SPECIES DIFFERENCES IN METABOLISM OF PANOMIFENE, AN ANALOGUE OF TAMOXIFEN

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ABSTRACT:
In vitro metabolism of panomifene (E-1,2-diphenyl-1-[4-(2-[2-hydroxyethyl-amino]-ethoxy)-phenyl]-3,3,3-trifluoropropene; panomifene-epoxide, E-1,2-epoxy-1,2-diphenyl-1-[4-(2-amino-ethoxy)-phenyl]-3,3,3-trifluoropropene; M1, M2, M3, M4, M5, M6, M7, M8, M9) were produced exclusively by the dog. The structure of M3 was identified by mass spectroscopy as 4-hydroxy-panomifene. Furthermore, human liver microsomes formed a metabolite (M8) that was not detectable in the mixtures with mouse, rat, or dog microsomes. Its structure is suspected to be an oxidized form of panomifene with a double bond in the side chain.

The structure of panomifene is analogous to tamoxifen, an antagonist currently used as a therapeutic agent against breast cancer, and there are some similar routes in their metabolism. The main difference is that the rate of tamoxifen biotransformation seems faster than that of panomifene. On the other hand, 4-hydroxy-panomifene is produced by only dog, while 4-hydroxylated derivative is one of the main metabolites of tamoxifen that has potent antiestrogenic activity and is considered to be responsible for the formation of DNA-adducts.

Materials and Methods

Chemicals. Panomifene was provided by EGIS Pharmaceuticals Ltd. (Budapest, Hungary).

Tamoxifen is a nonsteroidal estrogen receptor antagonist for the treatment of breast cancer, particularly in postmenopausal patients. Although the parent compound has potent antitumorigenic activity related to its antiestrogenic effect, it is increased by para-hydroxylation of the phenyl ring on carbon 1 of butene, resulting in the formation of 4-hydroxy-tamoxifen. It should be mentioned that the metabolic activation of tamoxifen is an advantage rather than a requirement for antiesrogenic activity (1). Furthermore, tamoxifen is metabolized extensively in the liver into several more products: tamoxifen N-oxide, N-desmethyl-tamoxifen, and 4-hydroxy-tamoxifen, but they do not seem to have potent biological activity (2, 3). N-Desmethyl-tamoxifen is mainly produced by CYP3A, but CYP1A enzymes also participate in demethylation step to some extent (4, 5), whereas the formation of the active metabolite, 4-hydroxy-tamoxifen, is catalyzed by CYP2C9, CYP2D6, and CYP3A4 in humans, resulting in quite high individual variability (6). Additional studies showed that tamoxifen and its main metabolites inhibit CYP3A (ethylnitrophen and aminopyrine N-demethylation) in vitro. Moreover, tamoxifen can also inhibit its own metabolism (7). On the other hand, the induction of CYP2B and CYP3A enzymes was observed in tamoxifen administered rats (8).

Although tamoxifen is the major drug used in the treatment of breast cancer, in rare cases it seems to increase incidence of endometrial and liver tumors in humans (9–11) and to induce hepatocellular carcinoma and mammary tumors in rats and ovarian tumors in mice (12–14). 4-Hydroxy-tamoxifen may be important in the activation of species that form DNA adducts (15). Because of these undesirable side effects, efforts are being made to develop new therapeutics with similar or more potent antiestrogenic activity and lower incidence of side effects. Panomifene is a triphenyl-ethylene type antiestrogen and has the alkylaminoethoxy side chain that is essential for antiestrogenic activity. In some respects its structure is analogous to tamoxifen. Panomifene contains a trifluoro-methyl-group instead of an ethyl-group (as tamoxifen) and there is a hydroxy-ethyl-group on the nitrogen, whereas there are two methyl groups on the nitrogen of tamoxifen (fig. 1). The aim of the present study was to reveal panomifene metabolism by mouse, rat, dog, and human liver microsomes and to try to compare the microsomal biotransformation of panomifene and tamoxifen.

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HPLC "A" mobile phase for HPLC analysis. An aliquot (20 µl) was injected onto the chromatograph (Isco, Model 2350 pumps and V4 UV detector) equipped with two solvent systems: "A" acetonitrile:water: 70% perchloric acid 5:5:0.5 (v/v) solvent mixture was eluted at a flow rate of 1.0 ml/min by a gradient method of Lowry (17) with bovine serum albumin as the standard.

**Preparation of Microsomes.** Experiments were carried out by using microsomes prepared from livers of NMRI mice (LATI, Gödöllő, Hungary), Wistar rats (LATI), and Beagle dogs (Institute for Drug Research Ltd., Budapest, Hungary). Human livers were obtained from kidney transplant donors. Permission of Local Research Ethics Committee was obtained to use human tissues. Human livers were perfused with Euro-Collin's solution (Fresenius AG, Bad Homburg v.d.H., Germany), excised, and frozen immediately in liquid nitrogen. Liver microsomes were prepared by differential ultracentrifugation (16). The protein content of microsomes was determined by the method of Lowry et al. (17) with bovine serum albumin as the standard.

**Panomifene Metabolism.** The microsomal metabolism of panomifene was conducted in 0.1 M Tris-HCl buffer (pH 7.4) containing an NADPH-generating system (0.4 mM NADPH, 4 mM glucose-6-phosphate, 2 mM MgCl₂, and 1 unit/ml glucose-6-phosphate dehydrogenase) and 1 mg/ml microsomal protein in a final volume of 2 ml. 0.05 mM [³H]-panomifene (68.8 MBq/µmol) was added and incubated aerobically at 37°C for 90 min. The reaction was stopped by the addition of 5 ml dichloromethane followed by vigorous mixing for 1 min. The extraction procedure was repeated twice more and the organic phases were combined.

**Thin Layer Chromatography of Metabolites.** The organic phase was evaporated to dryness and the residue was redissolved in 0.1 ml methanol for thin layer chromatography. Ten microliters of the samples were analyzed on 0.2-mm thick DC-alufolien Kieselgel 60 F₂₅₄ plates (Merck, Darmstadt, Germany) in chloroform:methanol:25% NH₄OH = 95:5:0.5 (v/v) solvent delivery system. Panomifene and its metabolites were detected and their amounts were calculated on the basis of digital autoradiography (Digital Autoradiograph LB-287, EG&G, Berthold, Wildbad, Germany).

**HPLC Analysis.** After extraction the residue was redissolved in 0.5 ml of HPLC "A" mobile phase for HPLC analysis. An aliquot (20 µl) was injected onto the chromatograph (Isco, Model 2350 pumps and V4 UV detector) equipped with a reversed phase column (Hypersil ODS 5 µm, 200 mm × 4 mm). Components of extracts were eluted at a flow rate of 1 ml/min by a gradient program with two solvent systems: "A" acetonitrile:water: 70% perchloric acid = 40:60:0.1 (v/v); "B" acetonitrile:water: 70% perchloric acid = 75:25:0.1 (v/v). pH of mobile phase was adjusted to 3.0 by the addition of triethylamine. Compounds were detected by UV absorbance at 236 nm.

**Identification of Panomifene Metabolites.** The chemical structures of metabolites produced from panomifene by liver microsomes were identified by mass spectroscopy. Electron impact measurements were carried out on an AEI-902 type (Manchester, UK) mass spectrometer (70 eV, 100 µA, 8 kV direct inlet, 180°C). The structures of the metabolites were confirmed by using panomifene derivatives as the standards.

**Results and Discussion**

**Biotransformation of Panomifene.** Metabolism of panomifene was investigated in hepatic microsomal systems, and the incubation mixtures were analyzed by thin layer chromatography and digital autoradiography or by HPLC with UV detection. The formation of metabolites was linear for up to 90 min, and the metabolites were not detected when microsomes or NADPH-generating system were omitted from the incubation mixture. Fig. 2 shows the thin layer chromatograms of the extracts of incubation mixtures in microsomes from several species (mouse, rat, dog, and human). Seven metabolites were detected on thin layer plates and termed M1 to M7 according to their distance from the start. The rates of metabolite formation by liver microsomes are expressed as nmol/mg protein/90 min (table 1). Biotransformation of panomifene resulted in the formation of hydroxy- and side-chain modified derivatives.

Mouse liver microsomes were rather slow in biotransformation of panomifene; only about 1% of the parent compound was metabolized during the 90-min incubation period. Three metabolites (M2, M6, and M7) produced from [³H]-panomifene were detected on TLC plate by autoradiography (fig. 2), and the rates of their formation seemed to be quite similar. Rf value of M6 was identical with the panomifene derivative that had lost the whole side chain, whereas the mobility of M7 was the same as the panomifene derivative with -(CH₂)₃- OH side chain.

The quantities of each metabolite formed during 90 min by rat liver microsomes varied between 0.4 and 0.6 nmol. Rat produced similar metabolic profile (M2, M6, and M7) as mouse. It should be mentioned that M2 and M7 were observed exclusively during in vitro metabolism by liver microsomes from the rodent species. The structure of M2 has not been determined, but M7 was identified on the basis of its chromatographic and mass spectroscopic behavior. In addition, during the incubation with rat liver microsomes one more metabolite, M5, was separated by TLC and was identified on the basis of Rf values as the panomifene derivative that had lost the hydroxyethyl group. M5 was not formed in detectable amounts by mouse liver microsomes, but it was also produced by dog and human ones.

Some of the metabolites detected in the incubation mixtures with dog liver microsomes (M1, M3, M4, M5, and M6) were quite different from those produced by mouse or rat microsomes. M1, M3, and M4 were exclusively produced by dog liver microsomes. M3 seemed to be 4-hydroxy-panomifene, since it showed the same Rf value as the standard 4-hydroxy derivative of panomifene. Dog microsomes produced quite “high” amounts of M3 (1.94 ± 0.18
nmol/mg protein/90 min) compared with the others. M5 (which had lost the hydroxyethyl group from the side chain of panomifene) and M6 (which had lost the total side chain) were formed not only by rat liver microsomes but by dog and human ones also, although their amounts were very low, about 0.2–0.3 nmol/mg protein/90 min. M7, the metabolite that had a shortened side chain (-O-(CH₂)₂-OH), did not form in detectable amount.

During the analysis by thin layer chromatography, low levels (un-
Mass Spectroscopic Analysis of Panomifene and its Metabolites. On the basis of electron impact measurement, panomifene showed a molecular ion [M+] at m/z 427, and the fragmentation of the side chain led to the main fragment peaks at m/z 396, 340 and 88, 74 (fig. 4). The cleavage of the double bound in the middle of the molecule resulted in fragments at m/z 270 or 239. The latter is considered to be formed by a loss of 31 mass unit, caused by a further fragmentation of the side chain.

The structure of M3 metabolite of panomifene formed in vitro by dog liver microsomes was suspected to be 4-hydroxy-panomifene because its Rf value on TLC plate was the same as the standard 4-hydroxy-panomifene. M3 was isolated from TLC plate, further purified by HPLC, collected, and analyzed by mass spectroscopy. The mass spectrum of M3 (fig. 5) obtained by electron impact mass spectroscopy (EI-MS) led to the peak at m/z 443 as the molecular ion [M+]•, whereas the side chain fragmentation resulted in the peaks at m/z 412 and 356. These fragments proved that the hydroxyethylamino-ethoxy group was metabolically unchanged in M3. The observation that molecular mass of M3 was 16 mass units higher than the

![Fig. 3. Panomifene metabolism by human liver microsomes analyzed by HPLC.](image-url)
Fig. 4. Electron impact mass spectrum of panomifene.

Fig. 5. Electron impact mass spectrum of M3 metabolite produced by dog liver microsomes.
parent compound suggested that it was a hydroxylated panomifene derivative. The fragment peak at m/z 284 characteristic of 4-hydroxypanomifene was formed by the cleavage of the double bond. The electron impact analysis of the standard 4-hydroxy derivative displayed the same M\(^{+}\) and fragments as the isolated M3 metabolite.

M6 was produced by liver microsomes from all the species investigated, and on the basis of R\(_r\) values it seemed to be identical with the standard panomifene derivative that had lost the total side chain. The mass spectrum of M6 (fig. 6) analyzed by EI-MS demonstrated a peak at m/z 340 as molecular ion [M\(^{+}\)] and a fragment at m/z 69 derived from the cleavage of -CF\(_3\). The chromatographic properties and the mass spectroscopic analysis confirm that the structure of M6 is a panomifene derivative with an -OH group instead of long side chain.

The mass spectrum of M7 (fig. 7) showed the molecular ion at m/z...
384 and fragments at m/z 340, 271, and 69. The side chain cleavage led to the fragment peak at m/z 340 and the loss of trifluoro-methyl moiety resulted in the fragments at m/z 271 and 69. The chromatographic properties predicted and the mass spectroscopic analysis confirmed that M7 contained -O-CH₂-CH₂-OH side chain, the same structure as of the standard pano-mifene derivative.

Efforts were made to identify the structure of M8 metabolite produced by human liver microsomes only (fig. 8). The mass spectrum of the isolated and purified compound showed peaks at m/z 427 and 425 that were thought to be identical with pano-mifene and its oxidized form with a double bound in the side chain, respectively. The fragments at m/z 396 and 74 might belong to the parent compound, while the peaks at m/z 394 and 72 might come from the oxidized form. It was not confirmed that the peaks at m/z 427 and 425 represented molecular ions or were the products of thermic decomposition during mass spectroscopic measurement.

Fig. 9 summarizes all our knowledge of biotransformation of pano-mifene revealed by hepatic microsomal system; it contains the identified and unidentified metabolites with species differences.

**Comparison of Pano-mifene and Tamoxifen Metabolism.** The rate of pano-mifene metabolism of laboratory animals and of humans by liver microsomes is slow compared with that of tamoxifen according to Mani et al. (4, 18). With a prediction from the structure, 4-hydroxy-pano-mifene could be expected, as in case of tamoxifen. It was produced by dog liver microsomes, although the microsomes of other species did not form 4-hydroxy-panomifene.

Because the side chain of pano-mifene is different from that of tamoxifen, N-monodealkylated metabolite cannot be expected during metabolism. However, metabolites formed by shortening of the side chain of pano-mifene were detected, and these reactions were similar to the formation of N-didesmethyl-tamoxifen, metabolite Y (-OH instead of dimethylamino-group), and E (-OH instead of the side chain) of tamoxifen (these latter are minor metabolites of tamoxifen) (2). Furthermore, we did not detect the presence of pano-mifene N-oxide.

Ruenitz et al. (19) found that the incubation of tamoxifen with liver microsomes yielded a metabolite, which, although not completely characterized, seemed to be the tamoxifen-epoxide. It was partly confirmed by the results of Mani and Kupfer (20) since a reactive tamoxifen* intermediate was produced by rat and human liver microsomes which bound irreversibly to microsomal protein. Epoxide formation from pano-mifene was excluded, since liver microsomes from none of the species investigated in our study produced a metabolite that showed the same chromatographic behavior as the standard pano-mifene-epoxide.

We can conclude that there are some similar routes of pano-mifene and tamoxifen metabolism (hydroxylation and various side chain shortening reactions) but that the rates of metabolism are different. On
the other hand, many differences were observed in panomifene biotransformation by the laboratory animals and man. The fact that 4-hydroxy-panomifene is formed by only dog microsomes can be an advantage, since in the case of tamoxifen its 4-hydroxylated metabolite is considered to activate the formation of DNA-adducts (15).

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**References**


