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In Vivo Study of the Effect of Antiviral Acyclic Nucleotide Phosphonate (*R*)-9-[2-(phosphonomethoxy)propyl]adenine (PMPA, tenofovir) and Its Prodrug Tenofovir Disoproxil Fumarate on Rat Microsomal Cytochrome P450

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Summary

The total content of rat liver microsomal cytochrome P450 (CYP) significantly decreased after repeated i.p. administration of the antiviral agent tenofovir ((*R*)-9-[2-(phosphonomethoxy)propyl] adenine) and tenofovir disoproxil at a daily dose 25 mg/kg, although the content of liver microsomal protein did not change. The decrease of the CYP content was accompanied by concomitant increase of the amount of inactive CYP form, cytochrome P420. This effect was confirmed by a parallel study of the activities of selected CYP forms, CYP2E1 (p-nitrophenol hydroxylation) and CYP1A2 (7-ethoxyresorufin deethylation). The activity (expressed relatively to the protein content) of both CYP forms decreased significantly following the decrease of the total CYP. On the other hand, the CYP2E1 activity expressed relatively to the decreasing total CYP content remained unchanged. However, CYP1A2 activity also decreased when calculated relatively to the total native CYP content indicating lower stability of this form. Semiquantitative RT-PCR showed no significant changes in expression of major rat liver microsomal CYP forms after tenofovir treatment. In conclusion, repeated administration of tenofovir in higher doses led to significant decrease of the relative proportion of active liver microsomal CYPs accompanied by a conversion of these enzymes to the inactive form (CYP420) maintaining the sum of CYP proteins unchanged.

Key words

Tenofovir • PMPA • Cytochrome P450 • CYP2E1 • CYP1A2

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Introduction

Adefovir and tenofovir, acyclic nucleoside phosphonates of the structure similar to natural nucleotides, are two recently introduced antiviral agents acting as inhibitors of replication of retroviruses and DNA-viruses (Holý 2003). Adefovir, 9-[2-(phosphono-methoxy)ethyl]adenine (PMEA), is used mainly for treatment of hepatitis B, whereas tenofovir, (*R*)-9-[2-(phosphonomethoxy)propyl]adenine (PMPA), is one of the drugs of choice for treatment of the HIV infection.

Studies on the involvement of the cytochrome P450 (CYP) enzymes in drug pharmacokinetics have been shown to be useful as they can help in understanding drug interactions based on induction or inhibition of particular CYP activities (Anzenbacher and Anzenbacherová 2001). Tenofovir pharmacokinetics and clinical pharmacology has been recently reviewed; however, little is known about its influence on the liver microsomal system of cytochrome P450 (Kearney *et al.* 2004). A possibility of reduction of the activity of one CYP enzyme (CYP1A2) was mentioned on the basis of the *in vitro* experiments (Branch *et al*. 2002). As the *in vivo* data on the effects of acyclic nucleoside phosphonates on liver microsomal CYP were scarce, it appeared to be useful to focus on this subject. Recently, a paper on the effects of adefovir on CYP system of rat liver microsomes *in vivo* has been published indicating a denaturation of liver microsomal CYP after i.p.

administration of higher doses of adefovir (Zídek *et al*. 2006). As the structures of adefovir and tenofovir are very similar to each other, it has been necessary to implement the data with those on tenofovir. This study hence deals with changes of properties of the rat liver microsomal CYP system resulting from application of tenofovir and its prodrug, tenofovir disoproxil fumarate with the methyl group introduced onto the side chain of the parent molecule (Fig. 1).

Methods

Materials

(*R*)-9-[2-(phosphonomethoxy)propyl]adenine (tenofovir, PMPA) was synthesized in the Institute of Organic Chemistry and Biochemistry (Prague) and (*R*)-9-[2-[bis[[(isopropoxycarbonyl)oxy]methoxy]phosphinyl] propyl]adenine fumarate (tenofovir disoproxil, bis(POC)-PMPA) was kindly donated by Gilead Sciences (Foster City, CA). Chemical structure of tenofovir disoproxil is shown in Figure 1. p-Nitrophenol, p-nitrocatechol, NADPH sodium salt, bovine serum albumin and other chemicals used for laboratory work were purchased from Sigma (St Louis, MO). Sodium dithionite was product of Merck (Darmstadt, Germany). All chemicals used were of reagent grade quality.

Treatment of experimental animals, preparation of liver microsomes

Female rats (Lewis inbred strain, body weight 144-155 g) were purchased from Charles River (Sulzfeld, Germany). The test substances, tenofovir and tenofovir disoproxil, were intraperitoneally injected during ten consecutive days at doses 5 or 25 mg/kg. After ten days the rats were sacrificed and livers were removed and weighted. The liver samples were frozen in liquid nitrogen and stored at –70 °C until used. The protocol of the experiment was approved by the institutional Ethics Committee. For preparation of liver microsomes, defined part of the liver was excised, the sample was rinsed in cold 0.25 M sucrose in 50 mM Tris-HCl (pH 7.4). The tissue was then homogenized and subjected to differential centrifugation to obtain the microsomal fraction according to standard procedures (Lake 1989).

Determination of total protein and cytochrome P450 (P420) content

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 ($y = 1.0628 + 0.0203$, $r^2 = 0.996$) within the concentration range of 0 to 1 mg/ml. Differences between determinations were below 15 %.

Content of CYP and its inactive form P420 was determined directly by using an established CO-difference spectroscopy method (Omura and Sato 1967). An extinction coefficient of 91 $cm^{-1}mM^{-1}$ 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^{-1} 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 $mM^{-1}cm^{-1}$.

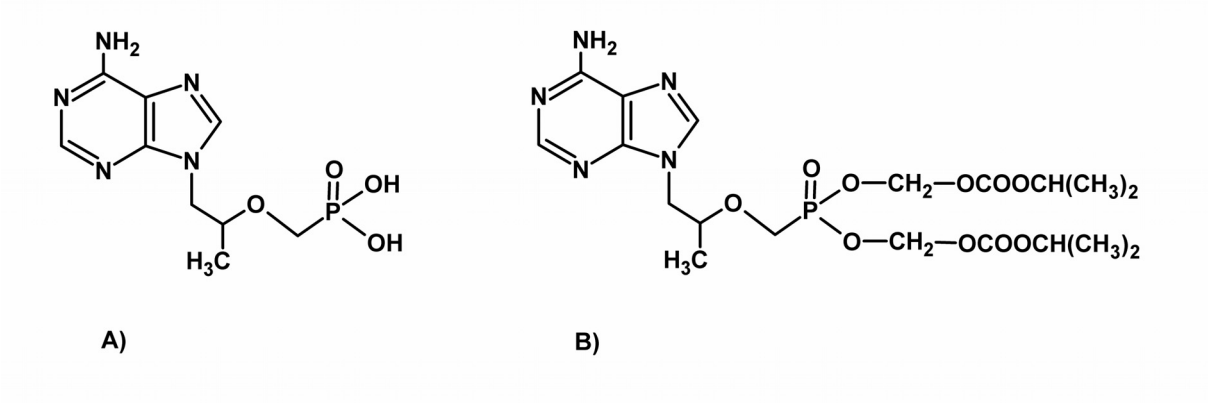

Fig. 1. Tenofovir (A) and tenofovir disoproxil (B)

Table 1. Influence of tenofovir and tenofovir disoproxil fumarate administration (25 mg.kg^{-1}) to the experimental animals on their body weight, on the relative weight of the liver and on the relative content of liver microsomal protein.

	Body weight (g)	Liver weight (% of body weight)	Microsomal protein (mg/g of liver)
Control	200 ± 4.5	4.18 ± 0.12	15.45 ± 0.20
Tenofovir	195 ± 5.7	4.14 ± 0.11	15.75 ± 0.50
Tenofovir disoproxil	197 ± 4.5	4.23 ± 0.15	16.00 ±0.38

Data are mean ± S. E.M.

p-Nitrophenol hydroxylation

A prototypic CYP2E1 activity, p-nitrophenol hydroxylation to p-nitrocatechol, was determined according to Chang *et al*. (1998) by spectrophotometric determination of the product formed using Shimadzu UV-2100 spectrophotometer. p-Nitrocatechol was used as standard with a calibration curve linear in the range of 0 to 20 nmol of p-nitrocatechol in the reaction mixture of 0.5 µl, i.e. up to the 40 µM molar concentration ($y = 0.0128x$ with $r^2 = 0.9989$). Enzyme activity was expressed both as nmol product/min/mg microsomal protein and as nmol product/min/nmol total CYP to obtain the specific CYP2E1 activity.

Determination of CYP1A2 activity

CYP1A2 activity was measured as 7-ethoxy-resorufin O-deethylation according to established procedure (Chang and Waxman 1998) based on spectrofluorometric detection of the product formed. A TECAN GENios absorbance/fluorescence/luminescence reader (Tecan Austria, Vienna) was used for fluorescence detection (excitation at 535 nm, emission at 595 nm). A linear calibration curve was obtained with resorufin as standard in the concentration range of 0 to 500 nmol ($y = 10.378x$; $r^2 = 0.9999$). Enzyme activity was expressed both as nmol product/min/mg microsomal protein and as nmol product/min/nmol total P450, i.e. as specific CYP activity.

Western blotting

Relative extent of CYP2E1 and CYP1A2 protein expression in microsomes was evaluated by Western blotting. The BioRad (Hercules, CA) MiniProtean equipment with Western blotting apparatus was used. Chemiluminiscence kit for Western blotting (Immun Star) was purchased from BioRad; nitrocellulose membrane was from Amersham Biosciences (Little Chalfont, GB). Rat anti human CYP2E1 and anti human CYP1A2 antibodies were from Daichi Pure Chemicals (Tokyo, Japan). Western blots were quantified using a standard curve with CYP content over a linear portion of the response curve (generally with 0.1 to 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 . Recombinant CYP1A2 and CYP2E1 (Panvera-Invitrogen, supplied by KRD, Prague, CZ) were used as standards.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Expression of seven different cytochrome P450 mRNAs was analyzed by a semiquantitative RT-PCR following the procedure described earlier (Zídek *et al*. 2006) based on the method of Morris and Davila (1996). 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 µg 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 with glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) commercially available primers (Clontech, Palo Alto, CA, USA). After initial denaturation (2 min, 94 °C), 25 amplification cycles were performed using a 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

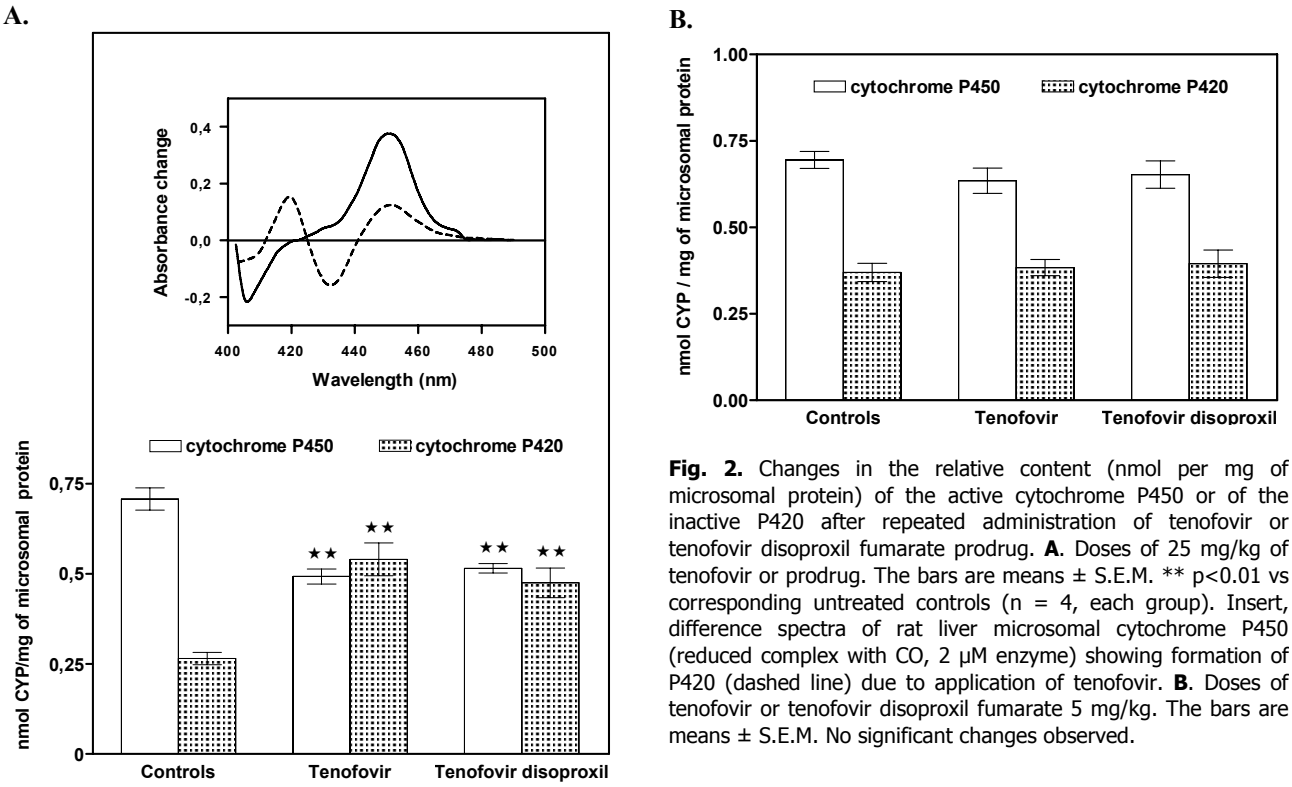

Fig. 2. Changes in the relative content (nmol per mg of microsomal protein) of the active cytochrome P450 or of the inactive P420 after repeated administration of tenofovir or tenofovir disoproxil fumarate prodrug. **A**. Doses of 25 mg/kg of tenofovir or prodrug. The bars are means ± S.E.M. ** $p<0.01$ vs corresponding untreated controls (n = 4, each group). Insert, difference spectra of rat liver microsomal cytochrome P450 (reduced complex with CO, 2 μM enzyme) showing formation of P420 (dashed line) due to application of tenofovir. **B**. Doses of tenofovir or tenofovir disoproxil fumarate 5 mg/kg. The bars are means ± S.E.M. No significant changes observed.

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 (1996). The method gave single products within a linear range of 20 to 30 cycles.

Data analysis

The data were evaluated by one-way analysis of variance (ANOVA) followed by multiple comparison Bonferroni test using Prism software (GraphPad, San Diego, CA).

Results

Although repeated administration of tenofovir and tenofovir disoproxil fumarate (25 mg/kg) to rats did change neither the body weight, the relative liver weight nor the amount of liver microsomal protein (Table 1), the content of rat liver cytochrome P450 (CYP) significantly decreased after 10 days to 70 and 72 % in the case of tenofovir and tenofovir disoproxil, respectively (Fig. 2A).

Concomitantly, the amount of inactive form of cytochrome P450, i.e. of the cytochrome P420 significantly increased. Formation of the inactive cytochrome P420 may be clearly seen in the respective absorption spectra (Fig. 2A, insert). As the amount of total microsomal protein remained unchanged (Table 1), increase of the amount of the inactive cytochrome P420 form occurred apparently at the expense of the native form. The application of tenofovir or tenofovir disoproxil in lower dose (5 mg/kg), however, did not cause any significant change of the absolute values of both cytochrome P450 and P420 remaining the proportion of the active and inactive form unaltered (Fig. 2B).

To answer the question whether the decrease of the total CYP amount is also accompanied by a corresponding decrease of activities of particular CYP enzymes, the activity of two CYP forms, namely, of the CYP2E1 (EC 1.5.99) and CYP1A2 (EC 1.14.14.1), was followed after i.p. application of higher dose of tenofovir (25 mg/kg). In parallel, the amount of the CYP2E1 and CYP1A2 protein was evaluated by Western blotting and the expression of the corresponding mRNAs was followed. No changes in the CYP2E1 and CYP1A2 mRNA as well as of other CYP mRNAs (Fig. 3) or of the CYP2E1 or CYP1A2 protein (results not shown) were observed after tenofovir administration.

On the other hand, the CYP2E1 activity expressed relatively to (unchanged) total microsomal protein decreased significantly as a result of tenofovir administration (25 $mg.kg^{-1}$) (Fig. 4A). However, when the same activity was calculated relatively to the amount of active CYP (i.e. as a specific activity of the CYP2E1),

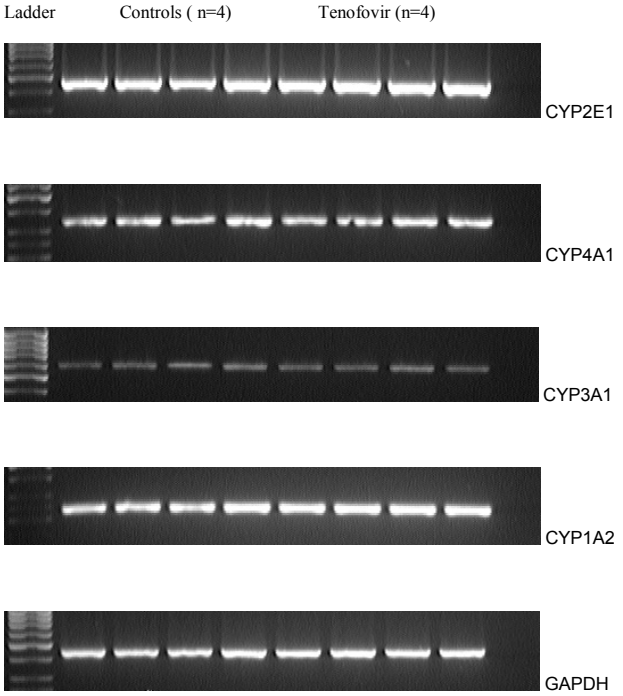

Fig. 3. RT-PCR analysis of expression of rat liver cytochromes P450. The rats were intraperitoneally treated with tenofovir (PMPA, 25 mg/kg) or with saline for 10 days. RNA was extracted from up to 30 mg of liver tissue and analyzed by RT-PCR. Expression of a housekeeping gene GAPDH was determined as a control (lower photograph). There was no detectable mRNA expression of CYP 2C11, CYP 3A2 and CYP 1A1 (data not shown).

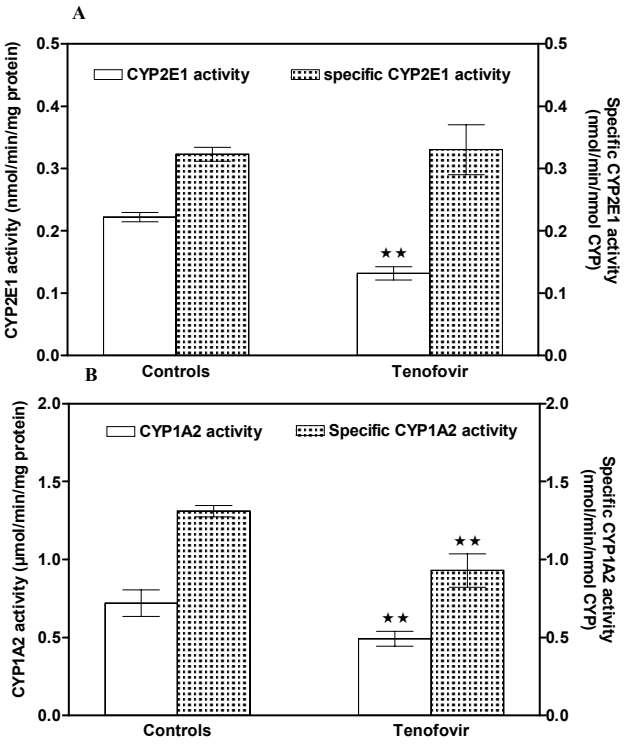

Fig. 4. Activities of CYP2E1 and CYP1A2 in rat liver microsomes after repeated treatment with 25 mg/kg of tenofovir. The bars are means ± S.E.M, n = 4-5, each group. **A**. CYP2E1 activity measured as 4-nitrocatechol formation from p-nitrophenol; the formation of the metabolite was considered significantly decreased in controls (** $p<0.01$, vs. untreated controls) when calculated per mg of microsomal protein while it remained unchanged when expressed as specific activity i.e. relatively to the content of native enzyme. **B**. CYP1A2 activity (7-ethoxyresorufin deethylation), both the relative as well as the specific activity significantly decreased (** $p<0.01$, vs. untreated controls).

no significant change due to administration of either tenofovir or its prodrug was observed. In other words, the relative proportion of the active CYP2E1 enzyme remained the same even when the total amount of native CYP decreased.

The activity of the second CYP form, CYP1A2, also significantly decreased after administration of tenofovir; in this case, contrary to CYP2E1, both the activity expressed either relatively to the total protein content or relatively to the content of active CYP in the samples (specific activity) was diminished due to the application of the drug (Fig. 4B). Hence, the rat CYP1A2 activity appears to be more susceptible to the effect of tenofovir.

Discussion

The results document that the denaturation of cytochromes P450 of the rat liver endoplasmatic reticulum (in other words, in the liver microsomal fraction) takes place after administration of higher dose of tenofovir or tenofovir disoproxil fumarate. The denaturation leads clearly to formation of the inactive P420 form, the sum of the active and inactive forms being unchanged. The process apparently affects the native, fully competent enzyme, i.e. the already expressed protein because the amounts of the sum of the total P450 and P420 protein and of the CYP proteins recognizable by the respective antibody as well as of their mRNA were not changed. This means that it is not caused by other effects as misfolding of the protein, or by an interference of the drug with the biosynthesis of CYP proteins. The interaction between the drug and the CYP enzymes must hence take place *in situ*. The mechanism of this effect may be difficult to explain. One possibility may be an influence of tenofovir (and of its prodrug, tenofovir disoproxil, providing there is still a part of it present in the hepatocyte) on phosphorylation pathways in the cell. Tenofovir is known to be converted to diphosphate by action of adenylate kinase and by nucleotide diphosphate kinase (Kearney *et al.* 2004). As the cAMP-dependent

protein kinase is known to label the CYP enzymes for destabilization (Jansson *et al.* 1990), an interaction of tenofovir with phosphorylation mechanisms in the hepatocyte may contribute to destruction of the native enzyme.

Interestingly, there are differences between individual CYP forms. CYP2E1 seems to be less prone to the denaturation (its activity decreases concomitantly with the amount of total active CYP), whereas rat CYP1A2 activity apparently decreases more quickly than total CYP content. In other words, specific CYP1A2 activity, expressed relatively to total active CYP, is not constant as it was in the case of the CYP2E1 but decreases significantly (Fig. 3B). In this respect it should be mentioned that in some species (minipig) CYP2E1 belongs to one of the most stable CYP forms which denature to the inactive P420 form only under high pressure (Anzenbacherová *et al.* 2005). On the other hand, rat CYP1A2 was shown to be more susceptible to protein degradation than other CYP enzymes (Guengerich 1978). Activities of CYP1A2 and CYP2E1 were chosen for this study as these two cytochromes P450 belong to CYP enzymes with highly conservative structure among species; as a result, also their activities are preserved in most of the species studied (Guengerich 1997, Court *et al.* 1997).

Decrease of the level of active hepatic CYP enzyme after tenofovir administration leads directly to a question on clinical relevance of the effect observed. Maximum serum tenofovir concentrations may reach up to 8.5 mg/l after infusion of 3 mg/kg/day in a dose-escalation monotherapy study (Kearney *et al.* 2004). Here, the dose of 25 mg/kg apparently interfered with the rat liver microsomal CYP system. To extrapolate the results obtained on CYP enzymes of the rat to the man is however difficult and far from being straightforward. For example, rat CYP1A2 is inducible by phenobarbital, which is not an inducer of the human CYP1A2 enzyme (Magnusson *et al.* 2006). In fact, phenobarbital is in most of the species a well-known inducer of another families of CYP enzymes and not of the human CYP1A2 form which is typically inducible by aromatic compounds (Anzenbacher and Anzenbacherová 2001, Guengerich 2005). Detailed studies on *in vitro* as well as *in vivo* interactions between this new class of antivirals and CYP enzymes are hence needed.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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