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Effect of long-term administration of a novel anticonvulsant drug candidate (TP-315) on living organism.

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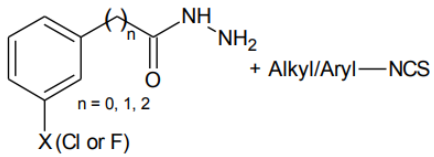
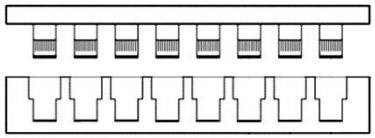
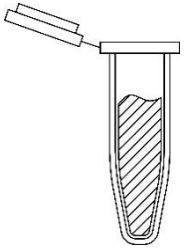


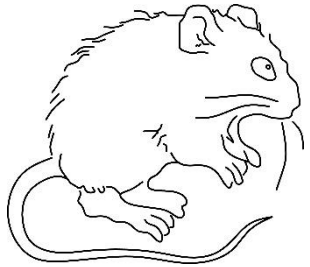
Synthesis of 1,2,4-triazole-3-thione derivatives



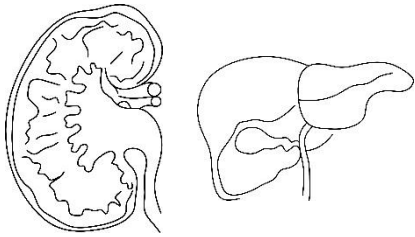
Parallel artificial membrane permeability ASSAY (PAMPA BBB)



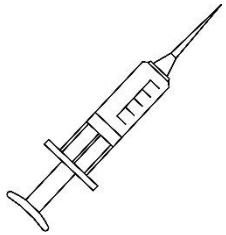
Determination of **CY**P450 activity with VIVID P450 assay kits



Experiments on adult male Albino Swiss mice



Liver and kidneys for histopathological examination



Blood for biochemical tests and HPLC analysis

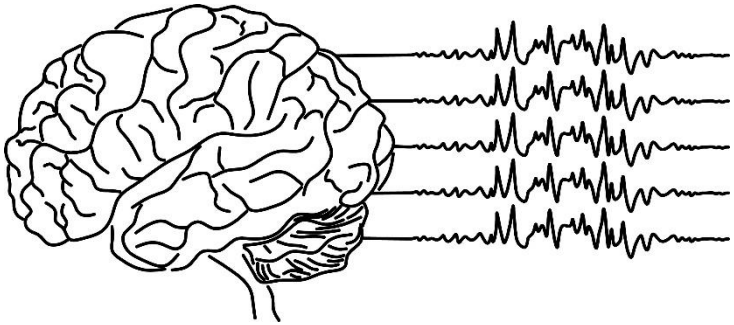
Abstract: Epilepsy is a chronic neurological disorder affecting nearly 65-70 million people worldwide. Despite the observed advances in the development of new antiepileptic drugs, still nearly 30% of patients suffer from the pharmacoresistant form of the disease. In our recent studies, we have identified 4-alkyl-5-aryl-1,2,4-triazole-3-thiones as a promising group of antiepileptic drug candidates acting on the voltage-gated sodium channels. Their anticonvulsant properties were proved in animal models of tonic-clonic generalized seizures (MES test) and in the 6 Hz model of pharmacoresistant epilepsy. Although several preclinical studies confirmed their favorable pharmacological and toxicological profile, little is known about the effects of long-term administration of such compounds on the living organism. In our current studies, using the combined results of PAMPA-BBB, MES and 6 Hz tests, the most promising drug candidate - 5-(3-chlorophenyl)-4-hexyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (TP-315), was selected for further experiments. After prolonged administration of TP-315 to adult Swiss-Albino mice, its effects on functional parameters of internal organs and cytochrome P450 enzyme system were evaluated. On the basis of both histopathological and biochemical examination, it was found that TP-315 does not exhibit hepatotoxic and nephrotoxic effects in mice. Moreover, TP-315 at the concentration determined in the blood of mice, did not significantly affect the activity of the studied CYP450 isoforms.

Keywords: epilepsy; antiepileptic drugs; 1,2,4-triazole-3-thione derivatives

Introduction

Epilepsy is one of the most common neurological diseases in the world. It is estimated that approximately 65 million people in the world, or approximately 1% of the population, suffer from epilepsy. Currently, the number of people suffering from the active form of epilepsy is around 5-10 people in 1,000 [1-3]. Treating epilepsy is primarily based on properly selected pharmacotherapy. Currently used drugs do not have the ability to inhibit epileptogenesis, they only show a symptomatic effect [4]. Currently, there are no drugs on the market that would effectively eliminate the symptoms of drug resistant epilepsy.

In our recent studies, we have demonstrated that 4-alkyl-1,2,4-triazole-3-thiones represent a group of promising antiepileptic drug candidates [5, 6]. Such compounds were active against tonic-clonic seizures and in an animal model of drug-resistant epilepsy. Moreover, we have also found that these compounds are able to improve the anticonvulsant effect of classical antiepileptic drugs [7-9]. However, it should be emphasized that all these previously performed studies concerned 1,2,4-triazole derivatives focused only on the preliminary screening of their anticonvulsant properties. In those studies, the effect of long-term use of the compounds on living organism has not been considered thus far.



The research goal of the studies was **assessment of the effect of the selected drug-candidate TP-315 (5-(3-chlorophenyl)-4-hexyl-2,4-dihydro-3H-1,2,4-triazole-3-thione) on functional parameters of internal organs, and the risk of interaction with drugs metabolized by the cytochrome P450 enzyme system.** The implementation of the studies was helpful to assess the possibility of long-term use of a selected compound through the prism of its impact on the liver and kidney functions, including the risk of interaction with drugs and substances metabolized by the cytochrome P450 enzyme system.

Results and discussion

Synthesis of 1,2,4-triazole-3-thione derivatives

The first stage of the studies was the synthesis of six derivatives of 1,2,4-triazole-3-thione (TP-10, TP315, TP-427, TPF-10, TPF-34, TPR-22) [Fig.1], that turned out to possess the most effective anticonvulsant properties in animal model of tonic-clonic generalized seizures (MES test) and in the 6 Hz model of pharmacoresistant epilepsy. They were synthesized as described in the published articles [5-11].

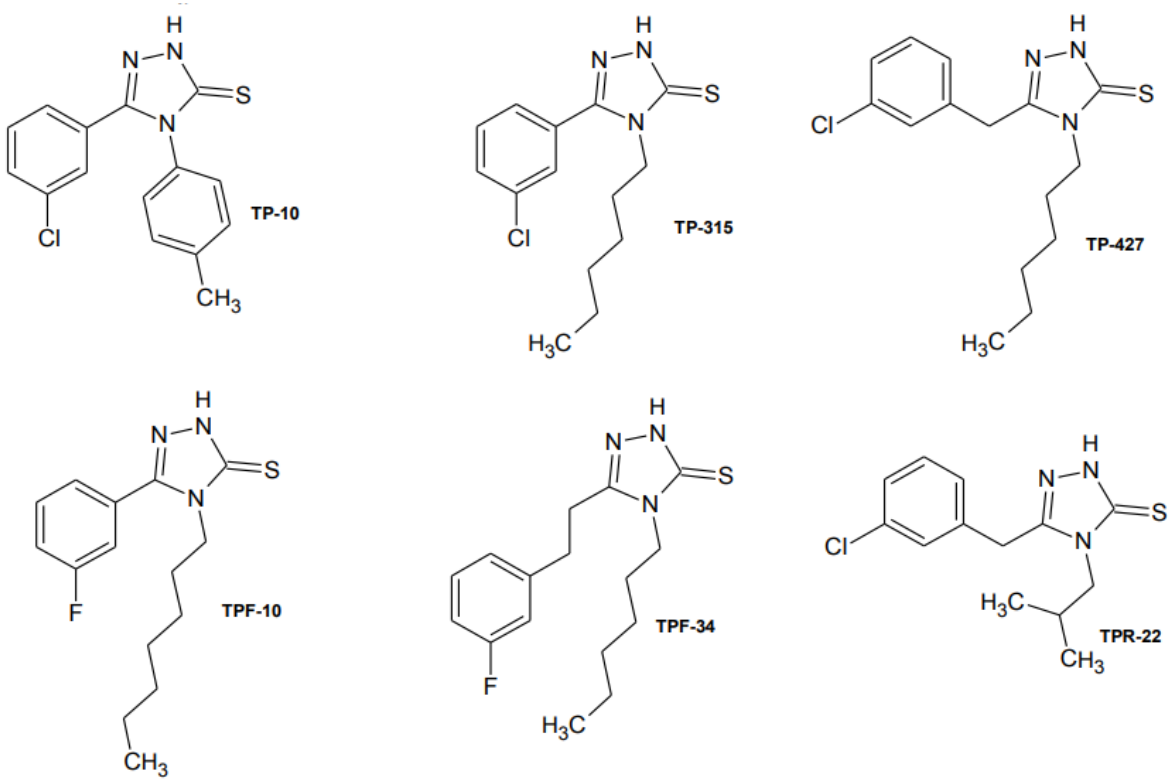


Figure 1. Chemical structures of 1,2,4-triazole-3-thione derivatives selected for further investigations.

Parallel artificial membrane permeability ASSAY (PAMPA BBB)

Blood-brain barrier (BBB) permeability of TP-10, TP-315, TP-427, TPF-10, TPF-34, TPR-22 compounds and valproic acid was investigated by using a PAMPA method (parallel artificial membrane permeability assay). The solutions of each compound were prepared in dimethyl sulfoxide (DMSO) at 4 mg/mL concentration and then diluted with Prisma buffer (pH = 7.4) to obtain the donor drug solution with the final nominal concentration of 20 µg/mL. After performing the experiment according to the manufacturer's procedure, the concentrations of TP-10, TP-315, TP-427, TPF-10, TPF-34, TPR-22 compounds and valproic acid were determined with an UV-reader (Multiskan GO, Thermo Scientific) (at 254 nm) in the donor and acceptor compartments.

Compound	Permeability coefficients ($P_e \times 10^{-6}$) ± SD [cm/s]
Valproic acid	15.37 ± 4.71
TP-10	40,2 ± 5,1
TP-315	2983.50 ± 21.10
TP-427	476.63 ± 39.59
TPF-10	8.19 ± 0.41
TPF-34	54.68 ± 2.14
TPR-22	34.43 ± 8.30

These results confirmed that six examined 1,2,4-triazole-3-thione derivatives can be classified as BBB+, since the compounds with Pe > 5.19 cm/s-characterized by good permeation through BBB [10].

TP-315 has the highest permeability across the blood-brain barrier in comparison to TP-10, TP-427, TPF-10, TPF-34, TPR-22 in vitro tests. TP-315 has also much higher permeability across the blood-brain barrier in comparison to valproic acid (a standard drug used to epilepsy treatment) (Tab.1.).

TP-315 was qualified for further experiments using adult male Albino Swiss mice.

Table 1. In-vitro measurement of the ability of the investigated 1,2,4-triazole derivatives to permeate through the blood–brain barrier (BBB). The permeability values (Pe) were calculated by using the following equation: where VD – donor volume, VA – acceptor volume, Cequilibrium – equilibrium concentration, CD – donor concentration, CA – acceptor concentration, S – membrane area, t – incubation time (180 seconds).

$$P_e = \frac{-ln\left(1 - \frac{C_A}{C_{equilibrium}}\right)}{S \times \left(\frac{1}{V_D} + \frac{1}{V_A}\right) \times t}$$

Experiments on adult male Albino Swiss mice

The experiments were carried out on adult male Albino Swiss mice weighing 20 ± 5g. The mice from the experimental groups were administered with TP-315, which in the PAMPA BBB assay showed the highest permeability across the blood-brain barrier. TP-315 was administered intraperitoneally (i.p.) for 14 days, at the ED50 dose (47,6 mg/kg body mass), determined in the previously conducted preliminary studies [5]. After completion of the experimental procedure (on 15th day - 24h after the last injection), the animals were decapitated. Their livers and kidneys were collected for histopathological examination while blood was drawn for biochemical tests and for HPLC determination of TP-315 in serum.

Determination of liver parameters (ALT, AST) and kidney parameters (urea, creatinine) in the samples from the examined and control mice

The liver parameters ALT (alanine aminotransferase) and AST (aspartic aminotransferase) were determined from the serum. AST is one of the most important indicators of hepatocyte necrosis. Simultaneous determination of AST and ALT allows to determine the degree of liver cell damage. Other serum parameters (i.e., urea and creatinine) determination enable to assess the renal functions.

The differences in ALT, AST, urea and creatinine concentration in the serum of the mice tested were not statistically significant in comparison to the mice from the control group (values significant with $p<0.05$, unpaired t-test) (Figure 2) On the basis of biochemical examination, it was found that TP-315 exhibit neither hepatotoxic nor nephrotoxic effects after long-term administration.

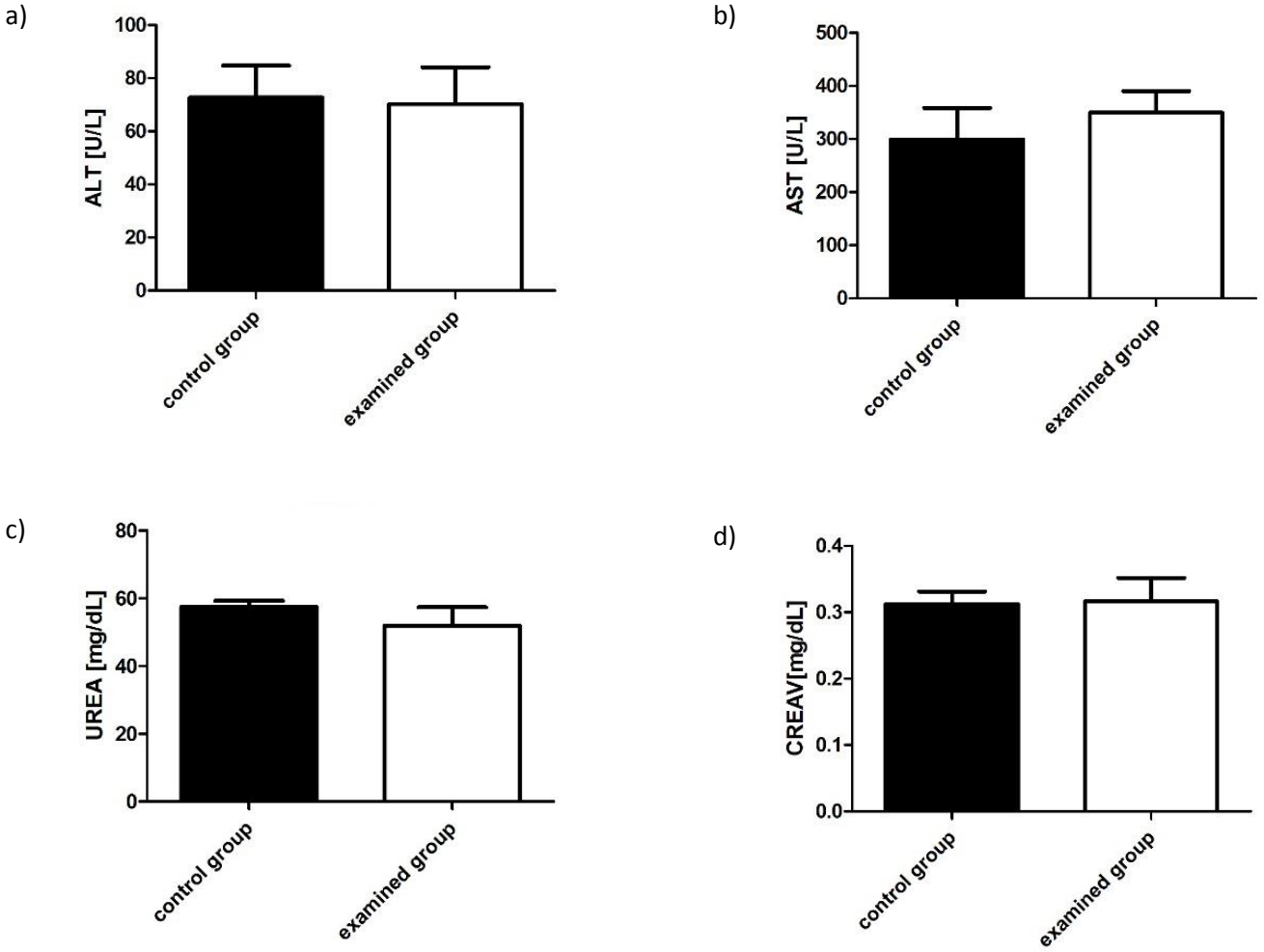


Figure 2. The differences in ALT (a), AST (b), urea (c) and creatynine (d) concentration in the serum of the mice.
The data were plotted as the mean value ± standard error (SD) and analyzed by means of GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical analysis was performed using unpaired t-test (significance was accepted at $p < 0.05$).

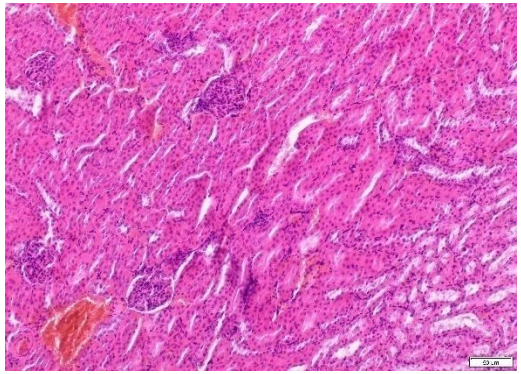


Figure 3. The histopathological structures of mouse' kidney tissues from control group (H&E staining).

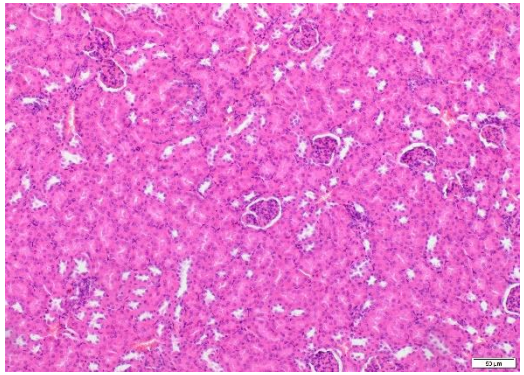


Figure 4. The histopathological structures of mouse' kidney tissues after TP-315 treatment (H&E staining).

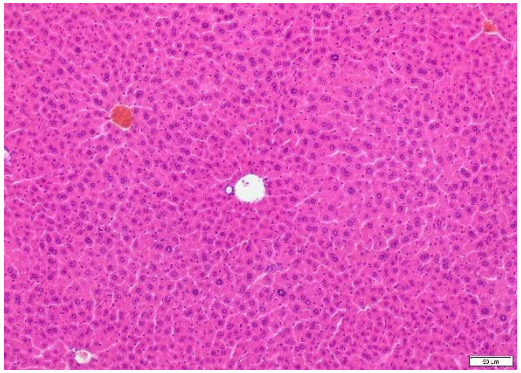


Figure 5. The histopathological structures of mouse' liver tissues from control group (H&E staining).

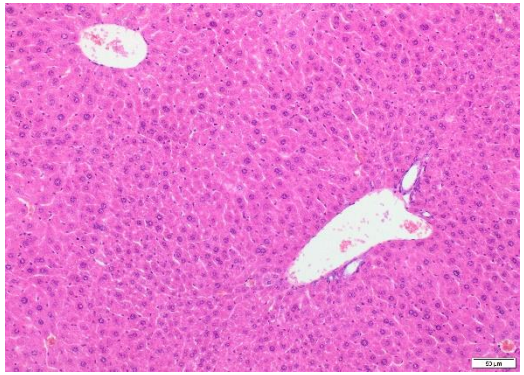


Figure 6. The histopathological structures of mouse' liver tissues after TP-315 treatment (H&E staining).

Histopatological examinations of liver and kidney samples collected from the examined and control mice.

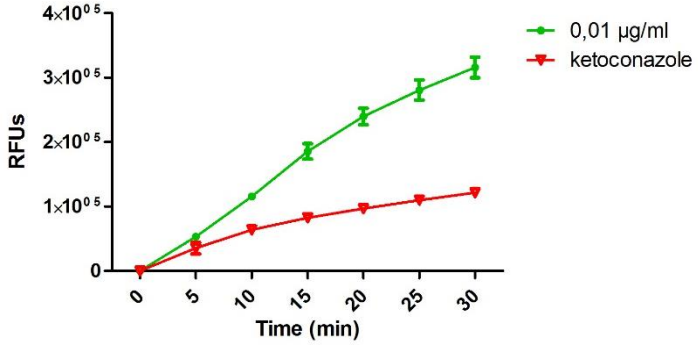
To obtain greater contrast allowing for more accurate analysis of individual tissue and cell structures, hematoxylin and eosin (H&E) staining was used.

There were no differences in the microscopic structure of kidneys from the control and experimental groups. The kidneys showed proper parenchyma shaping in both the cortical and medullary parts. The proximal convoluted tubules with single-layer cubic epithelium were in regularly array. Centrally, in the epithelial cells, a distinct nucleus was visible, surrounded by an eosinophilic cytoplasm. The light of the tubules was star shaped, covered by a striated border. The distal tubules of kidneys from examined group had a regular, round or oval lumen. The epithelial cells had poorly defined borders (Figure 4).

The microscopic image of the liver was similar in mice from the control group and the test group. It was a picture of a normal organ, without pathological changes. Hepatocytes with eosinophilic cytoplasm arranged in hepatic trabeculae. The hepatic trabeculae radiated towards the medial veins. The spaces showed cross-sections through arteries, veins and interlobular bile ducts (Figure 6).

On the basis of histopathological examination, it was found that TP-315 exhibit neither hepatotoxic nor nephrotoxic effects after long-term administration.

a)



b)

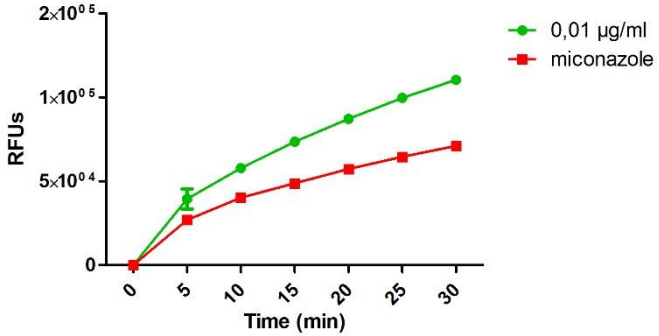


Figure 7. Screening of TP-315 on time-dependent inhibition of (a) CYP3A4 and (b) CYP2B6 at 0,01 µg/mL. Ketoconazole at 10 µM or miconazole at 30 µM were included as positive inhibitors. Data are presented as relative fluorescence units (RFUs). The data were plotted as the mean value ± standard error (SD) and analyzed by means of GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA, USA).

During enzymatic studies, TP-315 was examined in the concentration observed in serum of mice treated for 14 days with a fixed dose of 47.6 mg/kg. **Average concentration of TP-315 measured by HPLC coupled with fluorescence detection was 10,879 ± 0,824 ng/mL (mean ± SD).**

Determination of CYP450 activity with VIVID P450 assay kits

Possible inhibitory effect of the TP-315 on the catalytic activity of human cytochrome P450 enzymes was tested using Vivid P450 screening kits according to the manufacturer's standard procedure (Vivid BOMCC substrate CYP2B6 blue, Vivid BOMCC substrate CYP3A4 blue).

TP-315 at 0,01 µg/mL demonstrated an increase in fluorescence in CYP3A4 and CYP2B6 activity over time (Figure 7a-b).

These results showed that more fluorescence is generated than the positiv control (ketoconazole or miconazole), suggesting TP-315 at 0,01 µg/mL do not have an inhibitory effect on the respective enzymes.

Conclusions

- TP-315 has a high permeability across the blood-brain barrier in in vitro tests (approximately 195 times greater in comparison to valproic acid).
- On the basis of both histopathological and biochemical examination, it was found that TP-315 does not exhibit neither hepatotoxic nor nephrotoxic effects after long-term use in mice.
- TP-315 at 0,01 µg/mL has no inhibitory effect on the CYP2B6, CYP3A4 enzymes.

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Acknowledgments

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