***In vivo* effects of antiviral acyclic nucleoside phosphonate 9-[2-(phosphonomethoxy)ethyl]adenine (*adefovir*) on cytochrome P450 system of rat liver microsomes**

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Summary

Interference of antiviral agent *adefovir*, i.e. 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA) with microsomal drug metabolizing system was investigated in rats. The content of total liver cytochrome P450 (CYP) was lowered while that of its denaturated form, P420, was elevated in animals intraperitoneally treated with PMEA (25 mg/kg). Similar effect was observed after treatment with a prodrug of *adefovir*, *adefovir dipivoxil* (bisPOM-PMEA). The CYP2E1-dependent formation of 4-nitrocatechol from *p*-nitrophenol was diminished, though the specific activity of *p*-nitrophenol hydroxylase remained unchanged. PMEA had no influence on expression of CYP2E1 protein and mRNA and mRNAs of other P450 isoenzymes (1A1, 1A2, 2C11, 3A1, 3A2, and 4A1). It may be concluded that repeated systemic administration of higher doses of PMEA results in a partial degradation of rat CYP protein to inactive P420.

Introduction

Acyclic nucleoside phosphonates, synthetic analogues of natural nucleotides, are recognized antivirals inhibiting replication of both retroviruses and DNA-viruses. The major mechanism of their antiviral action is the inhibition of virus-induced DNA polymerases and/or of reverse transcriptases [1].

Many antiviral agents, including both protease inhibitors and reverse transcriptase inhibitors,

have been found to interfere with cytochrome P450 (CYP)-dependent drug-metabolizing system. Themselves, they are often substrates for various CYPs. On the other hand, the CYP enzymes can either be inhibited or induced by antivirals [2]. For example, the non-nucleoside reverse transcriptase inhibitor efavirenz is transformed to inactive metabolites by CYP3A4 and CYP2B6. At the same time, it enhances the activity of CYP3A4 and thus accelerates its own metabolism, as well as the metabolism of other drugs [3].

Concerning PMEA, only limited data are available on its possible involvement in CYP-dependent drug-drug interactions. There are only

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a few findings suggesting the dependence of the metabolic fate of PMEAs ester prodrugs on P450 mediated oxidation of the side chain [4]. The objective of this study was, therefore, to follow the interaction of PMEAs with the CYP system under conditions *in vivo* in rats.

Materials and methods

Compounds

9-[2-(Phosphonomethoxy)ethyl]adenine (PMEA; *adefovir*) and its bis(pivaloxymethyl) derivative (bisPOM-PMEA, *adefovir dipivoxil*) were synthesized in-house (Institute of Organic Chemistry and Biochemistry). The chemical structures of PMEAs and bisPOM-PMEA are shown in Figure 1. *p*-Nitrophenol, 4-nitrocatechol, NADPH sodium salt, phosphate buffered saline, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium dithionite was obtained from Merck Co. (Darmstadt, Germany).

Animals and treatment with PMEAs

Female rats of the Lewis inbred strain were purchased from Charles River Deutschland (Sulzfeld, Germany). Animals were intraperitoneally injected with PMEAs sodium salt (1 ml/kg body weight) on 2, 5, 10 and 18 consecutive days (25 mg/kg, each injection). The animals were sacrificed on day 19, i.e. 17, 14, 9, and 1 day(s), respectively, following cessation of the treatment. BisPOM-PMEA was applied for 10 consecutive days (25 mg/kg, each injection), and rats were sacrificed on day 19, i.e. 9 days following cessation of the treatment. All experimental protocols were approved by the institutional ethics committee.

Preparation of liver microsomes

Care was taken to excise approximately the same parts of liver tissue. They were rinsed in cold 0.25 M sucrose containing 50 mM Tris-HCl (pH 7.4) and blotted dry. Tissue were minced, homogenized in sucrose using a glass Potter Elvehjem homogenizer with a teflon pestle to obtain a 20% (w/v) homogenate, and centrifuged immediately at 9000 *g* (Janetzki K24) for 20 min at 4 °C. After separation of the nuclear and mitochondrial fraction, the supernatant was centrifuged at 105,000 *g* in a Beckman Optima MAX-E ultracentrifuge (Beckman MLS 50Ti rotor) for 60 min to separate the microsomes. The resulting supernatant was discarded and the microsomes were washed in an isotonic KCl solution and centrifuged at 105,000 *g* for 30 min at 4 °C. The microsomal fraction was resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA. Each ml of the suspension contained 2–3 mg of microsomal protein.

Protein determination

Total protein content was determined by bicinchoninic acid method with a standard BCA Protein Assay kit (Pierce, Rockford, IL). Determination was done in two parallels using a calibration curve ($y = 1.0628 + 0.0203x$, $r^2 = 0.996$) within the concentration range of 0–1 mg/ml. Differences between determinations were below 15%.

Cytochrome P450 determination

Content of CYP and its inactive form P420 was determined directly by using an established

Figure 1. Chemical structure of *adefovir*, i.e. 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA) and *adefovir dipivoxil* (bisPOM-PMEA) i.e. (bis)pivaloyloxymethyl ester of PMEAs.

Figure 2. Determination of the optimal number of PCR cycles for semiquantitative RT-PCR analysis of CYP2E1 mRNA expression. RNA extracted from liver of control and *adefovir* treated animals was reverse transcribed and then one fourth of cDNA was subjected to PCR. The number of amplification cycles was increasing from 20 to 30 cycles to test the linear conditions of PCR. Twenty five cycles were chosen for subsequent RT-PCR analysis of other P450 isoenzymes.

CO-difference spectroscopy method [5]. An extinction coefficient of 91/cm/mM for the absorbance difference between 450 and 490 nm (double-beam recording spectrometer Shimadzu UV-2100) was used for CYP determination and the CYP content was expressed as a nmol of cytochrome P450 per mg of microsomal protein (nmol/mg protein). Amount of the inactive P420 was calculated from the change in absorbance between 420 and 490 nm using specific P420 molar extinction coefficient 111/mM/cm.

p-Nitrophenol hydroxylation

The CYP2E1-catalyzed formation of *p*-nitrocatechol generated by the incubation of *p*-nitrophenol with rat liver microsomes was assayed according to the method described by Chang et al. [6] using Shimadzu UV-2100 spectrophotometer. The determination was done using a *p*-nitrocatechol standard with a calibration curve in the range of 0–20 nmol of *p*-nitrocatechol ($y = 0.0122x$ with $r^2 = 0.9985$). The *p*-nitrophenol hydroxylase activity was expressed as (a) nmol product/min/mg microsomal protein, and (b) nmol product/min/nmol total CYP, i.e. specific activity.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The expression of cytochrome P450 mRNA was analysed by a semiquantitative RT-PCR following the procedure described in [7]. Total RNA was extracted from 30 mg liver tissue using the RNeasy minikit and RNase free DNase set according to manufacturer's instructions (Qiagen, Hilden, Germany). 0.5 μ g of total RNA was reverse transcribed to complementary DNA. One fourth

of resulting cDNA was then amplified by PCR. PCR reactions were performed with commercially available specific primers for rat cytochrome P450 enzymes (Takara, Gennevilliers, France). As a control, cDNA was also amplified using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) commercially available primers (Clontech, Palo Alto, CA, USA). After initial denaturation (2 min, 94 °C), 25 amplification cycles were performed using a thermocycler Mastercycler gradient (Eppendorf, Hamburg, Germany). One cycle consisted of denaturation at 94 °C for 30 s, annealing at 56 °C (CYP primers) or 61 °C (GAPDH primers) for 30 s and extension at 72 °C for 30 s. After the last cycle, final extension at 72 °C for 7 min was carried out. The amplified DNA size was 450 bp for GAPDH and 331, 236, 248, 474, 579, 116 and 344 bp for CYP1A1, 1A2, 2C11, 2E1, 3A1, 3A2 and 4A1 enzymes, respectively. PCR products of predicted size were identified by electrophoresis on 1.5% agarose gel containing ethidium bromide. The gels were photographed in UV light. The results were interpreted on the basis of procedure described by Morris and Davila [7]. The method gave single products within a linear range of 20–30 cycles; determination of optimal number of PCR cycles for quantification yielded results shown in Figure 2.

Western blotting

The relative extent of CYP2E1 protein expression in microsomes was estimated by Western blotting. The BioRad (Hercules, CA) MiniProtean apparatus with Western blotting accessory was used. Chemiluminiscence kit for Western blotting (Immun Star) was purchased from BioRad and the nitrocellulose membrane was from Amersham

Biosciences (Little Chalfont, GB). Rat anti human CYP2E1 antibody was from Daichi Pure Chemicals (Tokyo, Japan). Western blots were quantitated using a standard curve with CYP content over a linear portion of the response curve (generally with 0.1–1.0 pmol of CYP) generated by scanning the blot with a computerized image-analyzing system (IMSTAR, Paris). Change of protein content in a spot was considered significant when the respective difference exceeded 20%. A recombinant CYP2E1 (Panvera-Invitrogen, supplied by KRD, Prague, CZ) was used as a standard.

Data analysis

The data were evaluated by means of one-way ANOVA and consequent Dunnett's multiple comparison test, using the Prism program (GraphPad Software, San Diego, CA).

Results

Compared to untreated control rats, no differences in body weight gain and relative liver weight were observed after repeated administration of PMEA. The content of total microsomal protein in control animals reached the value of 16.06 ± 0.63 mg/g of liver. It remained virtually unchanged irrespective of the length of PMEA treatment, including the group receiving 18 daily injections ($F_{(4,18)} = 2.38$, $p = 0.54$). Prolonged treatment with PMEA led to significantly depressed concentration of cytochrome P450 per mg of microsomal protein. On the other hand, the amount of microsomal P420 concentration was increased (Figure 3). Starting to be apparent with five daily injections onwards, the effects were statistically significantly correlated (Pearson $r = 0.985$, $p < 0.05$). The same effect on increased content of inactive P420 form was observed after administration of bisPOM-PMEA (Figure 4). Full recovery of CYP to its native P450 form has not been reached even within the interval of 28 days following cessation of PMEA treatment (Figure 5).

The activity of CYP2E1, *p*-nitrophenol hydroxylation, was determined to evaluate the effect of the active form of the drug, adefovir, to a specific CYP activity. Substantially inhibited production of 4-nitrocatechol from *p*-nitrophenol was found

Figure 3. Effect of repeated treatment of rats with PMEA (25 mg/kg, i.p.) on content of microsomal cytochromes P450 and P420. The bars are means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ vs. corresponding untreated controls ($n = 4-5$, each group).

Figure 4. Effect of repeated treatment of rats with PMEA and bisPOM-PMEA on content of liver microsomal cytochromes P450 and P420. The drugs (25 mg/kg, i.p.) were administered on 10 consecutive days and animals were sacrificed 9 days later. The bars are means $\pm$ SEM. ** $p < 0.01$ vs. corresponding untreated controls ($n = 4$, each group).

when the activity of *p*-nitrophenol hydroxylase was expressed as nmol 4-nitrocatechol/min/mg microsomal protein (Figure 6). Expressed in terms of specific activity of the enzyme, i.e. in nmol of 4-nitrocatechol produced per nmol CYP per min, no differences against controls were observed in microsomes from PMEA-treated rats, however (Figure 6).

The liver CYP mRNAs were assayed by RT-PCR in control rats ($n = 2$) and in animals given 10 daily injections of PMEA ($n = 3$). The treatment had no influence on expression of individual CYP enzymes (Figure 7) that otherwise

Figure 5. Duration of the effect of PMEA on increased content of inactive form of rat liver cytochrome P420. PMEA (25 mg/kg, i.p.) was administered on ten consecutive days. Animals ($n=4$, each group) were sacrificed at indicated time intervals posttreatment. The bars are means $\pm$ SEM. $**p < 0.01$ vs. corresponding untreated controls.

were constitutively present in controls. Moreover, neither the amount of immunoreactive CYP2E1 microsomal protein, detected by Western blotting, was altered after the PMEA treatment (Figure 8).

Discussion

Involvement of various CYPs in metabolism of compounds can result in clinically significant drug–drug interactions [8]. The orally bioavailable

prodrugs of acyclic nucleoside phosphonates such as 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA; *adefovir*), and 9-(*R*)-[2-(phosphonomethoxy)propyl]adenine [(*R*)-PMPA; *tenofovir*] are widely used for treatment of hepatitis B (Hepsera), and acquired immunodeficiency syndrome (Viread), respectively. Conceivably, they may be combined with other virostatics (e.g. emtricitabine) as well as with drugs of various therapeutic indications. In this study we have analysed possible *in vivo* interference of PMEA and bisPOM-PMEA with rat liver cytochrome P450 system. As a marker of CYP behaviour, the activity of the CYP2E1 was determined as its role in metabolism and activation of xenobiotics is well recognized [9]. Moreover, the CYP2E1 is a suitable model for CYP systems because this particular CYP form is considered to be relatively conserved across species and, hence, the results obtained with rat CYP2E1 may be at least to a certain extent extrapolated to human CYP2E1 [10, 11].

Our data demonstrate that PMEA does not influence important parameters of the liver CYP metabolizing system, such as expression of CYP mRNA and protein. Neither the total microsomal protein was changed by long-lasting PMEA treatment. On the other hand, both PMEA and bisPOM-PMEA considerably influenced the ratio between the active and denatured forms of CYP, i.e. P450 and P420, respectively. While the level of inactive

Figure 6. Formation of 4-nitrocatechol from *p*-nitrophenol by liver microsomes after repeated treatment of rats with PMEA. Formation of the metabolite was significantly decreased ($**p < 0.01$) when calculated per mg of total microsomal protein, while it remained unchanged when expressed as a specific activity of the enzyme, i.e. related to the total content of cytochromes P450. The bars are means $\pm$ SEM, $n=4-5$, each group.

Figure 7. RT-PCR analysis of expression of cytochromes P450. The rats were intraperitoneally treated with PMEA (*adefovir*, 25 mg/kg) or with saline for 10 days. The animals were sacrificed 9 days after cessation of the treatment. RNA was extracted from up to 30 mg of liver tissue and analyzed by RT-PCR. Expression of a housekeeping gene GAPDH was determined as a control. There was no detectable mRNA expression of CYP 2C11, CYP 3A2 and CYP 1A1 (data not shown).

Figure 8. Western blots of rat liver microsomal fractions (25 µg protein per lane) indicating the presence of CYP2E1 protein. The rats were given 10 daily injections of PMEA (25 mg/kg; i.p.), and sacrificed 9 days after cessation of the treatment. No differences in CYP2E1 expression was observed between PMEA-treated (lanes 1–4) and control (lanes 5–8) animals ($n=4$, each group).

P420 was about 25% of the total CYP in controls, it was approximately the same after five doses, and nearly as once higher than P450 after 10–18 injections of PMEA. It took more than 4 weeks to fully recover from the insult. As far as the excision of the liver samples and all steps in preparing microsomal fractions were identical in all experimental groups, it is unlikely that the augmented occurrence of P420

might be a technical artifact. An underlying mechanism of this effect, which may involve e.g. the protein phosphorylation acting as a functional switch [12], requires further elucidation.

Since the P420 is an inactive product of CYP, we analyzed whether and to what extent might these effects influence the CYP-dependent biotransformation of xenobiotics. To this point, we

investigated generation of 4-nitrocatechol from *p*-nitrophenol which is mediated by *p*-nitrophenol hydroxylase (CYP2E1). While the specific activity of CYP2E1 was the same in all groups, its activity related to the amount of microsomal protein was significantly suppressed. It is in good line with the finding of substantially lowered amount of active P450.

In conclusion, it can be suggested that PMEA (*adefovir*) may interact with the liver microsomal CYP system and hence also influence metabolic fate and biological effectiveness of some xenobiotics. Clinical relevance of the findings remains to be ascertained, especially in patients suffering with hepatitis B. Substantiated by our previous experiments showing outstanding anti-inflammatory potential of PMEA and bisPOM-PMEA in a model of human rheumatoid arthritis, adjuvant-induced arthritis in rats [13], we used relatively high dose of these agents. The dose of *adefovir dipivoxil* used for the treatment of hepatitis B infection is lower [14].

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