM concentrations can significantly impair the microsomal metabolism of the ionophore.

Results concerning the ability of various molecules in affecting the rate of microsomal MON O-demethylation provided indirect evidence of the involvement of P-450 3A in the in vitro metabolism of the ionophore. Indeed, when assayed in DEX microsomes at a concentration equal to the $IC_{50}$ value displayed by TIAM (25 μM), either KCZ, a selective P-450 3A inhibitor (Eagling et al., 1998), or MET, which is capable of binding both P-450 2B1 (Jonen et al., 1982) and KCZ, a selective P-450 3A inhibitor (Eagling et al., 1998), or MET, respectively. Among these drugs, the relatively unspecific P-450 inhibitor CRK (Kraner et al., 1994) exhibited a lower inhibition potency than TIAM, which, as mentioned above, can specifically bind P-450 3A. SMZ, which is not a P-450 inhibitor but a substrate of the constitutive P-450 2C11 in the male rat (Witkamp et al., 1993), did not affect the rate of MON O-demethylation. These results indicate that TIAM and, to a lesser extent, CRK, may interact with MON by lowering the rate of the hepatic microsomal metabolism of the ionophore, leading in vivo to a probable accumulation of the unmetabolized ionophore and, hence, to toxicosis. This mode of action could be reasonably excluded for SMZ, which apparently interferes with MON at another level.

The majority of our studies were performed using microsomes obtained from DEX-induced rats, because MON is not metabolized efficiently by uninduced preparations. Therefore, we cannot exclude that other subsets of constitutively expressed P-450s may be involved in the metabolism of the ionophore. Nonetheless, the results presented here support the concept that the P-450 3A subfamily plays a major role in the in vitro metabolism of MON in rat hepatic microsomes. Further work is in progress to verify whether this also could be the case in food-producing species. Moreover, although care should be taken in extrapolating data from in vitro experiments to living animals and from one species to another, it may be reasonably inferred that drugs capable of being either substrates of P-450 3A and/or inhibitors of P-450-mediated biotransformations would be expected to develop toxic interactions with MON and possibly other ionophores in target species. The P-450 3A subfamily is the major P-450 enzyme present in human liver and small intestine and is involved in the metabolism of approximately half of the drugs on the market (Ueng et al., 1997).

In this respect, the possibility that humans could ingest food of animal origin containing residues of MON or other ionophores should not be excluded a priori and should be avoided to prevent the onset of eventual drug-drug interactions.

References