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ORIGINAL ARTICLE

Correlation of inter-individual variations of amitriptyline metabolism examined in hairs with CYP2C19 and CYP2D6 polymorphisms

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Abstract The metabolite ratio of amitriptyline (AT), nortriptyline (NT) and its 10-hydroxy metabolites (E10-OHAT, Z10-OHAT, E10-OHNT and Z10-OHNT) was examined by liquid chromatography-mass spectrometry in hair samples of 23 white infants after long-term administration of AT. High inter-individual variation of the metabolite ratios were observed (e.g. NT/AT=0.8–8.1, E10-OHNT/Z10-OHNT= 1.6–10.3). The significance of these variations was proven by confirmation of the temporary stability of these ratios within a hair fibre. Moreover, an association of the metabolic phenotype with genetic disposition was observed. The genotypes of CYP2C19 (alleles *2, *3 and *4) and of CYP2D6 (*3, *4, and *6) were examined by conventional polymerase chain reaction genotyping experiments. The relative amount of demethylation (NT/AT) is clearly affected by the number of functional alleles of CYP2C19. The demethylation capacity of CYP2C19 poor metabolizers (3 individuals, compared to 15 extensive metabolizers) was 4.3 times depleted. Moreover, the selectivity of hydroxylation (e.g. E10-OHNT/Z10-OHNT) is significantly correlated with CYP2C19.

Keywords Amitriptyline · Pharmacogenetics · CYP polymorphism · Drug-facilitated crime · Segmental hair analysis

Introduction

Individual biotransformation of amitriptyline and genetic polymorphism

The impact of individual variations of drug biotransformation on pharmacological and toxicological effects is attracting increasing attention. Due to the genetic determination of many enzymatic biotransformation processes, the correlation of genetic disposition and metabolic phenotype is increasingly being investigated. The group of tricyclic antidepressants is of particular interest because of the high social prevalence, high dosages and complex metabolism. In particular, some of the classic antidepressants (e.g. amitriptyline, nortriptyline or doxepine) where found to show large inter-individual metabolic variations which proved to be due to genetic polymorphism of the enzymes involved in biotransformation [1–3]. The two predominant metabolic steps of amitriptyline (AT) biotransformation are demethylation to desmethylamitriptyline (nortriptyline, NT) and didesmethylamitriptyline and aliphatic ring hydroxylation to 10-OHAT and 10-OHNT. Hydroxylation yields relevant amounts of Z and E conformations (Fig. 1), each representing a mixture of (+) and (−) enantiomers. The *N*-demethylation of AT is described to be mainly governed by the CYP2C19 gene as observed in both in vivo and in vitro experiments [4–7], while an additional substrate concentration-dependent contribution of the CYP3A4 gene was confirmed by in vitro experiments [8].

The stereo-specific formation of E isomers of 10-hydroxyamitriptyline (E10-OHAT) and 10-hydroxynortrip-

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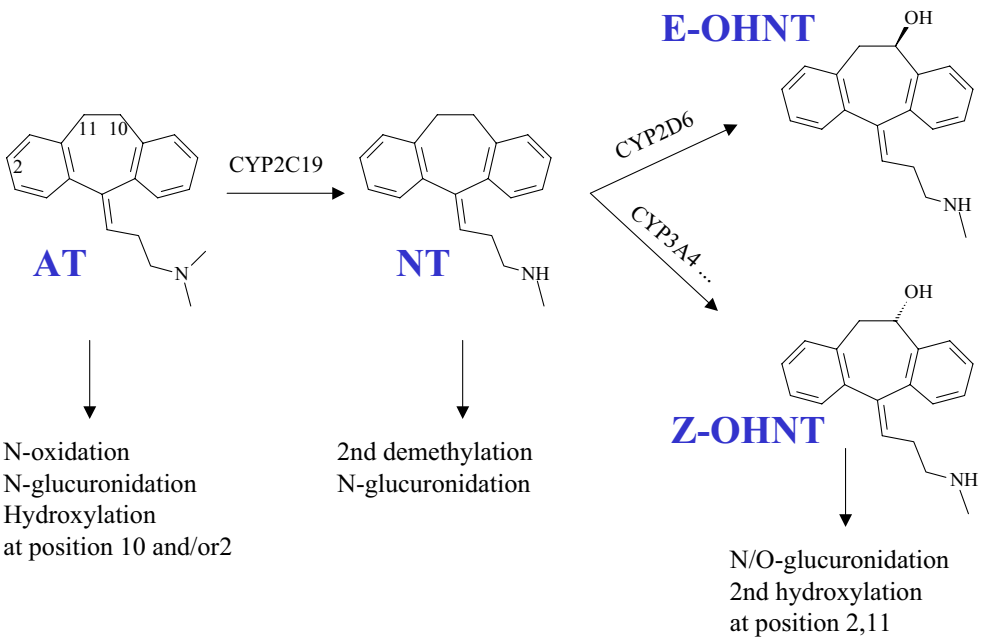

Fig. 1 Amitriptyline is mainly biotransformed to desmethyl and hydroxymetabolites by the enzymes CYP2C19 and CYP2D6. Other metabolic reactions (e.g. glucuronidation) can be ignored in hair analysis because respective compounds are not sufficiently incorporated into hair due to their high polarity

tyline (E10-OHNT) was attributed to a stereo-selectivity of CYP2D6 [4, 9, 10]. Moreover, the predominance of the (−) enantiomer in blood is due to a selective glucuronidation leading to a rapid renal excretion of the (+) enantiomer [11].

Polymorphism of CYP2D6 and CYP2C19

The CYP2D6 gene is highly polymorphic and encodes the best characterized cytochrome P450 enzyme, and more than 70 alleles have been identified. With regard to the enzyme activity of CYP2D6, four phenotypes can be distinguished: poor (no enzyme activity), intermediate (decreased enzyme activity), extensive (normal enzyme activity) and ultra rapid (increased enzyme activity) metabolizers. The poor metabolizer (PM, 7–10% of the European population) has no functional CYP2D6 gene, and in contrast a part of the ultra rapid metabolizers (UM, 7% of the European population) have three or more functional genes of this enzyme. The intermediate metabolizer carries two alleles which encode for enzymes with decreased activity. Whether individuals with one null allele and one wildtype allele are also intermediate metabolizers is controversial [12, 13].

The CYP2C19 gene also shows polymorphisms which influence the activity of this enzyme. There are three different phenotypes: poor (no enzyme activity), intermediate (decreased enzyme activity) and rapid extensive (normal enzyme activity) metabolizers. For rapid extensive metabolizers (RM), both alleles have no mutation, intermediate metabolizers (IM) have one functional and one dysfunctional allele, and the PMs (ca. 2% of the European population) have two dysfunctional alleles [14, 15].

Quantitative aspects of hair incorporation

There is a common consensus that lacking correlation between dose (serum concentration) of drugs and hair concentration is one of the most striking limitations in hair analysis. The substance incorporation into hair is generally controlled by two complementary pathways, i.e. inclusion into the hair bulb via blood or permanent absorption into the hair shaft from sweat and sebum. Both processes will usually coincide and cannot be sufficiently differentiated by analytical means (e.g. decontamination). Hair concentrations can therefore be affected by hair structure (pigmentation), individual physiological or pathological situations and are positively determined by the chemical structure of the compound. Hair incorporation is performed best by unpolar and basic drugs. Incorporation of polar metabolites is typically suppressed. In cases where metabolism leads to more polar and basic compounds, the general perspective is unclear. However, these limitations are not relevant within an individual hair sample, and hair profiling enables the reconstruction of administration cycles by examination of concentrations in adjacent segments of individual hairs or hair strands [16]. Moreover, corresponding metabolites are assumed to be similarly affected by external parameters, and concentration ratios may be used as reliable indicators for substance incorporation or administration pathways. The presence of relevant amounts of cocaine metabolites (e.g. benzoylecgonine/cocaine >0.05) may be used as a means to discriminate between external contamination and drug abuse. Similarly, significant differences of metabolite ratios in hair were assumed to be suitable to indicate individual variations of drug metabolism.

Study cohort

The illegal administration of drugs (unknown amounts) to a group of children by a child minder was suspected in a case of apparent accumulation of side effects. Hair samples appeared to be the only eligible specimen for investigations because the analyses started 3 weeks after the presumed termination of drug administration. Obvious drawbacks of these circumstances are lacking information on duration and amounts of drug intake. Side effects (e.g. diarrhoea, mouth dryness, dizziness, somnolence, constipation, speech disorders, anxiety, perspiration) were occasionally reported by the parents but are probably biased. The retention period of the affected children in the day care ranged from 4 weeks to 3 years. However, several diagnostic advantages result from their particular age (2–6 years) because the genetic disposition of metabolism is not affected by co-administration of other pharmaceutical substances, drugs of abuse, alcohol or nicotine. Moreover, an existing influence of age on pharmacokinetics can be neglected.

Materials and methods

Hair analysis

Sample preparation

Hair samples were collected according to the standard procedure for drug testing, i.e. by cutting hair strands close to the root and subsequent fixation. The initial weight of a hair bundle (default length 6 cm) was 50 mg. Samples were decontaminated by 5 min agitation in a gas-tight tube with 5 ml petroleum benzene (boiling range to 40°C, Merck, Germany), dried and cut into lengths of 1–2 mm. After adding 100 ng of the internal standard 5-(4-methylphenyl)-5-phenyl hydantoin [MPPH, research grade, Serva, Germany], the hair particles were extracted by 3 h ultrasonification at 55°C with 3 ml of methanol (for chromatography, Merck).

For hair profiling experiments, the amount of hair was reduced to individual hairs which were segmented into pieces of 5 mm. The segmentation of individual hairs required fixation using adhesive tape. The mass of single segments could not be examined by conventional laboratory devices; therefore hair concentrations were expressed as amount per centimeter (picogram per centimeter) to avoid the uncertainty of a conversion based on average hair masses (~1–6.4 μg/mm). The resulting hair snippets were transferred into vials containing 5 ng of the internal standard (MPPH) in 30 μl of a mixture of water and methanol (50:50, *v*/*v*). After 3 h ultrasonification at 55°C, the vials were analysed by liquid chromatography-mass spectrometry (LC-MS) at ambient temperature (20±2°C).

Liquid chromatography-electrospray-mass spectrometry

All analyses were carried out using an 1100 LC system (binary pump and autosampler, Agilent, CA, USA) coupled to an API 4000 mass spectrometer (Applied Biosystems, CA, USA), equipped with a Turbo-Ion-Spray (ESI) source. The instrument software Analyst (ver. 1.4.2) was used for data processing. Optimum ionisation and fragmentation conditions of AT, NT (Promochem, UK), hydroxyamitriptyline, E10-hydroxynortriptyline and Z10-hydroxynortriptyline (Sigma, MO, USA) are summarised in Supplementary Table 1.

Due to the identity of their mass spectra, the detection of the hydroxy-metabolites requires a chromatographic gradient separation of the conformers (Supplementary Fig. 1), which was performed on a Zorbax Eclipse RP 18 column (4.6*75 mm, 3.5 μm particle size). The mobile phase constituents were water (A, for chromatography, Merck) and acetonitrile (B, gradient grade, Baker, Germany) containing 2 mM ammonium acetate buffer (ACS certified, Baker), which were mixed to the following gradient: 0–1 min, 10%B; 1–6 min, 10–90%B; 6–7 min, 90%B.

Dealing with hair-profiling experiments, a rapid identification of AT and NT by separation on a Synergy Polar-RP (Phenomenex, CA, USA) narrow bore column (75*2.0 mm, 4 μm particle size) provided a sufficient retention using an isocratic mixture of A and B (50+50).

The mobile phase flow rate was consistently set to 700 μl/min, compatible with a source temperature of 650°C and source gas flow settings (nitrogen as sprayer and heater gas) of 50 psi. The injection volumes were 10 μl.

The concentration range and linearity of calibration and limits of detection (LOD) of the screening experiments are listed in Supplementary Table 2. The LODs of subsequent profiling experiments were 0.15 pg/cm (AT) and 0.16 pg/cm (NT).

A potential systematic influence of matrix effects could be excluded by comparison of matrix samples (18 random blank samples) with ten solvent calibrators, spiked to the same target concentrations of AT, NT, Z-OHAT and Z-OHNT. There was no significant variation of the relevant concentration ratios NT/AT (p=0.007), E/Z-OHAT (p=0.074) and OHAT/AT (p=0.008) between solvent and matrix samples.

Genotyping

DNA isolation

Genomic DNA from buccal swabs was isolated using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany).

Nucleotide numbering was consistent with the system recommended by the Human Cytochrome P450 (CYP) Allele Nomenclature Committee (http://www.imm.ki.se/CYPalleles/).

*Detection of the CYP2D6 alleles *3, *4 and *6*

The main mutations of the alleles CYP2D6*3 (2549A>del), *4 (1846G>A), and *6 (1707T>del) were identified using multiplex polymerase chain reaction (PCR). The sequence and concentration of each primer in the PCR used in this study are listed in Supplementary Table 2. The PCR was carried out in a total volume of 25 µl in the presence of 80 µM of each deoxyribonucleotide triphosphate (dNTP), 0.7 mM $MgCl_2$, 1 ng of genomic DNA as template and 1.25 U Ampli Taq Gold™ polymerase (Applied Biosystems). After initial denaturation at 94°C for 12 min, 35 cycles were carried out for 30 s at 94°C, 45 s at 68°C and 20 s at 72°C with a final elongation step for 60 min at 60°C. The PCR products were separated by capillary electrophoresis with the ABI PRISM® 310 Genetic Analyzer (Applied Biosystems).

TaqMan real-time PCR

The complete deletion (allele CYP2D6*5) or duplication of CYP2D6 was identified using TaqMan real-time PCR. The Quantifiler™ Human DNA Quantification Kit (Applied Biosystems) was used to detect the amount of human DNA, and quantification was carried out according to the manufacturer's instructions.

The sequences of the primers and probe to detect the amount of CYP2D6 used in this study are shown in Supplementary Table 3 and were used as described previously by Schaeffeler et al. [17]. The primers and probe were obtained from Applied Biosystems.

Real-time PCR was performed using the ABI Prism 7300 sequence detection system. Amplification reactions (25 µl) to detect the amount of CYP2D6 were carried out in duplicate using 1× TaqMan Universal Master Mix buffer (Applied Biosystems), 300 nM of each primer and 200 nM of the fluorogenic probe. Thermal cycling was initiated with a denaturation step of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and of 1 min at 60°C. The size of the PCR product for CYP2D6 was 89 bp. A standard curve was recorded and a no-DNA control was included in each experiment.

*Diagnosis of the CYP2C19 alleles *2, *3 and *4*

Genotyping of the CYP2C19 alleles *2, *3 und *4 was performed according to published methods [18, 19].

Results and discussion

Hair analysis

The primary identification of AT and NT in the total hair strands demonstrated that NT concentrations were significantly higher than the corresponding amounts of AT. This deviation to typical metabolite ratios determined in blood is probably due to the predominant incorporation of basic compounds (e.g. NT) into hair.

Moreover, apparent variations of the NT/AT ratio between individuals were observed. The stability of metabolite ratios (NT/AT) over a hair profile (Fig. 2) was confirmed by statistical evaluation of concentrations along a single hair [16]. These concentration ratios proved to be almost constant, whereas differences between individuals were far larger than analytical uncertainties or statistical variations within a hair fibre (Fig. 2). The same holds for the ratios of OHNT/NT or E10-OHNT/Z10-OHNT which are significantly different between individuals (Supplementary Table 4) but remain nearly constant within a profile.

Genotypes of CYP 2C19 and CYP2D6

No individuals without functional *CYP2D6* genes (PM), 9 individuals with only one functional allele (*CYP2D6*1*), 14 extensive metabolizers (EMs, two functional alleles) and no child with three functional alleles (UM) were observed during genotyping.

Three *CYP2C19* PMs (CYP2C19*2/*2), five heterozygous IMs with only one functional *CYP2C19* allele (4× *CYP2C19*1/*2* and 1× *CYP2C19*1/*4*) and 15 homozygous RMs were identified (Supplementary Table 4). This situation is biased by the fact that two of the three CYP2C19 PMs were specifically selected from the larger group and included into the study due to their exceptional metabolism. Moreover, six of the individuals were siblings, therefore the distribution of genotypes cannot be compared to average white populations.

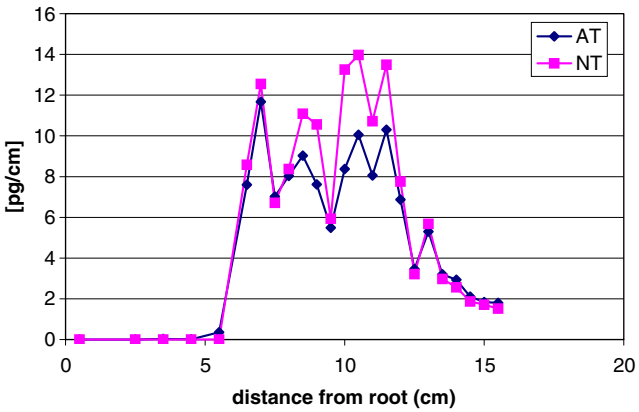

Fig. 2 Amitriptyline (AT) and nortriptyline (NT) concentration profiles examined in 0.5 cm segments of an individual hair (case number 10, total length 16 cm) exhibit a high correlation

Correlation of genotype and hair concentrations

N-demethylation of amitriptyline

The relative amount of NT compared to AT concentrations in hair ranged from 0.8 to 8.1. There was a clear and significant correlation of the NT/AT metabolite ratio with the number of functional alleles of CYP2C19. The same held for the corresponding ratio of hydroxy metabolites (OHNT/OHAT). The degree of demethylation was particularly decreased in individuals carrying two dysfunctional copies (PM) of the gene, whereas the virtual difference between IM and EM was not significant (Fig. 3, Supplementary Table 5). These observations are in good accordance to examinations of the polymorphism of AT in blood samples [6, 7].

Significant correlations of demethylation with any other parameter (gender, hair colour, total hair concentrations, CYP2D6) could be excluded.

Hydroxylation of nortriptyline and amitriptyline

There were large inter-individual variations of the amount (OHNT/NT=0.11–0.86, OHAT/AT=0.03–0.99) and stereospecificity (E10-OHNT/Z10-OHNT=1.6–10.3, E10-OHAT/Z10-OHAT=1.1–5.5) of hydroxylation of both AT and NT.

The amount of hydroxy-metabolites (relative to AT and NT) did not correlate with the genotype of either CYP2D6 or CYP2C19. This is in accordance with the suggestion that the difference between IM and EM phenotypes is not mainly attributed to the number of dysfunctional alleles. Phenotyping experiments [20] as well as observations of AT hydroxylation [3, 5, 6] demonstrated that the availability of two functional alleles does not result in significantly increased activity (p=0.761) of CYP2D6 compared to combinations of one functional and one dysfunctional allele.

The amount of the *trans*-isomer of E10-OHNT (relative to Z10-OHAT) was significantly depleted in subjects with two dysfunctional CYP2C19 alleles (Fig. 4) but independent of the CYP2D6 genotype. Although CYP2C19 is potentially capable of stimulating stereo-specific hydroxylation reactions (e.g. 16β-hydroxylation of steroids [21]), there is no experimental evidence for a direct contribution to hydroxylation of NT or AT [4].

Provided that elevated hydroxylation is associated with poor metabolic capacity of CYP 2C19 to compensate for the

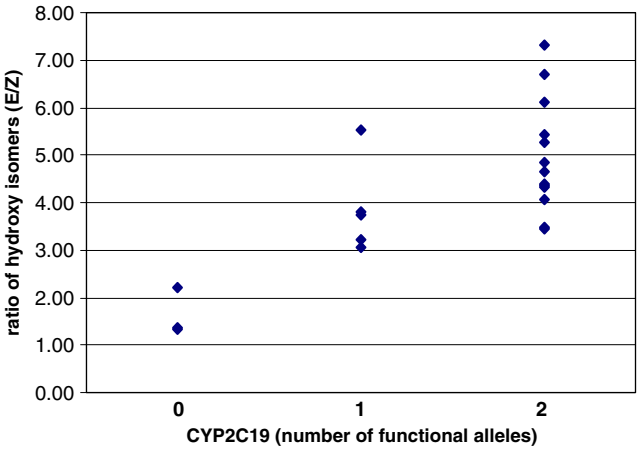

Fig. 4 The specificity of hydroxylation is significantly influenced by genetic polymorphism of the enzyme CYP2C19 (see Supplementary Table 5). The ratio of hydroxy isomers (E/Z) signifies the sum of *trans*-isomers (E10-hydroxynortriptyline+E10-hydroxyamitriptyline, E10-OHNT+E10-OHAT) divided by *cis*-isomers (Z10-hydroxynortriptyline+Z10-hydroxyamitriptyline, Z10-OHNT+Z10-OHAT)

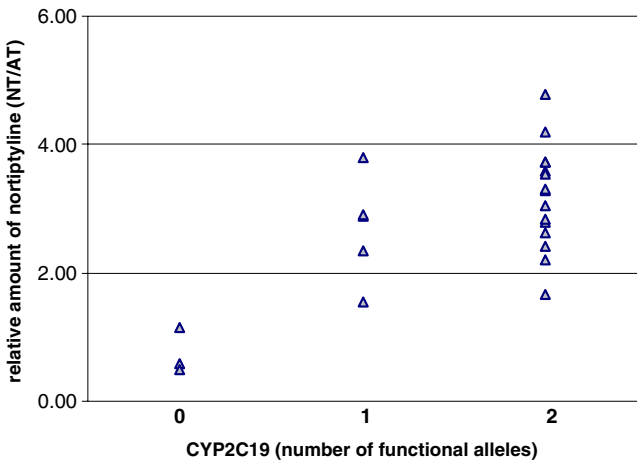

Fig. 3 The degree of demethylation of amitriptyline (the ratio of nortriptyline/amitriptyline, NT/AT) is clearly dependent on the genetic polymorphism of the enzyme CYP2C19

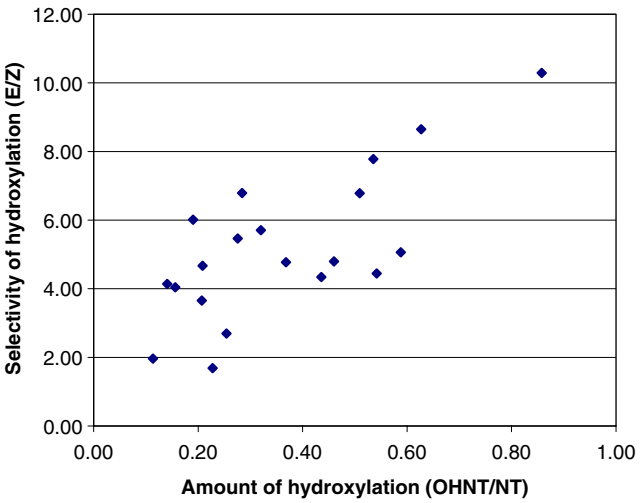

Fig. 5 The selectivity of hydroxylation (i.e. the ratio of E10-hydroxynortriptyline/Z10-hydroxynortriptyline, E/Z) is significantly (R^2=0.54, p<0.001, 23 cases) influenced by the total amount of hydroxylation

lack of demethylation (as suggested by Shimoda et al. [6]), it may be concluded that extensive hydroxylation is less selective due to the contribution of enzymes other than CYP2D6. This tendency seems to be confirmed by the significant correlation between the relative amount and specificity of NT hydroxylation (Fig. 5). On the other hand, there was no proof for a direct correlation between the lack of demethylation due to CYP2C19 mutations and increased amount of hydroxyamitriptyline in hair samples.

Conclusions

The comparison of hair concentrations of AT and its metabolites with the genotype of the individuals revealed no apparent relationship between CYP2D6 and the large inter-individual variation of hydroxylation. This is assumed to be due to the fact that outstanding genotypes (ultrarapid or poor metabolizers) were not present in the population tested and a different metabolic activity between individuals carrying one or two functional alleles of CYP2D6 is generally disputed.

In contrast, there was a clear correlation of CYP2C19 (number of functional alleles) with both metabolic pathways, demethylation and hydroxylation. The former, i.e. a significantly decreased amount of demethylation in CYP2C19 poor metabolizers, is in good accordance with metabolic studies in blood samples.

However, the conditions of the apparent influence of the CYP2C19 phenotype on hydroxylation stereo-specificity were less obvious. Indirect influences, e.g. the limited availability and capacity of enzymes and competition of substrates in consecutive metabolic reactions (*N*-demethylation of AT by CYP3A4 and/or CYP2C19, *N*-demethylation of NT by CYP2D6 and/or CYP2C19, hydroxylation of AT by CYP3A4 and/or CYP2D6) are assumed to be responsible for these phenomena.

The application of hair for investigation of metabolic phenotypes has clear limitations, e.g. comparatively low substance concentrations and the influence of chemical properties on hair incorporation. On the other hand, resulting hair concentrations are not affected by polar inactive metabolites (e.g. conjugates) and therefore appropriately reflect the average bioavailability. Hair analyses of metabolite ratios appear to be a useful option to collect retrospective information about long-term drug consumption and corresponding variations of the individual metabolism. The combination of toxicological findings with real-time PCR genotyping [22, 23] proved to be powerful to explain such individual variation. Antidepressants and neuroleptics appear to be particularly suitable target compounds due to the problems of drug compliance or illegal administrations, the relevance of individual variations in biotransformation and the high incorporation rate of these substances into hair.

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