Effect of Ivermectin on Induction of Cytochromes P450 in Male Rats

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Effects of the widely used antiparasitic drug ivermectin on cytochromes P450 were tested in adult male rats. Liver microsomes were prepared from animals after single or repeated treatments with various doses of ivermectin. The hepatic microsomal protein content and activities of cytochromes P450 towards alkoxyresorufins and testosterone as the specific substrate probes were measured and the results were compared with the data obtained in control animals. The therapeutic dose caused only a nonsignificant increase of the content of microsomal proteins and the activities under study, while the treatment with the higher doses led to significant increases of the concentrations of microsomal proteins and activities of 7-methoxyresorufin *O*-demethylase and 7-ethoxyresorufin *O*-deethylase. The results indicate the potential of ivermectin to induce the expression of cytochrome P4501A isoenzymes, which are known to be the biotransformation enzymes responsible for metabolic activation of promutagenes.

The generic name ivermectin has been assigned to the mass mixture of the 22,23-dihydroavermectines – H_2B_{1a} and H_2B_{1b} (w_r = 80 : 20) used widely as an antiparasitic drug with a broad spectrum of activity against several species of nematodes, arachnids, and insects parasitizing in farm animals, cloven-hoofed game, and several other species of animals [1]. The ivermectin is administered in animals parenterally (injections, pour-on solutions) or orally (granulate, paste, tablet). Based on the type of dosage form single or repeated doses are used. Its world-wide acceptance in health care of food and companion animals has made it a major commercial success. The ivermectin has also been found to be an effective microfilaricidal agent in human medicine [2].

The presence of drug residues in food products of animal origin depends on pharmacological properties of the drugs as well as on the physiological specificities of the species and breed of food animals [3]. Our previous measuring of residual concentrations in cloven-hoofed game treated orally with ivermectin yielded a rather unexpected result in that the withdrawal period after repeated administration of therapeutic doses (6 days) was shorter in comparison with a single administration [4]. To elucidate these findings, our investigations were focused on possible effects of ivermectin on activities of drug-metabolizing enzymes, since the length of the withdrawal period is determined mainly by the drug biotransformation pathways. The activities of the biotransformation enzymes can be influenced by many factors, the most important of which is enzyme induction, *i.e.* an increase in the expression of the gene encoding the enzyme as a response to the presence of a xenobiotic in the organism [5—7]. Ivermectin is a highly lipophilic substance with a long half-time period. This property indicates the possibility that ivermectin acts as an inducer of biotransformation enzymes.

The induction or suppression of drug-metabolizing enzymes is one of the important consequences of administration of pharmaceuticals. Possible induction should be tested carefully in all drugs prior to their release for clinical testing. Changes in patterns of drug biotransformation isozymes may essentially alter physiological processes and pharmacological or toxicological impacts of medication or exposure to environmental contaminants [7, 8].

Cytochromes P450 play the key role in the biotransformation of ivermectin [9]. In this study, 7-alkoxyresorufins and testosterone were used as the

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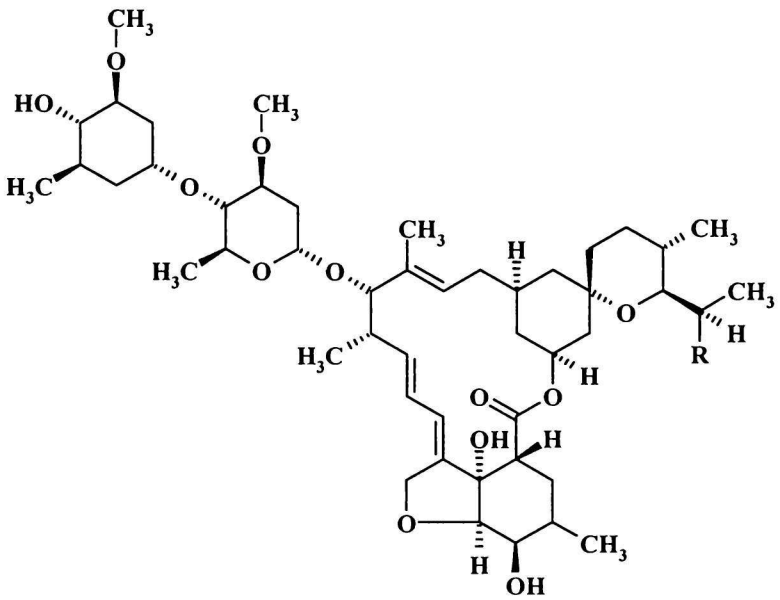

H_2B_{1a} R = CH_2CH_3

H_2B_{1b} R = CH_3

specific substrate probes to assess the possible influence of ivermectin administered in therapeutic or excessive doses on the activities of cytochrome P450 isozymes in rats.

EXPERIMENTAL

Ivermectin (IVOMEC inj., 50 cm^3, HC 30490) was purchased from MSD Agvet, Prague, Czech Republic. 7-Methoxy-, 7-ethoxy-, 7-pentoxy-, and 7-benzyloxyresorufins were obtained from Molecular Probes (Eugene, MI, USA), and the other specific substrates and products were supplied by Sigma. All the other chemicals were of the highest purity available commercially.

Animals and Preparation of Subcellular Fractions

Male adult rats (*Rattus norvegicus* var. alba, mass approximately 275 g) were obtained from the Research Institute for Pharmacy and Biochemistry (Prague, Czech Republic). The 16 animals were divided into four groups. The control group received six doses of water, group A was treated with a single dose of 35 mg of ivermectin in water suspension per 1 kg of rat body mass (35 mg kg^{-1}), group B with six therapeutic doses of 0.3 mg of ivermectin in water suspension per 1 kg of rat body mass (0.3 mg kg^{-1}), and group C with six successive doses of 10 mg of ivermectin in water suspension per 1 kg of rat body mass (10 mg kg^{-1}). All the animals were treated orally, fed on a standard diet with a free access to drinking devices, fasted 12 h before the end of the experiment, and sacrificed by decapitation under ether anaesthesia. Livers were collected and homogenized at $\rho = 0.3$ g cm^{-3} (1 g of liver tissue per 3 cm^3 of buffer) in 0.1 M sodium phosphate buffer, pH 7.4, using the Potter—Elvehjem homogenizer. The microsomal fraction was separated by ultracentrifugation of the liver homogenate as described [10, 11].

Enzyme Assays

The 7-ethoxyresorufin (EROD), pentoxyresorufin (PROD), methoxyresorufin (MROD), and benzyloxyresorufin (BROD) *O*-dealkylase activities were determined using fluorimetric determination of resorufin [12] at 30 °C with the final concentrations of the substrates 2 μmol dm^{-3}. The assays were conducted using the Perkin—Elmer luminescence spectrophotometer LS 50B with the excitation and emission wavelengths of 530 nm and 585 nm, respectively. The EROD, PROD, MROD, and BROD activities were calculated using the standard amount-addition technique.

The hepatic microsomal testosterone hydroxylase (TOH) activities were assayed using essentially the method according to [13]. The reaction mixture (total volume 1 cm^3) contained 0.1 cm^3 of microsomes, 10 mm^3 of solution of testosterone in methanol ($c = 25$ mmol dm^{-3}), 10 mm^3 of NADPH (103 mg cm^{-3} water) in 0.01 M phosphate buffer, pH 7.5. After 15 min of incubation at 37 °C, the reaction was stopped by the addition of 6 cm^3 of dichloromethane. After shaking

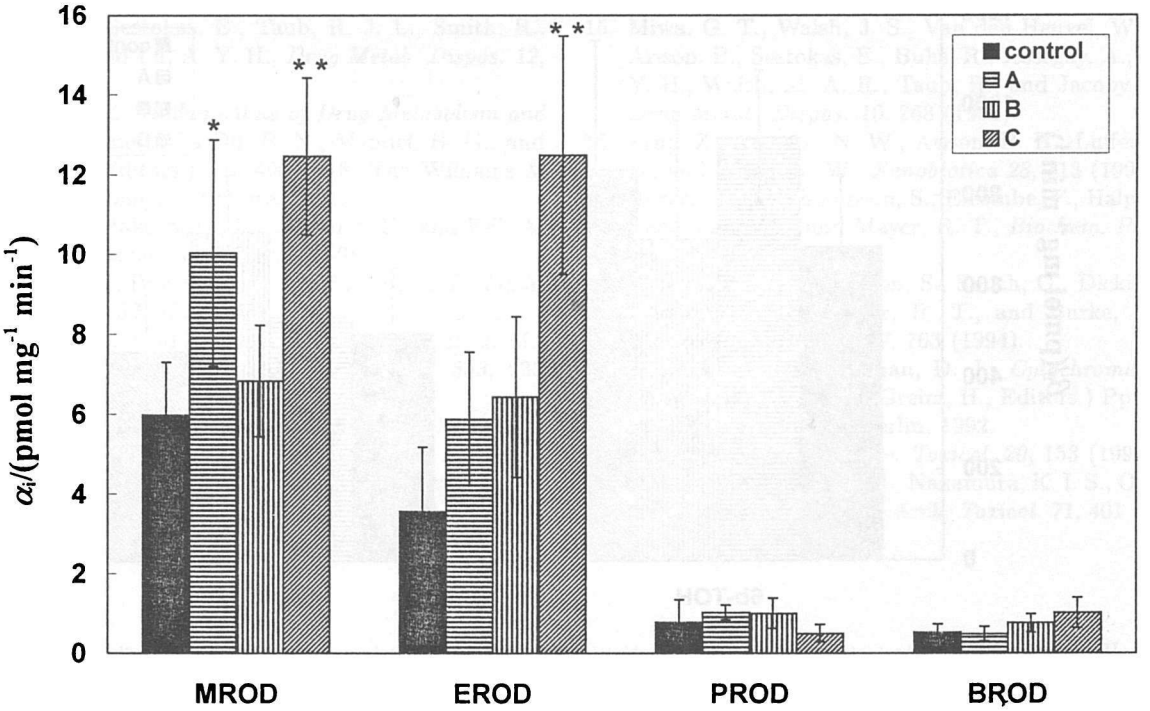

Fig. 1. Specific CYP activities α_i towards 7-alkoxyresorufins in hepatic microsomes of control or ivermectin-treated rats (A, single dose 35 mg kg^{-1}; B, six successive doses of 0.3 mg kg^{-1}; C, six successive doses of 10 mg kg^{-1}). Activities expressed as $\alpha_i = (n(\text{resorufin})/(m(\text{protein})\ t))/(\text{pmol mg}^{-1}\ \text{min}^{-1})$, $n = 4$, $^*p < 0.05$, $^{**}p < 0.01$.

and centrifugation, the organic phase was separated and dried. The samples were dissolved in a methanol—water solution ($\varphi_r = 1 : 1$) and the products were analyzed using a Waters HPLC system (NovaPak C18 column, 4 μm). The mobile phase consisted of 20 % methanol in 0.04 % acetic acid (A) and methanol with 0.04 % acetic acid (B); the gradient of the mobile phases was 15—40 % B in 33 min.

Protein concentrations were assayed using the bicinchoninic acid method [14].

RESULTS AND DISCUSSION

Long-term or repeated interactions of drugs or other xenobiotics with live organisms can result in the induction of enzymes, particularly those participating in the metabolism of the inducing agent. Investigations of the metabolism of ivermectin in microsomes of rats and steers [15] and swine [9] have identified 24-hydroxymethyl and de-3-*O*-methyl derivatives as the major metabolites. The structure of ivermectin and its major metabolites let us assume that cytochromes P450 (CYP) play the key role in its metabolism. Cytochrome P4503A4 (CYP3A4) has been identified as the predominant enzyme responsible for the metabolism of ivermectin in human liver microsomes [16].

The induction of the CYP isoenzymes can be assessed by measuring the enzyme activities towards specific substrates *in vitro*. In this study the effects of oral administration of ivermectin on hepatic microsomal CYP activities were investigated in control rats and in rats treated with a single dose (group A), repeated therapeutic doses (group B), and repeated 30-fold higher doses (group C).

Table 1. The Proteins Content in Rat Microsomes from Control Animals or Ivermectin-Treated Animals

Rat group	ρ^*(microsomal proteins) ± SD/(mg cm^{-3})
Control	8.98 ± 0.85
A	9.93 ± 0.97
B	7.88 ± 0.60
C	11.20 ± 0.84

*Average value of four experiments.
A = single dose 35 mg kg^{-1}, B = repeated doses 6 × 0.3 mg kg^{-1}, C = repeated doses 6 × 10 mg kg^{-1}.

Microsomal protein concentrations were determined as a rough prescreening marker of induction. A significant increase of the protein content was found in microsomes of animals of group C treated repeatedly with 10 mg of ivermectin per 1 kg of rat body mass (Table 1).

The activities towards substrates relatively specific for CYP isoenzymes were studied to evaluate possible effects of ivermectin on cytochromes P450. The following specificity patterns have been found for 7-alkoxyresorufins used as substrate probes for the determination of activities of individual CYP isoen-

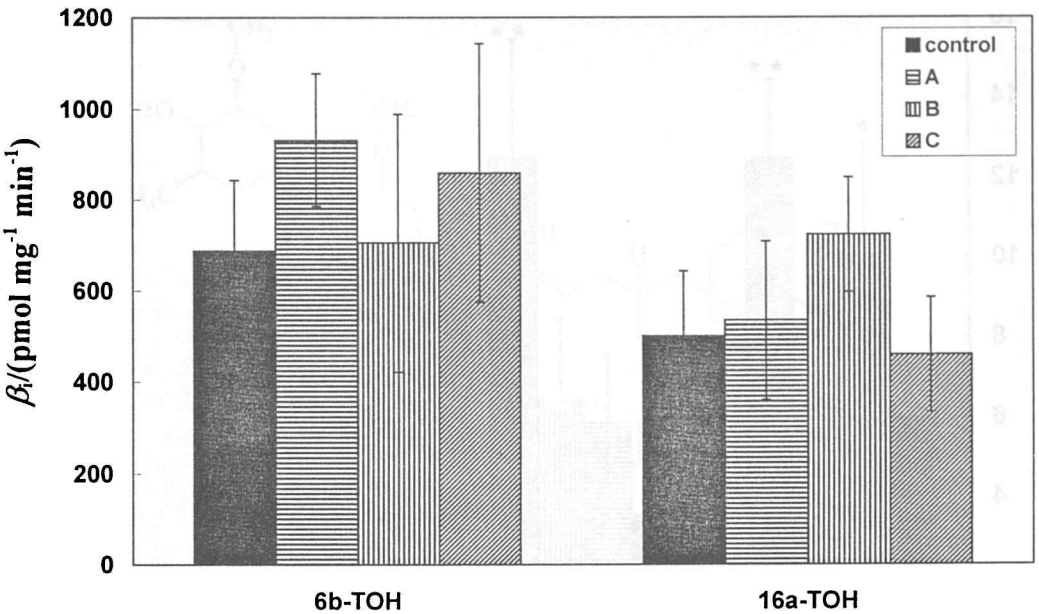

Fig. 2. Hepatic microsomal testosterone hydroxylase activities β_i in control and ivermectin-treated rats. 6b-TOH, testosterone 6β-hydroxylase activity; 16a-TOH, testosterone 16α-hydroxylase activity; for treatment and other abbreviations see Fig. 1. Activities expressed as $\beta_i = (n(\text{product})/(m(\text{protein})\ t))/(\text{pmol mg}^{-1}\ \text{min}^{-1})$, $n = 4$.

zymes [17, 18]: EROD is specific for CYP1A1 and less for CYP1A2; MROD is specific for CYP1A2 and less for CYP1A1; PROD is specific for CYP2B isoenzymes; BROD is specific for CYP3A and partly for CYP1A1 and CYP1A2. Therapeutic doses of ivermectin in rats induced only a nonsignificant increase of EROD and partly also the BROD activities, but the administration of the higher doses in groups A and C resulted in a significant induction of the MROD and EROD activities. The induction pattern of *O*-dealkylase activities is presented in Fig. 1.

Hepatic microsomal testosterone hydroxylations were not significantly influenced by ivermectin (Fig. 2). Testosterone 6β-hydroxylase and 16α-hydroxylase activities have been reported to be mainly associated with the CYP3A and CYP2 enzymes, respectively [19].

Our results indicate the ability of high doses of ivermectin to stimulate the expression of the CYP1A, but not of the CYP2B or CYP3A forms in rats. Cytochromes P4501A are the major isozymes involved in the biological activation of various environmental pollutants including procarcinogens, such as polycyclic aromatic hydrocarbons and arylamines [7]. The induction of CYP1A is obviously associated also with a complex of other toxic effects, such as activation of protein kinase c cascade and increased mitosis and cell proliferation [7, 20]. Therefore, exposure to high doses of ivermectin may increase the risk of mutagenesis and cocarcinogenesis. However, any extrapolation of the data obtained in rats to cattle and deer must be rather reserved taking into consideration species-specific differences in the quantity, activity, substrate specificity, and inducibility of biotransformation enzymes [8, 21]. Moreover, very little is known currently of the characteristics of biotransformation enzymes and their inducibilities in ruminants [3]. Anyhow, our results can be regarded as a warning that ivermectin can induce CYP activities, and underline the utility of continuing analogous studies in target animal species.

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