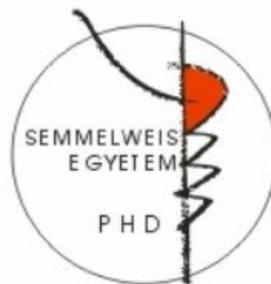


*IN VITRO* and *IN VIVO* FARMACOKINETIC  
EVALUATION OF MECHANISM OF TOFISOPAM-  
TACROLIMUS DRUG-DRUG INTERACTION

Doctoral theses

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## **Introduction**

The attentions of the patients, doctors, and the authorities are turn to the prevention of drug-drug interaction by the reason of the increasing number of the adverse events. Permissions of several drugs were withdrawn during last years, by the reason of severe adverse drug reactions caused by drug-drug interaction. In our observed three cases tofisopam increased the blood level of tacrolimus leading clinically relevant renal fuction decline as adverse drug reaction and necessitating reduction of the tacrolimus dose or discontinuation of the administration of tofisopam. This clinical observation raised the possibility of drug-drug interaction between the two adminisitered drugs, tofisopam and tacrolimus.

The patients after transplantation are required the immunosuppression therapy during their whole life, due to the prevention of rejection their transplanted organ. In such case where the continous chonic therapy need for the patients is essential to estimate the probability of adverse drug reaction before the new drugs are added to the others. The exact identifications of mechanism of interactions need to clarify the pharmacokinetic and clinical pharmacology properties of the drugs are involved into the interaction.

The knowing of the potential interaction of tacrolimus as narrow therapic range immunosuppression drug is elementary aspect in the treatment of transplanted paptients. Both the significant elevation or reduction in plasma level of immunosuppression drug due to the interaction of the tacrolimus, may lead to the impairment of transplanted kidney. The reduction of plasma concentration of the immunosuppression drug may cause the rejection of the graft, or the early lost of the transplanted kidney may occur. On the other hand, the elevated plasma level in consequence of tacrolimus interaction may lead to the reversible or irreversible lesion of the transplanted kidney function due to the familiar renal toxic effect of drugs belong to calcineurine-inhibitors.

According to the several authors, the temporary reduction in graft function may affect on the long term succes of kidney transplantation. The avoidance the drug-drug interaction during treatment of transplanted patients is mandatory, due to the changes in

the immunosuppression blood level may lead the graft lost. The potential interaction of tacrolimus is administered in observed clinical cases, are very well know, otherwise the interaction with tofisopam was published only in few cases. Joint administration of both drugs suggested that tofisopam inhibits the metabolism of the immunosuppressant hereby elevates the bioavailability of it widely. Lowering the dose of tofisopam or the leaving off tofisopam from treatment led the recurrence of tacrolimus blood level to normal range which enhanced the possibilities of drug-drug interaction.

The potential mechanism first of all was evaluated according to the literature data. Based on this evaluation the most potential possibility could be caused this interaction was the inhibition of CYP 450 3A4 enzyme by tofisopam.

Based on the pharmacokinetic properties of the tacrolimus and tofisopam we tried to provide a modell with *in vitro* and *in vivo* studies of the interaction's mechanism between the two drugs connecting to the CYP 450 3A4 enzyme.

### **The aims of the present work**

- In vitro measurement of tofisopam effect on the CYP-450 3A4 enzyme.
- Perform the tofisopam single dose human clinical study and evaluation of results.
- Perform the double-blind placebo controlled cross over human pharmacokinetic study to evaluate the effect of tofisopam on the oral dose pharmacokinetics of the hepatic CYP-450 3A4 probe drug ***alprazolam***
- Perform the double-blind placebo controlled cross over human pharmacokinetic study to evaluate the effect of tofisopam on the oral dose pharmacokinetics of the hepatic/intestine CYP-450 3A4 probe drug ***midazolam***.
- Evaluate of the results from two human interaction studies in order to define tofisopam inhibitory effect on CYP 450 3A4.

## **Materials and methods**

### ***In vitro* study**

The administered immunosuppressive agent is a substrate of the CYP3A4 system, so the effect of tofisopam on the CYP3A4 enzyme was investigated *in vitro* using human recombinant CYP3A4 supersome (Cat. No.: P 202). Benzyoxy-4-(trifluoromethyl)-coumarin (BFC) was used as substrate. Tofisoapam was added in concentration ranges of 0.1-0.25, 0.5-0.75 and 1-5  $\mu$ M.

The 150  $\mu$ l mixture for measurement of enzyme activity contained 1 pmol CYP-450 3A4 supersome (Human rekombinant CYP-450 3A4 supersome), and 50 mol/l benziloxi-4-(trifluorometil)-kumarin (BFC) substrate in 70 mmol/l natrium-phosphate pH 7,4 buffer.

After 10 minutes preincubation at 37 °C, the reaction was initiated by the addition of 1,5 mmol/l nikotinamid-adenin-dinukleotid-foszfát-dinátrium (NADPH), (Reanal, Budapest). After 10 minutes incubation at 37 °C temperature the 150  $\mu$ l 500 mmol/l tris-hidroximetil-aminometán (Tris bazis: Sigma Co. (St. Louis, USA), and acetonitril (Thomasker & Chemiris, Budapest) mixture in 40 % vs. 60 % was added to terminate the reaction. After 5 minutes 11500 rpm centrifugation, the 1800  $\mu$ l terminate solution was added to 200  $\mu$ l supernatant, and was measured the 7-hidroxi-4-(trifluorometil)-kumarin (HFC) (GenTest Corporation, Woburn, USA) level by spektrofluorimetry on 409 nm and on 530 nm.

### ***In vivo* human clinical studies**

All clinical studies were performed after the written aprovals of the Hungarian National Institute for Pharmacy, and the Clinical Pharmacological Ethical Committee of Scientific Health Council were obtained. The studies were conducted in accordance with the Declaration of Helsinki and recent guidelines and each volunteer provided informed consent before entering the study.

#### ***1. Tofisopam Single Dose Pharmacokinetic Study***

This was a single-centre, open-label pharmacokinetic study. Twelve healthy Caucasian non smoking male volunteers, ranging from 18 to

40 years of age, and body mass index within 19-26 kg/m<sup>2</sup> were enrolled in the study. Health was evaluated by medical history, physical examination, ECG and clinical laboratory tests including HIV, hepatitis B and C tests and urine drug screen within two weeks prior to inclusion to the study. The volunteers were not allowed to use any medication within 14 days before and during the study, only single doses of 500 mg paracetamol for the treatment of headache or other pain was permitted during the restriction period. Eligible subjects were admitted to the study site on the day before dose administration, and they stayed there up to 24 hours following the drug administration. On the morning of the treatment day, volunteers were asked for possible intercurrent illnesses, and if indicated, a physical examination was repeated. Before drug administration, blood drawing ("0" time-sample), and safety measurements, blood pressure (BP), and heart rate (HR) were performed. The subjects were given two tablets (2x50 mg tofisopam) between 8 and 9 a.m. after an overnight fast. Safety (BP, HR) measurements were performed 1.5 and 24 h after the single dose of tofisopam. Blood samples (á 7.5 ml) for tofisopam analysis were collected in the morning just before the tofisopam dose (0) and 0.25, 0.5, 0.75, 1, 1.33, 1.66, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 16, 24, 36, 48 hour after the tofisopam administration. Blood samples were collected into tubes (S-Monovettes, Sarstedt, Nuembrecht, Germany) contained anticoagulant (K-EDTA). Blood samples were centrifuged at 3000 rpm at room temperature and plasma separated within 15 minutes of veinpuncture. The separated plasma was aspirated off and filled into test tubes for plasma samples. Each tube was labelled with study code, subject identification (treatment number and initials), date and time of the sample and signature of the person taking the blood. The plasma samples were stored at -20°C or colder. Chromatographic analyses were carried out using HP 5890 Serious II gas chromatograph equipment with a Nitrogen-phosphorus detector. The column was a Supelco SPB™-(1 25m x 0.32mm x 0.25 µm) + HP retention gap (5m x 0.32 mm). The injector and detector were operated at temperature 270 °C and 280 °C, respectively. Analytical solution (4 µl) were injected in the split mode (split ratio according to the pressure program), and the oven temperature was programmed as follows: 260 °C for 10 min, increasing to 280 °C (40°C/min) and holding for 3.5 min. Helium was used as carrier gas and flow rate

during the isobar section was 2.8 mL/min and linear velocity was 59 cm/sec. Detector gases: Makeup gas flow rate: 30 mL/min, Air flow rate: 100 mL/min, Septum purge gas flow rate: 3 mL/min, Hydrogen flow rate: 3.8 mL/min. The run time was 14 minutes. The pharmacokinetic parameters were determined from the plasma concentration-time data using two compartment model and model independent methods using Kinetika™ Ver. 4.0.2 validated pharmacokinetic software package (Inna Phase Corp., Philadelphia, PA., USA). Only observed data was evaluated, no specific procedures were carried out due to missing data. Calculations of the pharmacokinetic variables were based on data sets with complete relevant data. The statistical analyses of safety parameters were performed according to the intention to treat (ITT) principle, i.e. all recruited study volunteers were included in the analyses. Descriptive statistics for statistical analysis was used for safety, demographic and pharmacokinetic parameters.

## 2 *Effect of tofisopam on the oral dose pharmacokinetics of the hepatic CYP-450 3A4 probe drug **alprazolam**.*

Sixteen Caucasian healthy male volunteers, ranging from 22 to 33 years of age and body mass index within 19-26 kg/m<sup>2</sup> were recruited to the study. Good health of the volunteers was judged by medical history, physical examination, electrocardiogram (ECG) and clinical laboratory tests including HIV, hepatitis B and C tests and urine drug screen within two weeks prior to inclusion to the study. The subjects smoked no more than 10 cigarettes per day. Concomitant medication with the exception of the occasional use of paracetamol was not allowed during the study. Following verbal and written information written informed consent was obtained from the subjects prior the screening examinations. The study was a double blind, randomised, two-way crossover design with three-week washout between the two nine-day treatment phases. The volunteers were confined trial site from the -1 day (day before the study drug intake) evening to the 2<sup>nd</sup> day morning; and from the 6<sup>th</sup> day evening to the 9<sup>th</sup> day morning in each treatment periods. Subjects received 100 mg (two 50 mg tablets) of tofisopam or matched placebo three times a day for 9 days in a random order. On day 7 of each treatment period subjects received a single 0.5 mg dose of alprazolam 1 hour after the morning

dose of tofisopam. Vital signs and ECG were checked at several time points during the study. A follow-up visit was performed 5-14 days after the second treatment period. For analysis of alprazolam venous blood samples of 7.5 ml were collected into tubes containing EDTA as anticoagulant before (0 hour) and 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 36, 48, 72 hours after the administration of alprazolam. Plasma trough concentration of tofisopam was measured on days 5, 6 and 7 before the administration of the morning dose of tofisopam. Sensitive validated capillary gas chromatography (GC) method with mass spectrometric (MS) detection was developed for determination of alprazolam in the human plasma. Midazolam was used as internal standard. The linear range of the assay was 0.1 to 20 ng/ml. The lower limit of quantification (LLOQ) for alprazolam was 0.1 ng/mL. The concentration of the tofisopam in the plasma was determined by a validated gas chromatography – nitrogenous phosphorus detection (GC-NPD) method. Girizopam, a compound having similar structure to tofisopam was selected as internal standard. The assay was linear in the 5-500 ng/mL. The lower limit of quantification (LLOQ) for tofisopam was 5 ng/mL. The pharmacokinetic parameters of alprazolam were determined from the plasma concentration-time data by model independent method using Kinetica™ Ver. 4.02 validated software package (InnaPhase, Champs sur Marne, France). The maximum plasma concentration ( $C_{max}$ ) and the time to  $C_{max}$  ( $t_{max}$ ) were obtained directly from the concentration-time profile. Area under the plasma concentration-time curve from time zero to the last measured time point ( $AUC_{0-t}$ ) was calculated with use of the linear trapezoidal rule. The area under the plasma concentration-time curve from time zero extrapolated to infinity ( $AUC_{0-\infty}$ ) was calculated from the equation:  $AUC_{0-\infty} = AUC_{0-t} + C_t/k_{el}$  where  $C_t$  and  $k_{el}$  represent the observed plasma concentration at the last measurable sampling time and the elimination rate constant, respectively. The elimination rate constant was determined as the negative slope of the log-linear part of the plasma-concentration-time curve using linear regression. Elimination half-life ( $t^{1/2}$ ) values were calculated as  $\ln(2)/k_{el}$ . The apparent oral clearance ( $CL/F$ ) was calculated as the administered dose divided by the  $AUC_{0-\infty}$ . The pharmacokinetic interaction between alprazolam and tofisopam was handled as a bioequivalence problem. The administration of alprazolam with tofisopam was considered as test treatment whereas

administration of alprazolam with placebo represented the reference treatment. Following log transformation,  $C_{\max}$ ,  $AUC_{0-t}$ ,  $AUC_{0-\infty}$ , MRT, CL/F of the test and the reference treatment were compared by using four-way analysis of variance (4-way ANOVA) for treatment, period, subject (sequence) and sequence. The 90% confidence interval (90% CI) around the geometric mean of the test/reference ratio was calculated. Interaction between the two drugs was indicated if the 90% CI for the ratio of geometric means was not contained in the equivalence limit of 0.80-1.25. In addition, the log transformed pharmacokinetic parameters of the test and reference treatment were compared using Shuirmann test. The  $t_{\max}$  values were analysed by the non-parametric Kruskal-Wallis test. The trough concentrations of tofisopam on days 5, 6 and 7 were compared by using one-way ANOVA.

### *3. Effect of tofisopam on the oral dose pharmacokinetics of the hepatic/intestine CYP-450 3A4 probe drug **midazolam***

To evaluate the clinical relevance of the CYP3A4 inhibiting effect of tofisopam, a clinical study was performed using alprazolam as a hepatic CYP3A4 probe drug. Tofisopam slightly increased the AUC and  $T_{1/2}$  of alprazolam after single oral dose and decreased the clearance, indicating modest inhibition of hepatic CYP3A4. The magnitude of inhibition was not sufficient to explain the pharmacokinetic interaction between tofisopam and tacrolimus. Tacrolimus is a substrate of the intestinal CYP3A4; therefore interaction between the two drugs may occur at the intestinal level. A pharmacokinetic/pharmacodynamic drug-drug interaction study was performed to evaluate the effect of tofisopam on the CYP 3A4 activity at the intestinal level. Midazolam was selected as a probe drug since the small intestine is a major place for pre-systemic, CYP3A-mediated metabolism of midazolam after oral administration], which was clearly demonstrated by the discovery of the metabolites during anhepatic period of liver transplantation. Sixteen Caucasian healthy male subjects were enrolled to this open-label, two-period, single treatment sequence study after obtained written informed consent. Subjects received a single dose of 7.5 mg midazolam on study period 1. After one-week wash-out period subjects received 100 mg of tofisopam three times a day for 9 days.

On day 7 of tofisopam treatment, subjects received a single 7.5 mg dose of midazolam 1 hour after the morning dose of tofisopam. For analysis of midazolam venous blood samples of 7.5 ml were collected into tubes containing EDTA as anticoagulant before (0 hour) and 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 12, 16, 24, 48, hours after the administration of single dose midazolam in period 1. In period 2 when midazolam administration was performed after tofisopam pre-treatment the blood samples collections was the same with two additionally sampling time as 72 and 96 hours post dose. Midazolam plasma concentrations were measured for 48 h after single dosing and for 96 h when midazolam was co-administered with tofisopam. Plasma levels were determined using capillary gas chromatography-mass spectrometric method. Alprazolam was used as internal standard. The lower limit of quantification (LLOQ) for midazolam was 0.5 ng/ml. The pharmacokinetic parameters for midazolam were determined by standard noncompartmental method using Kinetica<sup>TM</sup> Ver. 4.02 validated software (InnaPhase, Champs sur Marne, France). The 90% confidence interval (90% CI) around the geometric mean of the test (midazolam-tofisopam co-treatment)/reference (midazolam treatment) ratio was calculated for maximum plasma concentration ( $C_{max}$ ), the area under the plasma concentration time curve from zero to infinity (AUC), the elimination half-life( $t_{1/2}$ ), and for apparent oral clearance (CL/F). Interaction was indicated if the 90% CI for the ratio of geometric means was not contained in the equivalence limit of 0.80-1.25. In addition, the log transformed pharmacokinetic parameters of the test and reference treatment were compared using Shuirmann test. The  $t_{max}$  values were analysed by the non-parametric Kruskal-Wallis test.

#### *4. Statistical comparison of the results based on human interaction studies*

In the both human clinical study the volunteers received the tofisopam as inhibitory agent in the same dose (3x100 mg) and the same duration (9 days). On 7th day in both study the volunteers received the probe drugs (alprazolam or midazolam). The test/referen ratios from study results were compared and 95 % confidence interval of common pharmacokinetic parameters were

evaluated. Significant differences were indicated if the 95 % CI of upper and lower limits were not contained zero.

## **Results**

### ***In vitro* study**

Tofisopam in 0.1, 0.25, 0.5, 0.75, 1 and 5  $\mu\text{mol/l}$  concentrations inhibited dose dependently the enzyme activity. Activity inhibition rates were 4%, 29%, 40%, 56%, 61% and 94%, respectively and the  $\text{IC}_{50}$  was 0.8  $\mu\text{mol/l}$ . The  $\text{IC}_{50}$  of positive control substance ketoconazole was 0.03  $\mu\text{mol/l}$ . In *in vitro* experiments the inhibitory effect of tofisopam was lower than that of ketoconazole (potent CYP3A4 inhibitor) with an order of magnitude. According to the *in vitro* results it could be concluded that tofisopam is an inhibitor of CYP3A4 but to clarify the clinical importance of this inhibition, and provide the exact answer for the mechanism of the tofisopam-tacrolimus drug-drug interaction further human clinical data were needed.

### ***In vivo* human clinical studies**

#### ***1. Tofisopam Single Dose Pharmacokinetic Study***

Eighteen subjects were screened of which 12 were entered into the study. None of volunteers discontinued the study prematurely and all the 12 volunteers completed the study as planned. Considerable inter-individual variation was observed in the plasma concentration of tofisopam. The two-compartment model could be applied in half of the cases; accordingly data of 6 volunteers were included in the PK analysis using compartment model. For that reason, the results of the compartment analysis were utilised only as supportive data. All data from 12 volunteers were included in the pharmacokinetic analyses using non-compartment method. Analysing by non-compartment method ( $C_{\text{max}}$ ) after 100 mg dose of compound ranged from 40.9 to 655.6 ng/mL and ( $t_{\text{max}}$ ) after the dose ranged from 0.5 h to 3.5 h.  $\text{AUC}_{0-\infty}$  values were somewhat less variable it ranged from 193.7 ng\*h/mL to 780.0 ng\*h/mL. The  $\text{AUC}_{\text{Rest}}$  values for the parent compound were lower than the 20 % limit in all but one cases.

The  $AUC_{0-t}$  was ranged 164.6 ng\*h/mL to 744.2 ng\*h/mL. The  $t_{1/2}^{\beta}$ ,  $Cl_{oral}$  and  $V_d$ , after single dose, ranged from 2.02 h to 8.09 h; 178.8 to 516.3 L/h; and 523.8 to 5154.1 L, respectively.

In this pharmacokinetic study following intake of 100 mg of tofisopam, the plasma concentration values of tofisopam were quite variable. Volunteers that ingested 100 mg of tofisopam, the maximum measured plasma concentration ranged variable. The average value of  $C_{max}$  was somewhat higher than that of observed in most of the other studies. The time at which the first measurable concentrations of tofisopam were present indicates relatively rapid absorption of compound from gastrointestinal tract. At first sampling time point (0.25 h) tofisopam plasma concentrations level ranged from 5.22 ng/mL to 21.47 ng/mL. Half an hour after tablets intake, detectable tofisopam concentrations were present in the plasma sample of each volunteer, moreover in 3 volunteers the plasma concentration values reached the maximum in this time point. The relatively small  $t_{max}$  values, which with the short absorption half life ( $t_{1/2}^{ka}$ ) and absorption rate constant ( $k_a$ ) values are refers to a rapid absorption process. Only one volunteer showed a delayed absorption and  $C_{max}$  was reached 3.5 hours after oral administration. In the clinical and laboratory data there were no any explanation of this atypical absorption profile. The AUC value was higher than that of observed in most of the other studies. The difference may be consequence partly of the higher sensitivity of the current techniques.

The apparent volume of distribution ( $V_d$ ) of the drug was high (1679.3-1235.0 L), that indicates that tofisopam has higher concentration in extravascular tissue than in the vascular compartment. The distribution phase of the tofisopam was found to be relatively rapid according to the alpha and  $t_{1/2}^{\alpha}$  resulted from compartment analysis. Among the typical parameters of elimination, the oral clearance ( $Cl_{oral}$ ), elimination rate constant ( $\lambda$ ) and elimination half life ( $t_{1/2}^{\lambda}$ ) were calculated. In this study the oral clearance ( $Cl_{oral}$ ) of tofisopam was 279.2-121.4 L/h, which indicates considerable first pass metabolism. The elimination hybrid rate constant ( $\lambda$ ) of tofisopam was relatively small (0.198-0.081 h). The biological half-life of 4.12 h supports the t.i.d administration of tofisopam in the clinical practice. A rapid absorption and distribution, relative short biological half-life and considerable inter-individual variation in the plasma concentration levels of parent

compound were the main characteristics of the pharmacokinetics of tofisopam

## *2. Effect of tofisopam on the oral dose pharmacokinetics of the hepatic CYP-450 3A4 probe drug **alprazolam***

The effect of tofisopam on the hepatic CYP3A4 activity was evaluated by using alprazolam as a probe drug of the enzyme activity. Alprazolam is metabolised primarily by hepatic microsomal oxidation. As several in vitro and in vivo studies have suggested cytochrome CYP3A4 catalyses the 4 and alpha hydroxylation of alprazolam consequently alprazolam clearance and elimination half-life can be influenced by factors that alter hepatic oxidising capacity. In this study 100 mg tofisopam t.i.d., the highest recommended dose in the clinical practice increased the  $AUC_{0-t}$ ,  $AUC_{0-\infty}$  and  $t_{1/2}^{\beta}$  (90% CI are 1.14-1.35, 1.15-1.35 and 1.13-1.35, respectively) and decreased the CL/F (90% CI is 0.74-0.87). Whereas did not modify the  $C_{max}$  and  $t_{max}$  values of alprazolam. The changes in the AUCs,  $t_{1/2}^{\beta}$  and CL/F are considerable lower, than those of observed after the administration of the potent CYP3A4 enzyme inhibitors. Similar results were found using the Schuirmann test.

There were no significant differences in the trough concentration of tofisopam in the different days indicating that steady-state concentration of tofisopam was reached by the time of alprazolam administration. The modest inhibition of hepatic CYP3A4 enzyme does not seem to be sufficient to explain the pharmacokinetic interaction between tofisopam and tacrolimus. Several studies revealed that tacrolimus is a substrate of the intestinal CYP3A4 enzyme and the P-glycoprotein (PgP) drug transporter therefore interaction between tofisopam and tacrolimus at the intestine could be hypothesised.

In conclusion tofisopam slightly modify the single oral dose pharmacokinetics of alprazolam in the applied dose regimen indicating modest hepatic CYP3A4 enzyme inhibitory effect. Based on this data, the inhibitory effect of tofisopam on the metabolism of alprazolam seems to be considerable weaker, than that of the potent CYP 3A4 inhibitors. According to this modest effect, it can be concluded that the tofisopam inhibition is probably not irreversible on the hepatic level.

### *3. Effect of tofisopam on the oral dose pharmacokinetics of the hepatic/intestine CYP-450 3A4 probe drug **midazolam***

Sixteen subjects completed the study. The  $C_{max}$ ,  $AUC_{0-\infty}$  and  $t_{1/2}^{\beta}$  values of midazolam increased, and the  $CL_{oral}$  decreased significantly when midazolam was administered with tofisopam compared to the administration of midazolam alone. The results of the study indicate that the CYP3A4 inhibitory effect of tofisopam is more pronounced at the intestinal level than in liver.

The results showed that the 90% confidence intervals were 196.6-309.1% for  $AUC_{0-t}$ , 193.5-304.7% for  $AUC_{0-\infty}$  and 157.7-237.9% for  $C_{max}$ , all out of the predefined ranges for supporting equivalence, indicating considerable pharmacokinetic interaction between tofisopam being in steady state and midazolam given as a single dose. Similar results were found using the Shuirmann's test. Also statistically significant differences were seen in case of clearance and  $t_{1/2}^{\beta}$  when midazolam was administered with tofisopam compared to the administration of midazolam alone. Tofisopam did not modify the  $t_{max}$  values of midazolam. Based on the results, it can be concluded that significant pharmacokinetic interaction is present between tofisopam and midazolam as the 90% geometric confidence intervals for the test/reference ratios of the main target pharmacokinetic parameters ( $AUC_{0-t}$ ,  $AUC_{0-\infty}$ ,  $C_{max}$ ) were out of the acceptance limits for equivalence (80-125%). A possible explanation of findings from this study is that tofisopam acts competitively for not only the hepatic but also the intestinal CYP3A4 enzyme, which catalyses the metabolism of midazolam on both site. The in vivo significant inhibitory effect of tofisopam on the CYP3A4 in the gut wall was observed based on the 2 to 3-fold increases in AUC of midazolam together with the 1.5 to 2.5-fold increases in midazolam bioavailability. Consequently midazolam clearance and half-life can be influenced by factors that alter hepatic oxidising capacity. The elimination of midazolam is significantly slowing in case of co-administered with tofisopam. Tofisopam does not modify  $t_{max}$ . On the basis of the above mentioned results of the present study, it can be concluded that tofisopam has a significant pharmacokinetic and pharmacodynamic interaction on the single oral dose pharmacokinetic of midazolam due to the possible competitive interaction on hepatic and intestinal CYP3A4. Nevertheless, based

on the FDA recommendation tofisopam could be considered as a moderate inhibitor of CYP 3A4, because it increased the AUC of midazolam, a sensitive CYP3A4 substrate by more than two but less than five fold.

#### 5. *Statistical comparison of the results based on human interaction studies*

Based on the statistical results can be concluded that tofisopam administered in same dose and same duration caused the same effect on the elimination phase of the both probe drugs. Otherwise pronounced significant differences were demonstrated between the AUC and  $C_{\max}$  values ( $p < 0.0001$ ). Follow from all of this results, the significant differences between the  $C_{\max}$  and AUC values are the consequence of the tofisopam inhibitory effect at intestinal level. The explanation of distinction of hepatic/intestinal inhibitory effect is the level of tofisopam plasma concentration in hepatic cell is less than need for more pronounced inhibition.

#### **Conclusions**

- In case of clinical observation the 164-495 %-increase of plasma concentration and bioavailability of tacrolimus caused by tofisopam.
- According to our *in vitro* finding tofisopam inhibited dose dependently the enzyme activity on human recombinant CYP 3A4 supersome. Inhibitory effect of tofisopam was lower than that of ketokonazole (potent CYP 3A4 inhibitor) with an order of magnitude.
- Based on tofisopam-alprazolam interaction study, the tofisopam inhibited moderately (20 %) the CYP-450 3A4 enzyme at hepatic level. Alprazolam is metabolised primarily by hepatic microsomal oxidation. The cytochrome CYP 450 3A4 catalyses the 4 and alpha hydroxylation of alprazolam consequently alprazolam clearance and elimination half-life were changed influenced by tofisopam that alter hepatic oxidising capacity.

- The 20 % high inhibitory effect of nine days long repeated dose tofisopam treatment is closed out the tofisopam inhibition on CYP 450 3A4 enzyme with irreversible manner based on the result from tofisopam-alprazolam human interaction study.
- In vitro evaluation of tofisopam was stated that tofisopam metabolism connects to the CYP 450 3A4 isoenzyme. With this fact together with our results show that tofisopam inhibitory effect on hepatic CYP 450 3A4 enzyme is competitive. The amount of inhibition is 20 %.
- The magnitude of inhibition was not sufficient to explain the pharmacokinetic interaction between tofisopam and tacrolimus. Therefore, the effect of tofisopam on the pharmacokinetics of tacrolimus seems to be more pronounced, that it could be expected considering its effect on the pharmacokinetics of alprazolam.
- Tacrolimus is a substrate of the intestinal CYP3A4; therefore interaction between the two drugs may occur at the intestinal level. A pharmacokinetic/pharmacodynamic drug-drug interaction study was performed to evaluate the effect of tofisopam on the CYP 3A4 activity at the intestinal level.
- Based on results from tofisopam-midazolam interaction study, tofisopam inhibit the CYP450 3A4 enzyme at intestinal level as well. Midazolam was selected as a probe drug to model of tacrolimus since the small intestine is a major place for pre-systemic, CYP3A-mediated metabolism of midazolam after oral administration, which was clearly demonstrated by the discovery of the metabolites during anhepatic period of liver transplantation. The  $C_{max}$ ,  $AUC_{0-\infty}$  and  $t_{1/2}^{\beta}$  values of midazolam increased, and the  $CL_{oral}$  decreased significantly when midazolam was administered with tofisopam compared to the administration of midazolam alone.
- The results of the study indicate that the CYP3A4 inhibitory effect of tofisopam is more pronounced at the intestinal level than in liver.

- According to the literature data, tofisopam inhibits the CYP 450 1A1 enzyme as well which is partly responsible for tacrolimus metabolism. Together with our results, it seems that tofisopam inhibitory effect on this enzyme may play a role in increased levels of tacrolimus in clinical observations.
- Summarizing the results, in the observed clinical cases, the tofisopam–tacrolimus interaction was caused by tofisopam inhibitory effect on tacrolimus metabolism on CYP 450 3A4 enzyme at hepatic and also at intestinal level.
- Based on the FDA recommendation tofisopam could be considered as a moderate inhibitor of CYP 3A4, because it increased the AUC of midazolam, a sensitive CYP3A4 substrate by more than two but less than five fold.
- The concomitant administration of drugs metabolized by the CYP 450 3A4 with tofisopam needs carefulness and attention. The blood concentrations of drugs that are metabolized by CYP3A4 may be increased if they are used in combination with tofisopam.

### **Evaluation of the results from practical point of view**

1. Our results provided the evidence of the tofisopam–tacrolimus drug–drug interaction mechanism which is raised on the clinical observations.
2. We demonstrated with *in vitro* and *in vivo* techniques the existence of drug–drug interaction on CYP-450 3A4 enzyme.
3. Our results mean the predictions to drug interaction with other drugs metabolized by CYP 450 3A4 administered together with tofisopam treatment.
4. Our results support, that the knowing of the metabolism/pharmacokinetic properties of the immunosuppressant is essential for avoidance of drug–drug interactions that lead to the loss of transplanted organs.

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