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PHARMACOKINETICS AND DISPOSITION

Donepezil plasma concentrations, CYP2D6 and CYP3A4 phenotypes, and cognitive outcome in Alzheimer's disease

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Abstract
Purpose The purpose of the study is to evaluate whether donepezil (D) plasma concentrations and activity of CYP2D6 and CYP3A4 are associated with the therapeutic response of patients with mild to moderate Alzheimer's disease (AD).
Methods This study comprised 54 patients affected by probable AD in therapy with D 10 mg/daily for at least 3 months. Plasma concentrations of D and its three main metabolites (6DD, 5DD, DNox) were assayed with a novel high performance liquid chromatography (HPLC) technique. Cognitive progression was assessed at baseline and at 9 months of follow-up with the mini mental state examination (MMSE). The activities of the two cytochromes involved in D metabolism—CYP2D6 and CYP3A4—were evaluated according to their metabolic ratios in plasma or urine, after test doses of probe drugs (dextromethorphan and omeprazole).
Results A significant correlation was found between plasma levels of D and variations in MMSE scores after 9 months of therapy ($r^2 = 0.14$; $p = 0.006$). Neither the concentrations of D metabolites nor the metabolic ratios of CYP2D6 and CYP3A4 showed any correlations with cognitive variations. Low CYP2D6 activity and advanced age were associated with high D concentrations. Patients who were treated with CYP2D6 and P-glycoprotein (P-gp) inhibitors also had higher D plasma levels (mean difference = 19.6 ng/mL; $p = 0.01$) than those who were not.
Conclusions D plasma concentrations, but not cytochrome phenotyping, are associated with cognitive outcomes in AD patients.

Keywords Donepezil · Plasma concentrations · CYP2D6 phenotype · Alzheimer disease

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Introduction

Donepezil (D), an inhibitor of acetylcholinesterase (AChE), has demonstrated its efficacy in the cognitive improvement and stabilization of patients with Alzheimer's disease (AD) [1]. The drug is metabolized by hepatic P450 cytochromes, especially CYP2D6 and CYP3A4 isoenzymes [2]. This process leads to the production of three main metabolites: 6-O-desmethyl-donepezil (6DD) and donepezil-N-oxide (DNox), which are pharmacologically active [3, 4], and 5-O-desmethyl-donepezil (5DD). Current literature data indicate that 6DD plasma levels at steady state are about 20 % those of D [5]; no information is available about 5DD or DNox plasma concentrations. A positive correlation has been demonstrated between D plasma concentrations and variations in neurological scores in AD patients [6]. The therapeutic plasma concentration range recommended in the literature is 30–75 ng/ml [7]. However, according to a recent report, maximal improvement of symptoms can be achieved at D concentrations >50 ng/mL [8]. In addition, the cognitive response is not homogeneous among patients, but has a reported rate of responders between 40 and 50 % [9]. Various studies have attempted to establish whether D plasma concentrations and response to therapy are dependent on CYP2D6 and CYP3A4 genetic polymorphisms. A recent study states that CYP3A4 polymorphisms do not influence D plasma concentrations or clinical outcomes, although the polymorphism of P-

glycoprotein (P-gp) (the D extrusion system from the CNS) appears to show a trend toward a correlation [10]. CYP2D6 has many allele variants which influence enzyme activity, so that patients are classified as poor metabolizers (PM), intermediate metabolizers (IM), extensive metabolizers (EM), and ultrarapid metabolizers (UM) [11–13]. A 2006 study shows that EM heterozygous for a CYP2D6 defective allele had better clinical progression compared with homozygous EM and UM [11]. Pilotto et al. [12] demonstrated the prevalence of a CYP2D6 polymorphism with increased activity in non-responders, and Seripa et al. [13] found a higher frequency of enzymatic variants with low or absent activity among responders. A discrete proportion of responders, however, does not present particular polymorphic variants. In addition, some of the studies cited above may not be representative of the true AD population, since they dealt with highly selected subsets of patients, often with no confounding factors such as concomitant therapies or comorbidities. In this regard, a recent study on 129 patients treated with 10 mg D daily, while confirming a correlation between D clearance and CYP2D6 polymorphism, did suggest the importance of non-genetic factors, such as female gender and co-medication with inhibitors of CYP2D6 and CYP3A4 or inducers of CYP3A4 [14]. Our study had three main aims: (1) to assess the relative contribution of D and its main metabolites (6DD, DNox, 5DD) to the cognitive response, by monitoring plasma concentrations of all compounds; (2) to examine whether the enzyme activities of CYP2D6 and CYP3A4 (measured by phenotyping) are associated with D plasma levels and clinical outcomes; (3) to identify any pharmacokinetic interactions between D and co-administered drugs.

Methods

Patient recruitment

From November 2012 to June 2014, a total of 54 patients attending the Alzheimer Evaluation Unit, Geriatric Clinic, University of Padova (Italy), were enrolled in the study. Informed consent was obtained from all individual participants included in the study. Inclusion criteria were (1) Caucasian ethnicity; (2) age ≥65; (3) diagnosis of probable mild to moderate AD according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association Work Group (NINCDS-ADRDA) [15]; (4) therapy with D 10 mg/daily for at least 3 months; (5) no variations in any remaining pharmacological treatment in the 3 months prior to the study; (6) written informed consent. Patients were excluded if they had clinically significant or unstable medical illnesses, had undergone medical or surgical hospitalization within 1 month of the study, had allergies or intolerance to the drugs used to analyze the activity of cytochromes (dextrometorphan and omeprazole), or had severe urinary incontinence. Other types of dementia were excluded through the Hachinski Ischemic Score, clinical history, and neuroimaging. Mild cognitive impairment was also excluded, according to the Petersen criteria.

To study the influence of concomitant drugs on D plasma levels, co-medications were classified as CYP2D6 inhibitors or P-gp inhibitors, according to available in vitro and in vivo data [16–18].

Patient evaluation

All patients were evaluated for cognitive and functional status at the beginning of therapy with D, 3 months later, and then every 6 months. Both patients and their caregivers were instructed on how to conduct therapy and to contact our Alzheimer Unit for any intervening problem. Evaluation was performed with the following scales: MMSE [19], activities of daily living (ADL) [20], and instrumental activities of daily living (IADL) [21]. Since there are no recognized reference criteria in the literature, patients' clinical responses were evaluated according to our clinical practice, through any changes in MMSE scores at 9 months from baseline (ΔMMSE) [22]. Pharmacokinetic monitoring and cytochrome phenotyping were performed on the occasion of the same visit.

Determination of concentration of D and its metabolites

Between 8.30 and 9.30 am (12–15 h after last drug administration), a 5-ml fasting blood sample was drawn for assay of concentrations of D, 5DD, 6DD, and DNox. Blood samples were immediately centrifuged and plasma stored at −20 °C until drug assay. D and its metabolites were dosed by a novel HPLC technique with combined photometric and fluorimetric detection which, for the first time, has been shown to detect plasma concentrations of D, 5DD, 6DD, and DNox simultaneously. Details of the method are described elsewhere [23]. Detection limits of D, 5DD, 6DD, and DNox were 0.1, 0.07, 1.2 (photometric detection), and 0.3 ng/mL, respectively.

Determination of activity of cytochromes CYP2D6 and CYP3A4

As the basal rate of drug metabolism is determined not only by genetic constitution but may also depend on age, gender, diet, diseases, drug interactions, etc., the actual activity of the cytochromes involved in D metabolism was determined by phenotyping.

Phenotyping tests were performed before D administration and after drawing blood for plasma concentration monitoring. CYP2D6 activity was determined, after oral administration of 15 mg of dextromethorphan (DMT), by measuring the

concentrations of DMT and dextrorphan (DES, the metabolite produced by CYP2D6) in urine collected during the 8-h period post-dose. The metabolic ratio log(DMT/DES) is an accepted index of CYP2D6 in vivo activity [24].

To analyze CYP3A4 activity, 20 mg of omeprazole (OME) were given orally and concentrations of OME and its metabolite, omeprazole sulphone (SULF, produced by CYP3A4) were measured in plasma 3 h later, in order to calculate the metabolic ratio log(OME/SULF) [25].

All samples were stored at −20 °C until assayed. DMT, DEX, OME, SULF, and 5OH were assayed in biological samples with published HPLC methods, with minor modifications [26, 27].

The higher the metabolic ratios (MR), the lower cytochrome activities and related drug metabolism.

Statistical analysis

Data were analyzed with the software package SPSS version 17.0 (SPSS Inc., Chicago, IL). Normality was verified with the Kolmogorov-Smirnov test. For normally distributed variables, differences among groups were tested by Student's two-tailed *t* test. A one-way ANOVA followed by the linear trend test and/or multiple-comparison Tukey test was applied, if required. Correlations were evaluated by linear regression analysis and the coefficient of determination (r^2). Multiple regression analyses were performed to identify the biological variants which are associated with D plasma concentrations and changes in MMSE score at 9 months. Stepwise forward regression was performed, followed by multicollinearity analysis, when appropriate. Statistical significance was assumed for a p value <0.05.

The correlation between D concentration and MMSE score change at 9 months was chosen as primary endpoint. Assuming an effect size (r^2) of 0.15 (considered to be a medium value), a power of 0.8, and a probability of 0.05 [28], a minimum required sample size of 54 patients was calculated.

Lastly, to study the role of concomitant drugs on D concentrations, our patients were divided into three groups, depending on whether they were treated with potentially interacting drugs, as follows: (1) patients who simultaneously took both CYP2D6 and P-gp inhibitors; (2) patients who only took P-gp inhibitors; (3) patients who did not take any inhibitor.

Results

The demographic and clinical characteristics of the 54 patients are shown in Table 1. Seventeen were men (31.5 %) and 37 women (68.5 %), aged between 70 and 92 years (mean 80 ± 5 years). MMSE at baseline was 20.38 ± 3.11, and the mean change at 9 months was -0.10 ± 2.96 points. Patients' renal and hepatic functions were within normal ranges.

Table 1 Demographic and clinical characteristics of patients at baseline (mean ± SD)

Age (years)	80 ± 5
Gender	17 males (31.5 %); 37 females (68.5 %)
Education (years)	5.9 ± 3.4
Body weight (Kg)	65.6 ± 13.20
Height (cm)	159.1 ± 10.2
BMI (Kg/m^2)	26.2 ± 5.0
Creatinine (μmol/L)	84.3 ± 26.0
AST (UI/L)	22.8 ± 9.3
ALT (UI/L)	17.1 ± 6.0
γ GT (UI/L)	20.5 ± 17.2
MMSE baseline	20.4 ± 3.1
ADL baseline	5.6 ± 0.8
IADL baseline	3.5 ± 1.3 (males); 4.7 ± 2.7 (females)
MMSE change 9 months	−0.1 ± 2.9
ADL change 9 months	−0.6 ± 1.2
IADL change 9 months	−0.8 ± 1.5 (males); −1.3 ± 1.7 (females)

Plasma concentrations of D and its metabolites and their correlations with clinical outcomes

Mean plasma concentrations ± SD (ranges) of D, 6DD, 5DD, and DNox were 45.9 ± 21.0 ng/mL (10.0–105.9 ng/mL), 12.3 ± 10.3 ng/ml (1.2–36 ng/mL), 0.44 ± 0.64 ng/ml (0.07–2.8 ng/mL), and 6.9 ± 9.3 ng/ml (0.5–45.4 ng/mL), respectively.

Metabolite plasma levels were below detection limits in 3 patients for 6DD and 19 for 5DD. However, in 6 patients, concentrations of 6DD or DNox were similar to or exceeded those of D. Referring to the therapeutic range suggested by Baumann et al. (2004) (30–75 ng/ml), 20.4 % of our patients were under-dosed and 9.2 % were over-dosed.

With univariate analysis, a statistically significant correlation was found between D plasma levels and clinical response to the drug (Fig. 1; $r^2 = 0.14$; $p = 0.0061$). No significant correlations were found between the plasma concentrations of any metabolite and clinical outcomes.

Stepwise multiple regression analysis, including age, gender, basal MMSE, therapy duration, and plasma concentrations of D, confirmed that the only biological variant associated with ΔMMSE was the concentration of D (Table 2).

During follow-up, two patients developed transient side effects (nausea and hyperactivity), which disappeared without dose reduction. At the 9-month visit, the drug was stopped in one patient because of severe diarrhea (D plasma level: 77 ng/mL); the dose was reduced to 5 mg qd in another patient, due to onset of anxiety and insomnia (D plasma level: 40 ng/mL).

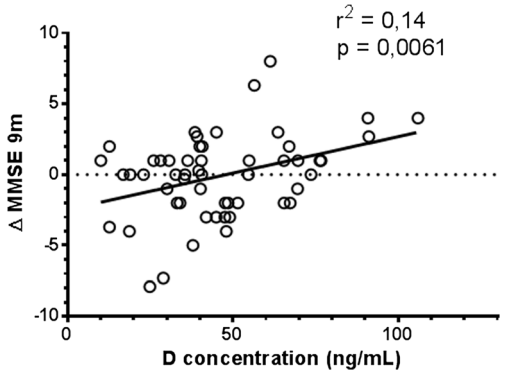

Fig. 1 Linear regression between plasma D concentration (ng/mL) and MMSE score change at 9 months from baseline (ΔMMSE)

CYP2D6 and CYP3A4 phenotypes and correlations with D plasma concentrations and clinical outcomes

The mean metabolic ratios of cytochromes were −1.37 ± 0.75 for CYP2D6 ($n = 47$) and 0.26 ± 0.46 for CYP3A4 ($n = 54$). CYP2D6 activity could not be measured in 7 patients, whose urinary 8-h collection could not be correctly performed due to partial urinary losses.

With univariate analysis, the metabolic ratios of CYP2D6 and CYP3A4 showed no statistically significant correlation with cognitive changes, although there was a weak but significant correlation ($r^2 = 0.11$; $p = 0.02$) between the CYP2D6 activity index and D plasma concentrations, consistent with the fact that low activity of CYP2D6 lessens the drug metabolized.

Stepwise multiple regression analysis, including age, gender, and metabolic ratios of CYP2D6 and CYP3A4, revealed that D concentrations were significantly correlated not only with CY2D6 activity ($p = 0.023$) but also with patients' age ($p = 0.0098$). Multicollinearity analysis showed that the two variables provided independent information (Table 3).

Concomitant drugs

The medications potentially inhibiting CYP2D6 and P-gp were paroxetine and sertraline; those inhibiting P-gp only were atorvastatin, simvastatin, and verapamil. Our population was divided into three groups according to concomitant drugs:

Table 2 Results of stepwise regression analysis, with MMSE score change at 9 months as dependent variable

	$r^2 = 0.14$; adjusted $r^2 = 0.12$; $n = 54$; $p = 0.0061$				
	beta	95 % CI	B	95 % CI	*p*
Intercept			−2.46	−4.24/−0.68	0.0091
D concentration	0.37	0.12/0.62	0.052	0.016/0.087	0.0061

group 1, taking CYP2D6 and P-gp inhibitors ($n = 12$); group 2, taking only P-gp inhibitors ($n = 12$); group 3, taking no inhibitors ($n = 30$). Mean D plasma concentrations showed a significantly decreasing trend from group 1 to group 3 (ANOVA; $p = 0.01$). D plasma levels were also significantly higher in group 1 than in group 3 (mean difference: 19.6 ng/mL; $p = 0.002$) (Fig. 2). Interestingly, the mean metabolic ratio of CYP2D6 was also significantly higher (mean difference: −0.63; $p = 0.007$) in group 1 than in group 3, confirming that CYP2D6 activity was reduced in patients taking CYP2D6 inhibitors. Mean age did not differ between groups (ANOVA, $p = 0.30$).

Discussion

The novelty of the present study is due to the measurement of steady-state plasma levels of D and its three main metabolites (6DD, 5DD, DNox) and to the determination of the in vivo activities of two cytochromes deemed responsible for D metabolism (CYP2D6 and CYP3A4) in subjects affected by AD, treated with D.

Donepezil is currently one of the EMA-approved drugs for AD treatment. Some controlled randomized trials show that this drug has a good safety range, but that its efficacy profile varies according to patient. The reasons for this are not yet completely clear. In this study, we hypothesize that concentrations of D and its metabolites are correlated with the clinical response to D, so that it may be useful as a parameter for monitoring therapy.

Our results confirmed that plasma concentrations of D are positively correlated with changes in cognitive scores [6, 29]. Instead, we observed that the plasma concentrations of the three main D metabolites were lower than those of the parent drug (on average, 33 % for 6DD, 18 % for DNox, 1.7 % for 5DD) and did not correlate with MMSE variations at the 9-month follow-up. In particular, plasma concentrations of 5DD—the only D metabolite without proven pharmacological activity—were extremely low and virtually undetectable in about one third of patients. Therefore, on the whole, our findings indicate that D metabolites do not substantially contribute to any drug effect at a daily dose of 10 mg, and that measuring their concentrations does not provide information about clinical outcomes. Other possible variability factors—such as age, gender, basal MMSE, and therapy duration—did not prove to be determinants of the D response.

From a therapeutic point of view, our findings raise the possibility that patients with negative variations in MMSE at 9 months and sub-therapeutic D plasma concentrations might have done better if the D dose had been higher. This would imply considering a higher D dose in those patients with mild to moderate AD who do not respond satisfactorily to D 10 mg/day and do not have adequate

Table 3 Results of stepwise regression analysis, with donepezil plasma concentration as dependent variable

	$r^2 = 0.24$; adjusted $r^2 = 0.21$; $n = 47$; $p = 0.0024$					Multicollinearity	
	beta	95 % CI	B	95 % CI	p	Tolerance	VIF
Intercept			−55.0	−139.0/29.0	0.21		
Age	0.36	0.098/0.61	1.41	0.39/2.43	0.0098	0.994	1.01
CYP2D6 MR	0.31	0.052/0.57	8.93	1.49/16.4	0.023	0.994	1.01

VIP variance inflation factor

plasma concentrations. In addition, even patients who show cognitive improvement on D may improve further if the dose is increased. Hefner et al. [8] did in fact show that patients who were "very much improved" had D plasma levels >50 ng/mL, and Yang et al. [30] reported that a mean D concentration of 75 ng/mL was needed to improve long-term memory in AD patients. With regard to this, in 2010, the FDA approved a new, once-daily 23-mg formulation for moderate to severe AD, and a subsequent study by Cummings et al. [31] demonstrated that D 23 mg/day elicits greater cognitive effects when compared with 10 mg/day; the higher incidence of adverse reactions initially recorded (nausea, vomiting, diarrhea, vertigo) substantially decreased as therapy continued.

All these observations clearly indicate that the D dose must be tailored to individual patients according to therapeutic plasma monitoring, cognitive changes, and side effects.

At variance with some published data on the effect of the CYP2D6 genotype on the D response [11–14], we did not find a significant correlation between CYP2D6 phenotype and clinical outcome ($p = 0.11$). This is not surprising, since our small population may not have included individuals with very slow or very fast CYP2D6 genotypes, and non-pharmacological factors may have had a greater impact on patients' responses. Other genetic studies have also failed to demonstrate a correlation between CYP2D6 mutated alleles and response to D [32, 33]. Thus, if a correlation does exist, it is probably weak.

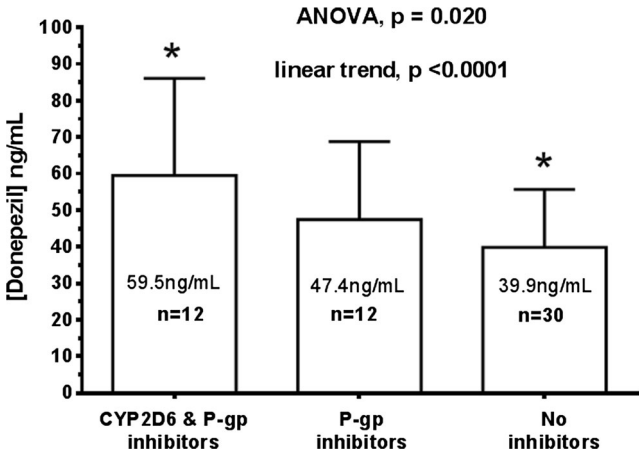

Fig. 2 Relationship between concomitant administration of CYP2D6 or P-gp P interacting drugs and mean plasma D concentration (ng/ml). * = groups with significant differences

Conversely, D plasma concentrations were significantly correlated with CYP2D6 activity ($r^2 = 0.11$; $p = 0.02$). In addition, patients who were treated with drugs known to inhibit CYP2D6 and P-gp (paroxetine and sertraline) showed 49.2 % higher D plasma levels than patients who were not. Matching the causal role of CYP2D6 inhibition, the activity of the cytochrome was lower in the former group of patients. A trend toward higher D levels was also observed in patients taking P-gp inhibitors, although significance level was not reached. This confirms the usefulness of monitoring D plasma concentrations in elderly AD patients who are often on poly-therapy and, in particular, those who take psychotropic drugs.

Multiple regression analysis identified CYP2D6 activity and age as the only significant variants associated with D levels (adjusted $r^2 = 0.21$; $p = 0.0023$). In any case, most interindividual D variability remained unexplained. Some contribution from P-gp activity cannot definitely be excluded by our data, in view of the trend toward higher D plasma levels in patients taking P-pg inhibitors.

In summary, our results confirm the association between D plasma concentration and clinical responses in AD patients, but they exclude any substantial therapeutic contribution by the two active metabolites, 6DD, and Nox. In turn, D plasma concentration appears to be directly correlated with the CYPD6 phenotype and patient's age.

Study limitations

The main limitation of this study is that the coefficient of determination ($r^2 = 0.14$) was slightly lower than that hypothesized in calculating sample size ($r^2 = 0.15$): this implies that the power of the test was 0.775 instead of 0.8. Larger, more highly powered studies are needed to confirm our preliminary results and to identify new markers of the D response.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was approved by the Ethics Committee of Azienda–ULSS 16, Padova Protocol n° 8793). All procedures in this study were in accordance with the 1964 Helsinki Declaration and its later amendments.

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