

Effect of Common *NAT2* Variant Alleles in the Acetylation of the Major Clonazepam Metabolite, 7-Aminoclonazepam

M. Olivera, C. Martínez, G. Gervasini, J.A. Carrillo, S. Ramos, J. Benítez, E. García-Martin and J.A.G. Agúndez*

Department of Pharmacology, Medical School, University of Extremadura, Badajoz, Spain

Abstract: We investigated the role of *NAT2* on clonazepam acetylation, using transiently expressed human *NAT2* alleles. The *NAT2***B* and the *NAT2***6A* variant alleles cause a 20 and 22-fold reduction in the V_{max} , respectively. We conclude that *NAT2* is responsible for 7-aminoclonazepam acetylation and that *NAT2* gene polymorphisms impair such metabolic pathway.

Key Words: *NAT2*, acetylation, polymorphism, pharmacogenomics.

INTRODUCTION

N-Acetylation by the hepatic arylamine N-Acetyltransferase-2 (*NAT2*, EC 2.3.1.5) is a major route in the metabolism and detoxication of several drugs and foreign chemicals. The genetic polymorphism of the *NAT2* enzyme was detected in 1960 [1].

About a half of Caucasian individuals are classified as slow acetylators (SA) and they show impaired metabolism of many therapeutically useful arylamine and hydrazine drugs [2]. The determination of *NAT2* genotype or phenotype has been proposed to predict adverse reactions in patients with tuberculosis receiving isoniazid [3], and prior to the concomitant administration of procainamide and phenytoin because the slow acetylation status has been related to the risk to develop agranulocytosis in patients receiving a combination of these drugs [4]. In addition it has been shown that *NAT2* polymorphisms modify the doxycycline-rifampin interaction that occurs in individuals treated simultaneously with these drugs, and that cause an inverse correlation between doxycycline and rifampin plasma levels. Among individuals classified as rapid acetylators rifampin plasma levels are greater whereas doxycycline levels are lower as compared to slow acetylators [5]. These effects together with the high frequency for individuals with impaired *NAT2* metabolism [6] make *NAT2* a relevant target for pharmacogenomic tests in clinical practice.

Clonazepam (CZP) is an effective benzodiazepine antiepileptic drug which in humans is primarily metabolized to 7-aminoclonazepam (7ACZP) by nitroreduction, and then N-acetylated to 7-acetamidoclonazepam (7AACZP) [7, 8]. Until now, indirect evidence based on a bimodal distribution of the 7ACZP-acetylation capacity in human liver cytosol suggests that the polymorphic *NAT2* enzyme is involved in 7ACZP acetylation [9]. Preliminary data obtained *in vivo* indicate that individuals classified as slow acetylators excreted in urine more 7ACZP and less 7AACZP than rapid

acetylators, thus supporting this hypothesis [10, 11]. However, drug-acetylating enzymes other than *NAT2*, such as *NAT1* have been shown to be polymorphic [6], and therefore the origin of bimodality on the acetylation of 7ACZP is not unambiguously attributable to the *NAT2* enzyme.

The aim of this study is to elucidate the role of the *NAT2* enzyme on 7ACZP acetylation in man. For this we have used a simplified system based on the use of expressed human *NAT2* enzymes in mammalian cells lacking intrinsic 7ACZP-acetylation capacity. The human *NAT2* enzymes *NAT2***4* (wild-type) and the two most common mutated enzymes (*NAT2***5B* and *NAT2***6A*) were expressed in COS-I cells and analyzed for 7ACZP acetylation capacity. These three allelic variants account for over 87% of human *NAT2* alleles in Caucasian individuals [12].

MATERIALS

Clonazepam and metabolites were kindly donated by Roche. S.A. (Madrid, Spain). Sulfamethazine, acetylcarnitine and carnitine acetyltransferase were purchased from SIGMA Chem Co. (Madrid, Spain). These and all other chemicals used in this study were of analytical grade. Water was filtered through a Milli Q water system (Millipore Corp., Bedford, Mass. USA).

METHODS

Human *NAT2* genes were obtained after PCR amplification from genomic DNA of individuals identified as homozygous for *NAT2***4*, *NAT2***5B* and *NAT2***6A* as described elsewhere [13]. The corresponding amplified DNA fragments were tested for seven known mutations by mutation-specific PCR, by using the method described in [14]. Every amplified DNA fragment was inserted into the p91023 expression vector using the *EcoRI* enzyme. The inserted 1.3 kb fragments contained the entire coding region of the gene, located in exon 2, and 5' and 3'-flanking regions as described elsewhere [15]. Monkey kidney COS-I cells were transiently transfected by using the three constructs, and mock-transfected by the use of the expression vector with no insert. Prior to the selection of COS-I cells for the expression of the *NAT2* genes, the lack of intrinsic 7ACZP-acetylation capacity in the cytosol of COS-I cells was checked. Forty to

*Address correspondence to this author at the Departamento de Farmacología, Facultad de Medicina, Universidad de Extremadura, Avda de Elvas s/n, E-06071, Badajoz, Spain; Tel: +34924289458; Fax: +34924289676; E-mail: jagundez@unex.es

fifty hours after transfection, the COS-I cells were harvested and cytosols were obtained under conditions that assured the stability of the NAT2 enzyme [16].

N-acetyltransferase activity was checked in the cytosolic preparations by following the method of Grant *et al.* [16] as follows: 25 μ l of a cytosolic preparation was mixed with 25 μ l of a solution containing 10 mM TrisHCl, 1 mM EDTA, 1 mM DTT, 50 mM KCl, 2.5 mmol KOH (pH 7.0) and with 20 μ l of 450 mM acetyl-CoA. The reaction was started with the addition of 20 μ l of a solution containing 5.4 mg acetylcarnitine and 24 mU carnitine acetyltransferase and the substrate, either sulfamethazine or 7-aminoclonazepam at final concentrations ranging from 3 to 500 μ M. Reactions were carried out at 37 °C over 30 min and were stopped by the addition of 10 μ l of 15% HClO₄. Samples were centrifuged for 10 min in a microfuge at maximum speed. For sulfamethazine analysis, 50 μ l of the supernatant was injected onto an Spherisorb CN 5 μ 4.6 x 250 mm column (Sugelabor, Madrid, Spain) and the elution of sulfamethazine and N-acetyl sulfamethazine was monitored at 254 nm. The flow rate was 1.5 ml/min and the mobile phase consisted of acetonitrile 15% in 19.8 mM phosphate buffer. Sulfamethazine and the acetylated metabolite eluted at 9.6 and 12.4 min, respectively. The HPLC analysis for clonazepam metabolites was based on that reported for midazolam [17], with minor modifications as follows: 50 μ l of the supernatant was injected onto an Ultrasphere IP 5 μ 4.6 x 250 mm column (Sugelabor, Madrid, Spain) and the elution of 7ACZP and the acetylated metabolite were monitored at 250 nm. The flow rate was 0.7 ml/min and the mobile phase consisted of acetonitrile 30% in sodium acetate 100 mM (pH 4.7). 7ACZP and the acetylated metabolite eluted at 6.8 and 6.4 min, respectively. Parent drugs and metabolites were quantitated from peak areas compared with those of standard curves prepared of parent or metabolites. All measurements were done within the linear range of the standard curves and the interday and intraday variation was in all cases under 5%.

All the assays included samples that were stopped at time = 0 and blanks without cytosols and/or without substrate. All the experiments were done at least in triplicate and all measurements were done under linear conditions for incubation time and for cytosolic protein concentration. Kinetic data were evaluated according to standard procedures by graphical analysis of Lineweaver-Burk, Dixon and Hill's plots. All

the results given are mean \pm SD of three or more measurements made under identical conditions.

The comparison values were calculated by using the statistical package SPSS 11.0.1. (SPSS Inc. Chigaco, Ill, USA). The t test was used for comparison of kinetic parameters of samples transfected with different sequences.

RESULTS

NAT2 enzyme activity was analyzed in cytosolic preparations from untransfected COS-I cells by using the NAT2-specific substrate sulfamethazine. Table 1 shows that untransfected and mock-transfected cells lacked intrinsic acetylation capacity, whereas transfected cells displayed acetylation enzyme activity. This activity was dependent on the mutations present in the transfected genes. The Km values were similar and in the multiple comparison analyses did not reach statistically significant differences. However the Vmax values did display statistically significant differences and the Vmax/Km values indicated that variant alleles cause approximately a 90% and an 88% decrease in the intrinsic clearance of the enzyme for the alleles NAT2*5B and NAT2*6A, respectively.

When cytosolic preparations were analyzed for 7ACZP acetylation (Table 2) it was evident that untransfected and mock-transfected cells lacked 7ACZP acetylation activity whereas transfected cells were able to acetylate 7ACZP. This indicates that 7ACZP acetylation is carried out at least in part by the human NAT2 enzyme. In addition, Table 2 shows that common mutations at the NAT2 gene significantly modify the Vmax values and that alleles NAT2*5B and NAT2*6A cause a 95% and a 91% decrease, respectively, in the intrinsic clearance of the enzyme. In addition, a statistically significant modification in the Km was observed for the variant allele NAT2*6A.

DISCUSSION

This study confirms that the human NAT2 enzyme is capable of catalyzing the acetylation of 7ACZP. The most common mutations affecting the NAT2 gene cause the same alterations in 7ACZP acetylation (major changes in the Vmax, and little effect in the Km) as in the acetylation of sulfamethazine, which is considered a substrate specific for the NAT2 enzyme.

Table 1. Sulfamethazine Acetylation Capacity in Cytosolic Preparations from COS-I Cells

	NOT TRANSFECTED	MOCK-TRANSFECTED	NAT2*4	NAT2*5B	NAT2*6A
Km (μ M)	--	--	85.4 \pm 8.06	103.3 \pm 23.9 p = 0.025	67.7 \pm 23.7 p = 0.038
Vmax (nmol/min per mg)	Not detectable	Not detectable	38.2 \pm 13.3	4.1 \pm 2.5 p < 0.001	2.6 \pm 2.0 p < 0.001
Vmax/Km	--	--	0.451 \pm 0.157	0.039 \pm 0.022 p < 0.001	0.052 \pm 0.047 p < 0.001

Values represent means \pm SD of at least three independent experiments. p values for variant alleles were obtained by comparison with the NAT2*4 (wild-type alleles) by using the t test for independent samples. The significance limit corresponding to multiple comparison analyses for two factors was p = 0.025.

Table 2. 7ACZP Acetylation Capacity in Cytosolic Preparations from COS-I Cells

	NOT TRANSFECTED	MOCK-TRANSFECTED	NAT2*4	NAT2*5B	NAT2*6A
Km (μ M)	--	--	370 \pm 58	320 \pm 52 p = 0.092	216 \pm 112 p = 0.004
Vmax (Arbitrary units)	Not detectable	Not detectable	21.9 \pm 8.27	1.07 \pm 0.52 p < 0.001	0.96 \pm 0.51 p < 0.001
Vmax/Km	--	--	0.059 \pm 0.022	0.003 \pm 0.002 p < 0.001	0.005 \pm 0.003 p < 0.001

Values represent means \pm SD of at least three independent experiments. p values for variant alleles were obtained by comparison with the NAT2*4 (wild-type alleles) by using the t test for independent samples. The significance limit corresponding to multiple comparison analyses for two factors was p = 0.025.

Clonazepam is effective in the treatment of seizures, panic disorders and as coadjuvant therapy in the treatment of obsessive-compulsive disorders, acute mania or acute agitation and depression [18], as well as cochleovestibular disorders [19]. In addition, a rise in clonazepam abuse has been detected in the United States [20] and in France [21]. Detection of 7ACZP has been used by law enforcement agencies because after a single oral dose of 3 mg clonazepam, in most individuals the major clonazepam metabolite, 7ACZP, can be detected in urine up to 21 days after administration [22, 23]. Our findings suggest that detectable levels of 7ACZP in urine could be greatly influenced by the NAT2 genotype, and therefore studies need to be conducted to analyze whether the NAT2 genotype should be considered in determining the best time interval for urine collection.

Besides common adverse effects for benzodiazepines, adverse effects of clonazepam include toxic retinopathy [24], behavioral side effects [25], or interaction with oxycodone [26]. The present study raises the question of whether these adverse effects are related to clonazepam itself or are due to clonazepam metabolites. According to published data [1,5], about half of Caucasian subjects would have impaired 7ACZP metabolism and therefore elevated, possibly toxic, levels of clonazepam. The clinical relevance of these findings and the capacity of genotyping techniques to predict 7ACZP acetylation *in vivo* deserve further studies.

ACKNOWLEDGEMENT

Grants SAF 2003-00967 from the Ministerio de Ciencia y Tecnología, FIS 05/1056 from the Fondo de Investigación Sanitaria, Instituto de Salud Carlos III, Madrid, and SCSS 0502, 0507 and 0549 from Junta de Extremadura, Mérida, Spain.

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