Clinical significance of molecular diagnostic methods in children's individual drug treatment

Ph.D. thesis

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1. Introduction

One percent of Hungarian pediatric population has been reported to suffer from epilepsy, but most of them are treated successfully with anticonvulsants. One of the first choices of antiepileptic therapy is valproic acid (VPA), which is generally well-tolerated, and rarely induces serious side effects. Rare complications may occur in patients treated chronically with VPA, including hepatotoxicity, hematologic disorders, hyperammonemic encephalopathy or neurological toxicity. The risk of serious adverse effects is increased in children, especially in those younger than 2 years of age. The mechanism of VPA- induced toxicity is not clearly understood, but both the parent compound and some of its unsaturated metabolites have been associated with mitochondrial dysfunction and cytotoxicity. In adults, the majority of VPA dose is eliminated as glucuronide conjugate in the urine. Mitochondrial β-oxidation is the second major route of biotransformation, forming 2-ene-VPA, 2,4-diene-VPA and 3-keto-VPA. The cytochrome P450 (CYP) mediated branch of VPA metabolism is the formation of 4-ene- VPA and hydroxylated metabolites (3-, 4-, and 5-hydroxy-VPA metabolites). Kiang *et al.* have demonstrated that CYP2C9 is the major enzyme in CYP-mediated metabolism of VPA, accounting for about 10-15% of the administered dose, whereas CYP2A6 and CYP2B6 play a minor role in VPA metabolism. Although CYP-mediated pathways contribute to a minor part of VPA metabolism in adults (<20% of the administered dose), the CYP-catalyzed oxidation may become the principal route of the metabolism in those special cases when glucuronidation or mitochondrial β-oxidation pathways are compromised or poorly developed, for example, in children. Shifting the metabolic pathways may account for the age-related differences in the incidence of VPA-induced adverse effects.

Hepatic glucuronidation is known to be developmentally regulated. UGTs (uridine 5'diphospho-glucuronyl transferases) involved in VPA glucuronidation are expressed under the adult levels until sometime after 10–15 years of age. VPA and some of its metabolites are considered to be the inhibitors of mitochondrial β-oxidation. CYP-dependent metabolism in children exceeds adult activities, and decreases to adult levels by puberty. As a consequence, larger amount of VPA dose is liable to CYP2C9-dependent metabolism in pediatric patients than in adults. Furthermore, the genetic and nongenetic factors, influencing CYP2C9 activity, can increase the predisposition to VPA-induced

serious adverse reactions; thus, recognition of risk factors can contribute to the avoidance of adverse events.

There have been several clinical studies, investigating relationship between VPA pharmacokinetics and patients' *CYP* genotypes, although clear evidence for the association between VPA serum concentrations and *CYP2C9* genotype has been rarely provided. Statistically significant, but relatively small differences in plasma concentrations of VPA have been observed in patients with *CYP2C9*3* allele comparing to those with two wild type alleles. Although polymorphic *CYP* alleles result in nonfunctional CYP enzymes and permanent poor metabolism, the individuals with functional wild type alleles may become transient poor metabolizers as an effect of internal (e.g., diseases, hormonal status, age) or environmental factors (e.g., nutrition, medication). This means that *CYP* genotype determines the potential for the expression of functional or nonfunctional CYP enzyme. For example, a patient with *CYP2C9*2/*2* or *CYP2C9*3/*3* basically displays poor metabolism of CYP2C9 substrates, whereas a subject carrying *CYP2C9*1/*1* possesses the potential for having functional CYP2C9 enzyme. However, nongenetic factors, such as co-medications or co-morbidities give rise to altered phenotypes. Thus, *CYP2C9*1/*1* genotype, predicted to be translated to an extensive metabolizer phenotype, may be switched into poor metabolism due to phenoconversion, which eventually influences the patient's response to VPA. Furthermore, the genotype-phenotype mismatch results in more poor metabolizers than it would be predicted from *CYP2C9* genotype.

A patient's CYP-status can be estimated by the evaluation of *CYP* genotypes and current CYP expression. The complex diagnostic system CYPtest^{TM} can determine drug-metabolizing capacity by combining *CYP* genotypes and current CYP expression in leukocytes. CYP2C9 mRNA levels in leukocytes of those subjects who do not carry loss-of-function mutations in *CYP2C9* gene was proven to reflect the hepatic tolbutamide hydroxylation activity selective for CYP2C9. A preliminary *CYP2C9* genotyping for *CYP2C9*2* and *CYP2C9*3* can identify the genetically determined poor metabolism of CYP2C9 enzyme, and then CYP2C9 expression in leukocytes of patients with wild type alleles (*CYP2C9*1/*1*) can estimate a reduced or even increased CYP2C9 activity resulted by nongenetic variations.

2. Objectives

The conventional VPA therapy is symptom-driven, which means, we increase VPA dose till seizure-free period, and decrease dose, if we see side effects.

Our present work aimed to prospectively investigate whether preliminary assaying of CYP2C9-status (CYPtest^{TM}) and CYP2C9-status guided VPA therapy have potential clinical benefit for pediatric patients, and whether personalized drug therapy can reduce the risk of VPA misdosing-induced adverse reactions.

Our basic questions were:

- Is there any importance of CYP2C9 expression in VPA pharmacokinetics in children? Can CYP2C9 genotyping alone predict poor VPA metabolism?
- Is the CYP2C9 polymorphism of the Hungarian epileptic children similar to those of the Caucasian population?
- Is there any correlation between CYP2C9-status (genotype and expression) and blood levels of VPA?
- Can patients' CYP2C9-status predict optimal dosage requirements of VPA?
- Is CYP2C9-status guided VPA therapy effective in pediatric patients?
- Is CYP2C9-status guided VPA therapy reduce the incidence of side effects?

3. Methods

According to our aims, we have planned two studies. First, we investigated the children's CYP2C9 genotype and expression, and attempted to demonstrate relationship between CYP2C9-status and blood levels of VPA.
In the second study, we wanted to know, whether preliminary assaying of CYP2C9-status and CYP2C9-status guided VPA therapy have potential clinical benefit for pediatric epileptic patients.

Pediatric patients suffering from epilepsy diagnosed with partial or generalized seizures were enrolled in both of the studies carried out at Heim Pál Children's Hospital and at the 2nd Department of Pediatrics, Semmelweis University (Budapest, Hungary). We recruited novel epileptic patients, younger than 15 years of age. The patients on non-VPA therapy or on multidrug therapy were excluded from the study. The patients were also excluded if their VPA therapy was interrupted. The parents or representatives of each pediatric patient gave their informed consent to participate in this study. The studies were approved by the Hungarian Committee of Science and Research Ethics, and the Committee of Ethics of Heim Pál Children's Hospital.

First study

In this study, we investigated CYP2C9-status of pediatric patients younger than 15 years of age and its influence on the steady-state serum concentrations of VPA as well as on patients' dose-requirements. We attempted to provide evidence for that *CYP2C9* genotype is not the only determinant factor in CYP2C9 metabolizer status of a patient, but the expression rate of the wild type gene can highly influence a patient's CYP2C9 metabolizing capacity and his/her response to a drug.

Pediatric patients (n = 50) suffering from epilepsy diagnosed with partial or generalized seizures were enrolled in the study, who were CYP2C9 tested at the beginning of antiepileptic therapy. The patients' demographic data, as well as the details of anticonvulsant therapy were recorded. The patients (boys/girls: 20/30) were at the average age of 6.75 years (range: 0.5–15 years), and all of them belonged to the Caucasian white population. Blood samples for CYP2C9 testing were taken before the beginning of

anticonvulsant therapy. The patients were not given any other medication, but VPA as monotherapy, and the target dose was adjusted to the patients' body weight according to the clinical protocol. The therapy was initiated at low dosages (10–15 mg/kg), and the target doses were subsequently titrated until optimal clinical response was achieved, generally within 5–10 days. Blood samples for drug assays were taken 2 and 4 weeks after the beginning of VPA treatment. The sampling at the 2nd week was applied for checking VPA serum concentration, and the dose was modified if the exposure exceeded the target range of VPA concentration. The serum levels measured at the 4th week were considered to be the stable steady-state concentrations, whereas the doses applied for the stable VPA concentrations were considered to be the maintenance doses.

Second study

In this study, we prospectively investigated whether preliminary assaying of CYP2C9-status and CYP2C9-status guided VPA therapy have potential clinical benefit for pediatric patients, and whether personalized drug therapy can reduce the risk of VPA misdosing-induced adverse reactions.

Pediatric patients (N = 99) with epilepsy diagnosed with partial or generalized seizures were enrolled in the study. We recruited patients, younger than 15 years of age, who were newly diagnosed with epilepsy and were to have VPA therapy. The patients were excluded if their VPA therapy was interrupted. The patients' clinical data were collected between 2006 and 2014. Patients before 2010 were on conventional VPA therapy (control group, 47 patients; boys/girls: 31/16; average age of 8 years). From 2010, each child (N=52 boys/girls: 24/28; average age of 6.25 years) was CYP2C9 tested at the beginning of antiepileptic therapy, and the dosage of VPA was assigned by the results of the first research. The patients' demographic data and the details of anticonvulsant therapy were recorded. The patients were not given any other medication, but VPA as monotherapy (Convulex or Depakine). The hematologic (red and white blood cell and platelet counts) and biochemical parameters (serum alkaline phosphatase, aspartate transaminase, alanine transaminase, gamma-glutamyl transferase, calcium, and phosphorus) were checked at the beginning of the anticonvulsant therapy and monitored regularly. Blood ammonia level was not routinely assayed, only if indicated by any symptom. All signs of adverse reactions due to the VPA treatment were reported, and were classified as mild (somnolence, fatigue, enuresis, or hair loss) or severe

(hyperammonemia, hematologic disorders, or confusion) side effects.

CYPtesting

The patients' CYP2C9-status was determined by CYP2C9 genotyping and by assaying CYP2C9 expression in leukocytes before the beginning of anticonvulsant therapy. Genomic DNA and leukocytes were isolated from the samples of peripheral blood according to the methods described by Temesvári et al. (2012). CYP2C9 genotyping was carried out by hydrolysis single nucleotide polymorphism (SNP) analysis for *CYP2C9*2* and *CYP2C9*3* using TaqMan Probes (BioSearch Technologies, Novato, CA, U.S.A.). For assaying CYP2C9 expression, total RNA was isolated from leukocytes, RNA was reverse-transcribed into single-stranded complementary DNA (cDNA), and real-time polymerase chain reaction (PCR) with human cDNA was performed using a Universal Probe Library (UPL) probe for CYP2C9 (Roche Diagnostics, Mannheim, Germany). The quantity of CYP2C9 messenger RNA (mRNA) relative to that of the housekeeping gene glyceraldehyde 3–phosphate dehydrogenase was determined. Three categories of CYP2C9 expression were distinguished to describe low, normal, and high expressers. The cutoff values for the CYP2C9 mRNA levels in leukocytes were established previously on the basis of the cutoff values for the hepatic CYP2C9 activity (tolbutamide hydroxylation), allowing a distinction between low, normal (medium), and high expressers ($5x10^{-6}$ and $2.5x10^{-5}$, respectively).

Serum VPA assay

The blood samples for drug assays were taken from the patients before the morning dose, and VPA concentrations were assayed every second week after the beginning of VPA treatment. The steady-state serum concentration of VPA was determined by the fluorescence polarization immunoassay method (AxSYM Valproic Acid Assay; Abbott Laboratories, Abbott Park, IL, U.S.A.). The VPA concentrations between 40 and 100 μg/ml were considered to be in the reference range. The 2-week sample was used for checking VPA serum concentration, and the dose was modified if exposure was outside the target range. The serum levels measured at the fourth week were considered to be the stable steady-state concentration, and the dose prescribed at this time was considered to be the maintenance dose.

VPAdosing

In CYPtest group (N = 52), the patients' anticonvulsant therapy was guided by their CYP2C9-status: (1) non-VPA therapy was proposed for the children with two mutated CYP2C9 alleles, and (2) VPA therapy adjusted to the patients' CYP2C9-status (CYP2C9 genotype and CYP2C9 expression) was applied in children with one or two wild-type alleles. Normal dose (30–40 mg/kg) was given to the normal CYP2C9 expresser patients carrying CYP2C9 homozygous wild genotype (*CYP2C9*1/*1*), reduced dose (10–20 mg/kg) was administered to the children with heterozygous genotypes (*CYP2C9*1/*2* or *CYP2C9*1/*3*), or to low CYP2C9 expressers, whereas increased dose (>40 mg/kg) was targeted in high expresser patients with *CYP2C9*1/*1* genotype. In the control group (N = 47), the target dose of VPA was adjusted to the patients' body weight (20–40 mg/kg) according to the standard clinical protocol, and was modified if the signs of adverse reactions or nonresponsiveness required.

Statistical analysis

First study

The serum concentration values of VPA were normalized by the dose and the body weight, and expressed as (μg/ml) × (mg dose/kg body weight)$^{-1}$. The data of normalized VPA concentrations and dose-requirements for the optimal therapeutic level in the groups with various CYP2C9-statuses were expressed as the median (and range). Between-group differences were calculated by the use of Kruskal-Wallis test followed by Dunn's multiple comparisons test. A P-value of ≤ 0.05 was considered statistically significant.

Second study

Statistical analysis of biochemical and hematologic parameters as well as VPA serum levels was carried out using GRAPHPAD INSTAT (v3.05, GraphPad Software, San Diego). Parameter distributions were analyzed by Kolmogorov-Smirnov test. Between-group differences were calculated by Mann-Whitney U-test. The benefit of CYP2C9-status guided VPA therapy over the classical dosing was also evaluated, comparing the ratio of patients with therapeutic VPA serum levels, normal hematologic, or biochemical parameters in the CYPtest group with those in control group. The frequencies of adverse events were also compared. The differences between the two groups were calculated by the

Fisher's exact test. A P-value of ≤0.05 was considered statistically significant.

4. Results

CYP-status of pediatric patients

Of 58 pediatric patients aged between 0.5 and 15 years, all, except for one, expressed at least one functional *CYP2C9* allele, and 70% of patients carried *CYP2C9*1/*1* genotype. The patient with two loss-of-function alleles was not enrolled in the further investigation, since he was on non-VPA therapy. 30 % of patients carried one of the polymorphic variant alleles (*CYP2C9*2* or *CYP2C9*3*). The frequencies of *CYP2C9*2* and *CYP2C9*3* alleles in patients (9 and 6%, respectively) were similar to those in Caucasian (white) populations (11 and 7%, respectively). CYP2C9 expression assays revealed that almost half of the patients (44%) were normal CYP2C9 expressers, and substantial portion of the patients (56%) were low expressers. Two of the children displayed high CYP2C9 expression, and switched to another antiepileptic drug therapy (because of VPA inefficiency). Besides two high expresser patients, 5 other children were excluded from the further evaluation because of missing data, or starting non-VPA therapy.

On the basis of CYP2C9-status (*CYP2C9* genotypes and CYP2C9 expression), the remaining 50 patients were grouped into two main categories – homozygous wild (*CYP2C9*1/*1*) and heterozygous *CYP2C9*1/mut* genotypes (*CYP2C9*1/*2* or *CYP2C9*1/*3*), – and subdivided into two subgroups: normal (medium) and low CYP2C9 expressers. Although patients with two wild type alleles are generally considered to be extensive metabolizers, merely 12 children of 35 patients with *CYP2C9*1/*1* genotype were found to be normal CYP2C9 expressers, whereas the other 23 patients were low expressers, predicting poor CYP2C9 metabolism. This is particularly unusual, since CYP expression of children is usually significantly higher than that of adults. Furthermore, the group of patients with heterozygous *CYP2C9*1/mut* genotypes comprised both low and normal CYP2C9 expressers (4 and 11 patients, respectively). It is not surprising, since the mutant alleles are transcribed into CYP2C9 mRNA; however, their expression rates are modified by nongenetic factors, such as nutrition, food additives or hormonal status, similarly to the wild type allele. Co-medication as a nongenetic factor can be excluded, since the children on multidrug therapy were not enrolled in the present study.

Patients' VPA exposure and dose-requirement

The statistical analysis displayed significant association between the patients' CYP2C9-status and the steady-state serum levels of VPA normalized by the dose and the body weight. The normalized serum VPA concentrations were significantly lower in the normal expresser patients with *CYP2C9*1/*1* genotype (2.12 (μg/ml)×(mg dose/kg bw)$^{-1}$) than in low expressers (5.13 (μg/ml) × (mg dose/kg bw)$^{-1}$) or in patients carrying any polymorphic CYP2C9 alleles (*CYP2C9*2* or *CYP2C9*3*) (4.33 (μg/ml) × (mg dose/kg bw)$^{-1}$) for normal CYP2C9 expressers and 5.54 (μg/ml) × (mg dose/kg bw)$^{-1}$ for low expressers). The low expressers and the patients with polymorphic *CYP2C9* alleles showed about two- to threefold higher normalized serum VPA levels as compared with normal expresser patients carrying *CYP2C9*1/*1* genotype. The difference in normalized serum concentrations was not statistically significant between the patients with heterozygous genotypes (*CYP2C9*1/*2* or *CYP2C9*1/*3*) and those low expressers with two functional alleles (*CYP2C9*1/*1*). Moreover, no significant difference in normalized serum levels was observed between normal and low expressers with heterozygous *CYP2C9* genotypes.

According to the clinical practice, VPA serum concentrations ranged between 40 and 100 μg/ml are considered to be therapeutically optimal in the management of epilepsy. The low expresser patients or subjects with heterozygous genotypes required significantly lower dose of VPA for the optimal serum level than normal expressers carrying *CYP2C9*1/*1* genotype. The dose-requirement of VPA for the target serum level was similar for the low expressers and for the patients carrying polymorphic *CYP2C9* alleles (17.8 mg/kg for low expressers carrying *CYP2C9*1/*1*; 16.7 mg/kg for normal expressers with heterozygous genotype; 13.8 mg/kg for low expressers with heterozygous genotype). The conventional clinical practice is to target the VPA dose of 30 to 40 mg/ kg in children. The conventional dosing approach was appropriate for normal CYP2C9 expresser patients with *CYP2C9*1/*1* genotype, comprising 24% of the children in the study. The *CYP2C9* genotype controlled VPA dosing would have targeted reduced VPA dose for 30% of the patients, for those carrying heterozygous *CYP2C9*1/mut* genotypes. However, low expressers with *CYP2C9*1/*1* genotype also required reduced VPA dose for the optimal serum concentration. CYP2C9 phenoconversion substantially increased the number of children (to 76%) on reduced VPA dose.

Multiple comparison analysis showed that CYP2C9-status (*CYP2C9* genotype and

CYP2C9 expression) influenced the serum concentrations of VPA as well as the dose-requirements for the optimal serum concentration in pediatric patients. However, low CYP2C9 expression in patients with homozygous wild genotype seemed to display similar effects on VPA exposure and dose-requirement to those carrying polymorphic *CYP2C9* alleles (*CYP2C9*2* or *CYP2C9*3*). Consistently, the serum VPA concentration and dose-requirement of the children carrying two wild type *CYP2C9* alleles (*CYP2C9*1/*1*) were found to be influenced by the CYP2C9 expression, whereas loss-of-function mutations in *CYP2C9* gene resulted in poor metabolism of VPA independently on the degree of CYP2C9 expression.

Potential benefit of CYP2C9-status guided VPA therapy

In CYPtest group of the second study, antiepileptic therapy was adjusted to the patients' CYP2C9-status, whereas the children of the control group were on conventional antiepileptic therapy. One patient in CYPtest group showed homozygous mutation, and was excluded from the study.

For 74.5% of the patients in the CYPtest group (N=51), who were low CYP2C9 expressers or normal expressers with mutant *CYP2C9* alleles, reduced VPA dose (10–20 mg/kg) was targeted, whereas normal target dose (30–40 mg/kg) was administered to the normal expresser patients with *CYP2C9*1/*1* genotype (23.5% of the patients) and increased dose (>40 mg/kg) was applied to the high expresser child carrying *CYP2C9*1/*1* genotype. Despite the fact that reduced VPA dose was administered to most of the children in the CYPtest group, the therapeutic efficacy (seizure frequency) was similar to those in the control group of patients on conventional VPA therapy. Although the average VPA serum concentrations in the CYPtest group did not differ significantly from those in the control group, the CYP2C9-status guided VPA dosing substantially reduced the number of patients displaying VPA serum levels out of the therapeutic range (9/51 vs. 21/47). Moreover, the deviation of VPA concentrations from the therapeutic range (40–100 µg /ml) was significantly lower in the CYPtest group than in the control group.

For recognizing the early signs of adverse reactions, biochemical and hematologic parameters were followed in both groups from the beginning of anticonvulsant therapy. Before the beginning of antiepileptic therapy, the liver function parameters, serum levels of calcium and phosphorus, as well as red blood cell, white blood cell, and platelet counts were in the reference ranges in all patients. One month after the beginning of VPA

therapy, no significant alterations were observed in red or white blood cell and platelet counts. Biochemical parameters of patients in both groups were also in reference ranges, except for the alkaline phosphatase. The patients in the control group displayed a significant increase in serum alkaline phosphatase activity, which is generally associated with either hepatotoxicity or bone metabolic disorders. Nevertheless, the levels of neither serum transaminases and gamma–glutamyl transferase nor calcium and phosphorus in control patients differed from those parameters in the CYPtest group. Serum alkaline phosphatase levels show great variation with age in children; however, the substantial increase within 1 month was attributed to VPA treatment rather than to the transient hyperphosphatasemia. The age of the control patients was considered similar to that of the children in the CYPtest group, whereas serum alkaline phosphatase activities exceeded the normal range in almost half of the control patients and merely in 4% of the CYPtested patients. Furthermore, VPA-associated serious adverse effects were observed more frequently in the control group than in the CYPtest group. Hyperammonemia (>50 μmol/L), the most frequent side effect in control patients (17%), was always accompanied by elevated levels of alkaline phosphatase (higher than 750 unit/L or even >1,200 unit/L) and with other adverse reactions, such as somnolence, fatigue, consciousness, or behavior disturbances. Furthermore, serum VPA concentrations in the patients with increased ammonia levels were found to be higher than 100 μg/ml. It should be mentioned that the prevalence of mild side effects, including weight gain, hair loss, enuresis, and somnolence, was significantly less in the CYPtest group (P=0.05).

5. Conclusions

The optimal serum concentration of VPA is strongly influenced by the patients' VPA metabolizing capacity, which is also critical to avoid the therapeutic failure or toxicity of VPA. According to our knowledge, CYP-mediated oxidation is not the major route of VPA metabolism in adults; however, our present work clearly demonstrated that CYP2C9 played a prominent role in children younger than 15 years of age.

Comparing to the conventional clinical practice, the *CYP2C9* genotype-based medication may bring some benefit to children on VPA therapy; however, metabolic activity of CYP2C9 is often overestimated by the prediction from the patient's *CYP2C9* genotype. The major source of overestimation is CYP2C9 phenoconversion that can be attributed to the CYP2C9 downregulation by cytokines in epilepsy. Thus, prospective investigation of pediatric patients' genetic and nongenetic variations in CYP2C9 allows prediction of potential 'poor metabolizers' carrying *CYP2C9* alleles with loss-of-function mutations or displaying low CYP2C9 expression. CYP2C9-status controlled medication may facilitate the improvement of the individual VPA therapy, leading to the dosage optimization for a more effective therapy and minimizing the risk of side effects. CYP-status guided VPA therapy has been demonstrated to be able to reduce the side effects and improve the safety of anticonvulsant therapy in one of the most vulnerable patient populations.

We concluded from the results:

- *CYP2C9* genotyping of pediatric patients was able to predict VPA poor metabolism in approximately 30% of patients. Inferring patients' valproate metabolizing phenotype merely from *CYP2C9* genotype results in false prediction. Additional information on CYP2C9 expression is very important in epileptic children.
- The frequencies of *CYP2C9*2* and *CYP2C9*3* alleles in Hungarian children suffering from epilepsy were similar to those in Caucasian (white) populations.
- Phenoconversion of CYP2C9 was demonstrated (expression was downregulated) in two third of epileptic children without polymorphic allele, probably due to cytokine release in epilepsy.
- There is a significant association between the patients' CYP2C9-status and the steady-state serum levels of VPA normalized by the dose and the body weight.

These findings also confirm that the major enzyme of VPA metabolism in children is CYP2C9.

- Our major findings indicated that personalized anticonvulsant therapy can be applied in pediatric patients by revealing the allelic variants in *CYP2C9* gene and the current CYP2C9 expression in patients' leukocytes.
- CYP2C9-status guided VPA treatment is clinically as effective as conventional antiepileptic therapy.
- The tailored VPA treatment can contribute to the avoidance of misdosing and potential adverse reactions in pediatric patients.

6. Bibliography of the candidate's publications

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