N-ACETYLATION OF ETAMICASTAT, A REVERSIBLE DOPAMINE-ß-
HYDROXYLASE INHIBITOR

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Etamicastat: in vitro N-acetylation

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List of Abbreviations:

AP-ESI Atmospheric pressure-electrospray ionization; BIA 5-961 (R)-N-(2-(1-(6,8-difluorochroman-3-yl)-2-thioxo-2,3-dihydro-1H-imidazol-5-yl)ethyl)acetamide; DBH, dopamine-ß-hydroxylase; DBP, diastolic blood pressure; LC/MS, liquid chromatography- mass spectrometry; ISTD, internal standard; NE, norepinephrine; NAT, N-acetyltransferases; SIM, Selected ion monitoring; SBP, systolic blood pressure;
Abstract

Etamicastat, (R)-5-(2-aminoethyl)-1-(6,8-difluorochroman-3-yl)-1H-imidazole-2(3H)-thione hydrochloride, is a reversible dopamine-ß-hydroxylase (DBH) inhibitor that decreases norepinephrine (NE) levels in sympathetically innervated tissues. Following in vivo administration N-acetylation of etamicastat was found to be a main metabolic pathway. The purpose of the current study was to characterise the N-acetylation of etamicastat by N-acetyltransferases (NAT1 and NAT2) and evaluate potential species differences in etamicastat N-acetylation using a sensitive and specific LC/MS assay. Marked differences in etamicastat N-acetylation were observed among the laboratory species and humans. After oral administration, the rat, hamster and humans presented the highest rates of etamicastat N-acetylation, whereas almost no acetylation was observed in the mouse, rabbit, minipig and monkey, and no acetylation in dog. In in vitro studies, rats and humans showed similar acetylation rates, whereas no acetylation was detected in the dog. Studies performed with human recombinant NAT1 and NAT2 enzymes revealed that both were able to conjugate etamicastat, though at different rates. NAT1 had lower affinity as compared to NAT2 (Km, 124.8 ± 9.031 µM and 17.14± 3.577 µM, respectively). Comparing etamicastat N-acetylation by human single donor enzymes and sulfamethazine, a selective substrate to NAT2, a significant correlation (r²=0.65, p<0.05) was observed. No correlation was observed with p-aminosalicyclic acid, a NAT1 selective substrate. In conclusion, results herein suggest that NAT2 and, to a lesser extent, NAT1 contribute to etamicastat N-acetylation. Furthermore, the high interspecies and intra-species differences in N-acetylation should be taken into consideration when evaluating the in vivo bioavailability of etamicastat.
Introduction

*N*-Acetyltransferase (NAT) is one of the major hepatic phase II enzymes involved in drug metabolism. NAT, a cytosolic protein that is expressed in a wide variety of tissues, plays an important role in the *N*-acetylation of drugs containing aromatic amine and hydrazine groups, converting them to aromatic amides and hydrazides, respectively.

Humans express two functional NAT isoforms, NAT1 and NAT2. NAT1 is widely distributed in the organism, whereas NAT2 has a restricted tissue distribution with higher levels of expression in the liver and intestine (Husain et al., 2007; Meyer, 1994). *N*-acetyltransferases exhibit different substrate specificities; in humans, isoniazid and sulfamethazine are efficiently *N*-acetylated by NAT2, whereas *p*-aminobenzoic acid is a substrate for NAT1 (Meyer, 1994; Stevens et al., 1999). It is recognized that human NAT1 and NAT2 loci are highly polymorphic, with more than 25 alleles identified in each locus (Hein et al., 2000a; Hein et al., 2000b; Stanley and Sim, 2008; Walraven et al., 2008). As an important metabolizing enzyme in humans, the polymorphisms in human NAT expression, especially NAT2, raise concerns about drug-drug interactions related to drug metabolism during clinical use (Dorne, 2004; Spielberg, 1996). Slow- and rapid-acetylators of both forms of NAT1 and NAT2 have been identified in humans (Grant et al., 1991; Meyer, 1994). In addition to NAT polymorphisms, species differences in drug *N*-acetylation have also been described (Gao et al., 2006; Glinsukon et al., 1975; Sharer et al., 1995), which could introduce interspecies variability in drug metabolism, raising concerns on the use of certain animal species for metabolic profiling of new compounds.

Etamicastat (also known as BIA 5-453; Figure 1), is a novel peripheral selective DβH inhibitor in development by BIAL-Portela & Cª, S.A. (S. Mamede do Coronado, Portugal) as a new putative drug therapy for cardiovascular disorders. Etamicastat acts mainly at the periphery by decreasing noradrenaline levels in sympathetically...
innervated tissues (Bonifácio et al., 2009) and showed to reduce both systolic (SBP) and diastolic (DBP) blood pressure, alone or in combination with other antihypertensive drugs, and to reduce noradrenaline urinary excretion in spontaneously hypertensive rats (SHR) (Igreja et al., 2008; Igreja et al., 2011), with no significant changes in heart rate (HR). In humans, etamicastat was well tolerated following single oral doses in the range 2 mg to 1200 mg (Rocha et al., 2012) and multiple once-daily oral doses in the range 25 to 600 mg (Nunes et al., 2010). Studies of etamicastat in healthy subjects showed extensive N-acetylation of etamicastat to the inactive metabolite BIA 5-961, and a large interindividual variability in pharmacokinetic parameters of both etamicastat and BIA 5-961 (Nunes et al., 2011; Nunes et al., 2010; Rocha et al., 2012; Vaz-da-Silva et al., 2011). A pharmacogenetic investigation showed that such variability was dependent upon differences in individual NAT2 genotypes (i.e. single nucleotide polymorphisms, SNPs) evaluated by PCR/RFLP (Cascorbi et al., 1995) leading to phenotypic differences in the N-acetylation metabolizing ability (i.e. rapid or poor acetylator status) (Nunes et al., 2011; Nunes et al., 2010; Rocha et al., 2012; Vaz-da-Silva et al., 2011).

The purpose of the present study was to characterize the potential interspecies differences in the N-acetylation of etamicastat and characterize the role of NAT1 and NAT2 in the N-acetylation of etamicastat.
Materials and Methods

Chemicals

Etamicastat ((R)-5-(2-aminoethyl)-1-(6,8-difluorochroman-3-yl)-1,3-dihydroimidazole-2-thione hydrochloride; Etamicastat N-acetylated (BIA 5-961, (R)-N-(2-(1-(6,8-difluorochroman-3-yl)-2-thioxo-2,3-dihydro-1H-imidazol-5-yl)ethyl) acetamide) were synthesized in the Laboratory of Chemistry, BIAL (S. Mamede Coronado, Portugal), with purities >95%. All other chemicals were purchased from SIGMA-Aldrich (St. Louis, MO).

Recombinant human Arylamine N-Acetyltransferase 1 4, Wild-Type Allele (NAT1) and Arylamine N-Acetyltransferase 2 4, Wild-Type Allele (NAT2) expressed in baculovirus-infected insect cells were purchased from BD Gentest (Woburn, MA). Pooled human, monkey, dog and rat liver S9, cytosolic fraction and cytosolic fraction from single donors (HH18, HH31, HH35, HG42, and HH47) were purchased from BD Gentest (Woburn, MA). The protein contents were used as described in the data sheets provided by the manufacturers.

Laboratory animals

Adult male Wistar rats (150 to 200 g bodyweight), CD1 mice (20-25 g bodyweight) and Syrian hamsters (85 to 130 g bodyweight), supplied by Harlan (Barcelona, Spain), were kept 5 per cage under controlled environmental conditions (12 h light/dark cycle, room temperature 22±1 °C and humidity 50±5 %) with free access to food and tap water.

Female Himalayan rabbits (2.22 to 3.17 kg bodyweight), supplied by Charles River Germany (Niederlassung Kisslegg, Stolzenseeweg 32-36, 88353 Kisslegg, Germany), were kept individually in stainless steel cages under continuously monitored environmental conditions (room temperature range: 18 ± 3 °C; relative humidity range:...
30 - 70%), with 12-hour fluorescent light/12-hour dark cycle with music during the light period. Pelleted standard food was available ad libitum.

Male and female (nonpregnant) Göttingen SFP minipigs (males, 19–20 kg; females, 14–15 kg bodyweight), supplied by Ellegard Göttingen Minipigs ApS (Breeding Centre, Sorø Landevej, 302 DK-4261 Dalmose, Denmark), were housed in individual pens under continuously monitored environmental conditions (room temperature of 20-23 °C; relative humidity range: 30 - 70%), with 12-hour fluorescent light / 12-hour dark cycle. Animals were given 400 g pelleted pig diet presented twice daily.

Male and female (nulliparous and nonpregnant) Cynomolgus monkey (Macaca fascicularis) (males, 2.11–2.52 kg; females, 1.86–2.24 kg bodyweight), supplied by Harlan Laboratories SrL (Via Enrico Fermi, 8 20050-Correzzana, Milan, Italy), were housed in group cages, each cage housing animals of the same sex under continuously monitored environmental conditions (room temperature of 20-24 °C; relative humidity range: 40 - 70%), with 12-hour fluorescent light/12-hour dark cycle. Animals were given 180 g/day/animal pelleted standard monkey diet.

All animal procedures were conducted in the strict adherence to the European Directive for Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (86/609CEE), Portuguese legislation and the rules of the “Guide for the Care and Use of Laboratory Animals” 7th edition, 1996, Institute for Laboratory Animal Research, Washington, DC. The number of animals used was the minimum possible in compliance with current regulations and scientific integrity.

**Human subjects**

Young human healthy volunteers were enrolled in the study while participating in a single-center, entry-into-man, phase 1, double-blind, randomized, placebo-controlled
study as previously described (Rocha et al., 2012). Etamicastat 1200 mg was administered with 250 mL of water, in the morning, after an overnight fast and subjects remained fasted at least 4 hours post-dose. A normal sodium diet (NaCl = 7g/day) was provided and no concomitant medication was allowed during the study. The clinical part of the study was conducted in accordance to the principles of the Declaration of Helsinki and the Good Clinical Practice Guidelines. An Independent Ethics Committee (CCP Ouest VI, Brest, France) reviewed and approved the study protocol and the subject information. Written informed consent was obtained for each subject prior to enrollment in the study.

Sample handling

Animals were fasted the night before administration. Etamicastat (mice, hamsters and rats 100 mg/kg; dog 20 mg/kg; rabbit 60 mg/kg; minipig 120 mg/kg; monkey 75 mg/kg) was given orally (p.o.) as a solution in water. Blank plasma was obtained from animals not subject to any treatment. In the experiments designed to evaluate in vivo etamicastat N-acetylation, samples were collected from anaesthetized dogs, mice, hamsters and rats at 3 and 9 h post-dosing and from anaesthetized rabbits, minipigs and monkeys at 2 and 24 h post-dosing. Human samples were collected at 2 and 24 h time points for comparison with the laboratory animals. Blood was collected and kept on ice until centrifuged at 1,500 g for 10 min at 4ºC. To an aliquot of 100 µl of plasma, 100 µl of acetonitrile containing a concentration of 500 ng/ml of the internal standard were added. After protein precipitation at room temperature, plasma samples were filtered using a 0.2 µm filter, samples were then injected into LC/MS.

Etamicastat N-acetylation in vitro by different species

Briefly, N-acetylation by human, monkey, dog and rat S9 fraction and pooled human cytosol was measured using an incubation mixture (100 µl total volume) containing 2 mg/ml total protein, 5 mM MgCl₂ and 0.25 mM Acetyl-CoA in 50 mM.
phosphate buffer pH 7.5. After 5 min pre-incubation reactions were initiated with 10 µM etamicastat. Reaction mixtures were incubated for up to 60 min and terminated with 100 µl 1 % formic acid in acetonitrile. All incubations were performed in a water bath shaking at 37°C. After removal of the protein precipitates by centrifugation for 10 min at 15,000 g, the supernatant was filtered through 0.2 µm Spin-X filters (Corning, NY) and injected onto a LC-MS.

### Kinetics of etamicastat N-acetylation in human pooled cytosol

Rates of N-acetylation were determined in human pooled cytosol, as described above with etamicastat concentrations ranging 5-500 µM. Experimental assay conditions for the determination of N-acetylation rates in human pooled cytosols were previously optimized by evaluating time (0 to 120 min) and protein dependency (0.5 to 3 mg/ml) of the enzymatic assay. Reactions were then carried out with 2 mg/ml total protein and an incubation time of 15 min. The reaction was terminated by adding ice-cold acetonitrile 1 % formic acid. After precipitation the samples were vortexed, centrifuged and supernatants filtered through Spin-X filters (0.2µm, Costar). The supernatant was injected in LC-MS.

### Etamicastat in vitro N-acetylation by human single donor cytosolic fraction

N-acetylation by human single donor cytosolic fraction was measured using the conditions described above. After pre-incubation with cytosols from single donors, the reaction was initiated by adding 500 µM etamicastat and the mixture was incubated for up to 15 min.

### Kinetics of etamicastat N-acetylation by NAT1 and NAT2

The acetylating reaction for recombinant NAT1 and NAT2 fractions was performed in duplicate in a final reaction volume of 100 µl, consisting of 0.1 mM acetyl-CoA, an acetyl-CoA regenerating system composed of 5 mM acetyl-DL carnitine and 1 unit of carnitine acetyltransferase per millilitre of assay buffer (250 mM triethanolamine-HCl, 5 mM dithiothreitol, pH 7.5) and NAT1 and NAT2 at the concentration of 0.04
µg/ml. The rate of N-acetylation was determined with etamicastat concentrations ranging from 5 to 500 µM and 5 to 2000 µM, for NAT1 and NAT2, respectively. The reaction was terminated by adding ice-cold acetonitrile 1 % formic acid. After precipitation the samples were vortexed, centrifuged and supernatants filtered through Spin-X filters (0.2µm, Costar). The supernatant was injected into LC-MS. The reaction was evaluated for the linearity of the product formation with respect to the incubation time (0 to 60 min) and to protein concentration (0.1 to 10 µg/ml).

**LC-MS analysis**

The analysis of the extracted plasma samples was performed using LC-MS/MS (Quattro Ultima, Waters) with positive (etamicastat) and negative (BIA 5-961) ion detection. Separation was performed on a Symmetry C8, 3.5 µm, 0.46 cm x 15 cm column (Waters) using water:acetonitrile 0.1% formic acid (20:80, v:v) as mobile phase. Electrospray ionisation was used with a capillary current of 2.8 kV. The multiple reaction monitoring pair was m/z 312→283, collision 25 eV and a cone voltage of 25 V for etamicastat; m/z 352→184, collision 30 eV and a cone voltage of 30 V for BIA 5-961; and m/z 402→120, collision 25 eV and a cone voltage of 25 V for internal-standard, an etamicastat similar molecule, BIAL’s proprietary compound. The analysis of samples extracts from in vitro studies was performed using LC-MS (Agilent, AP-ESI, 1100 Series, Agilent Technologies) with negative ion detection. Separation was performed on a Zorbax SB-C18, 3 µm, 30 x 4.6 mm, column (Agilent ) using a mobile phase A (water containing 0.1 % formic acid (v:v)) and B (methanol containing 0.1% formic acid (v:v)), with isocratic conditions of 50 % of A and 50 % of B. Selected ion monitoring (SIM) with the detection of each compound of interest was used for quantification: m/z 352 (BIA 5-961) and 412 (etamicastat). For maximal sensitivity, the fragment energy was set to 120 V and further settings were 3500 eV for the capillary voltage, 350ºC nebulizer gas temperature and 40 psi nebulizer pressure.
Data Analysis

Kinetic parameters of etamicast N-acetylation were obtained by fitting velocity data to the following models (equations 1 and 2) with GraphPad Prism (CA, USA):

Michaelis-Menten equation,

\[ v = \frac{V_{\text{max}} \cdot S}{K_m + S} \]

where \( v \) is the rate of the reaction, \( V_{\text{max}} \) is the maximum velocity, \( K_m \) is the Michaelis constant and \( S \) is the substrate concentration.

Houston & Kenworthy equation,

\[ v = \frac{V_{\text{max}} \cdot S}{K_m + S + \left( \frac{S^2}{K_{si}} \right)} \]

where \( K_{si} \) is the constant describing the substrate inhibition interaction. All data is reported as mean±S.E.M..
Results

Etamicastat N-acetylation in laboratory animals and humans

Upon oral administration the levels of etamicastat and its N-acetylated metabolite were quantified in the rat, mouse, hamster, rabbit, dog, minipig, monkey and human plasma at two time points (Figure 2). The circulating levels of etamicastat and its N-acetylated metabolite were markedly different across species. In the dog, only etamicastat could be detected at 3 and 9 h after oral administration of the parent compound. In the mouse, rabbit, minipig and monkey, plasma levels of the N-acetylated metabolite of etamicastat (BIA 5-961) were considerably lower than of the parent compound. The highest levels of N-acetylated etamicastat were detected in the rat, hamster and human healthy volunteers, comprising between 40% and 63% of the circulating etamicastat in hamsters and rats, respectively. In the humans similar levels of etamicastat and its N-acetylated metabolites were detected at 2 and 24 h post-dosing.

Etamicastat N-acetylation by different species in vitro

To evaluate the in vitro N-acetylation by different species, etamicastat was incubated with liver enzymes from rat, dog, monkey and human with and without acetyl-CoA, a necessary cofactor for NAT activity. As shown in Figure 3, no N-acetylation of etamicastat was detected in the absence of acetyl-CoA. In the presence of acetyl-CoA there were marked inter-species differences in etamicastat N-acetylation. N-acetylated etamicastat was detected in human, rat and monkey, but the levels of N-acetylated compound in the monkey were less than one tenth of those in the rat and in humans. No N-acetylation of etamicastat was observed in the dog.

Etamicastat N-acetylation by human cytosol

Kinetic analysis of etamicastat N-acetylation was performed in human liver cytosolic fraction pools. As shown in Figure 4, preparations displayed typical
hyperbolic kinetics. The apparent kinetic parameters derived from the curve fitted to the Michaelis-Menten equation are listed in Table 1. The intrinsic clearance (Cl_{int} = V_{max}/K_m) of etamicastat in human liver cytosolic fraction pools was 0.127 µl mg prot⁻¹ min⁻¹.

**Inter individual differences in etamicastat N-acetylation**

N-acetylation of etamicastat was measured in the cytosolic fractions from 5 (NAT1) or 4 (NAT2) human donors (HH18, HH31, HH35, HG42 and HH47) chosen to provide differences in their catalytic activities of the NAT1 and NAT2 enzymes (Table 2). As shown in Figure 5, the N-acetylation rate of etamicastat was different for the different donors. No positive correlation was obtained between etamicastat N-acetylation by human liver cytosol single donors and NAT1 activity against the NAT1 typical substrate p-aminosalicylic acid. In contrast, a significant correlation (r² =0.65, P<0.05) was obtained between the N-acetylation rate of etamicastat and that of sulfamethazine, a NAT2 typical substrate (Figure 6).

**Kinetics of etamicastat N-acetylation by recombinant NAT**

The characterization of the kinetics of etamicastat N-acetylation was performed for both NAT1 and NAT2. Each enzyme was incubated with different concentrations of etamicastat (5-500 µM to NAT2 and 5-2000 µM to NAT1) and the initial rates determined. The experimental data from NAT1 was fitted with Michaelis-Menten equation, and the data obtained from NAT2, showing an obvious substrate inhibition profile, was fitted with the Houston & Kenworthy equation. The resulting curves are shown in Figure 7 and the apparent kinetic parameters K_m and V_{max} derived from these curves, are depicted in Table 1. The enzyme with the highest affinity for the N-acetylation was NAT2, with a K_m of 17.14± 3.577 µM. NAT1 had lower apparent affinity with a K_m of a 3399 ± 312.0 µM. Accordingly it is possible to assume that NAT2 is the most important enzyme involved in the conjugation of etamicastat in the liver, since NAT1 has much lower affinity.
Discussion

Etamicastat is a reversible dopamine-ß-hydroxylase (DBH) inhibitor, which undergoes N-acetylation in humans markedly influenced by NAT phenotype. NAT N-acetylation is mainly described for drugs containing aromatic amine and hydrazine groups and etamicastat, although not containing any of these groups, was shown to be highly N-acetylated in the aminoethyl group (Nunes et al., 2010). Therefore a full understanding of the contribution of NATs enzymes in the etamicastat N-acetylation is essential for the evaluation of the inter-individual variability of etamicastat and its acetylated metabolite observed in clinical trials (Rocha et al., 2012).

In the study presented herein the kinetic parameters for etamicastat N-acetylation by recombinant NATs where determined and compared to those obtained with human liver cytosol in an attempt to elucidate which enzymes contribute to the etamicastat N-acetylation in vivo. N-acetylation of etamicastat in different species and the human intra-individual N-acetylation variability were also evaluated using in vitro and in vivo experimental models.

The results revealed marked differences in etamicastat N-acetylation among the species studied. The rat, hamster and man were the species showing the highest etamicastat N-acetylation rates, while mice, rabbit, minipig and monkey had almost no acetylated derivative, which accounted to less than 5% of the parent compound. N-acetylation of etamicastat in the hamster and in the rat accounts to approximately 40% and 63% of the parent compound, respectively. In humans the levels of the N-acetylated metabolite were similar to those of circulating etamicastat. These data suggest the presence of differences in etamicastat pharmacokinetics, metabolism and/or absorption among species. The levels of etamicastat in circulation may depend on 1) the relative rate of N-acetylation, 2) the route and the amount of etamicastat absorbed and excreted, and the 3) extent of other metabolic pathways. In agreement with the in vivo studies, in vitro studies showed different etamicastat N-acetylation
profiles among rats, dogs, monkeys and humans. With the exception of dogs, etamicastat N-acetylation was observed in all pre-clinical species examined, with a similar extent in rat and human S9 fractions, but being minor in monkeys. The N-acetylation pathway of etamicastat was absent in dogs, because NATs are not expressed in this species (Gao et al., 2006; Otsuka et al., 1983; Savidge et al., 1998; Sharer et al., 1995). The substantial differences observed between species on etamicastat N-acetylation were expected, since species differences and polymorphisms have already been observed in NAT expression (Gao et al., 2006). Additionally, it has been reported that different factors including genetic variation, age, gender and tissue type may alter NAT activity in mice, syrian hamsters and humans (Estrada et al., 2000; Levy et al., 1992). Furthermore, although rats, humans and monkeys express NAT, the relative expression and activity levels could affect the etamicastat metabolism resulting in interspecies differences. Interspecies scaling is often used to estimate the appropriated dosage for humans, based on the pharmacokinetics of drugs in animals (Mahmood et al., 2003). For etamicastat, however, the differences in N-acetylation between species could affect the accuracy of this approach. Moreover, the known genetic polymorphism of human NAT suggests caution must be taken due to inter-individual variability (Hein et al., 2000a; Hein et al., 2000b; Stanley and Sim, 2008; Sugamori et al., 2003).

To characterise in vitro N-acetylation of etamicastat pooled human cytosol and recombinant NATs were used. Pooled human liver cytosol N-acetylates etamicastat with a $K_m$ 124.8±9.0 µM and an intrinsic clearance of 0.127 µl mg.prot⁻¹.min⁻¹. Studies performed with human NAT1 4 and NAT2 4 showed that both enzymes were able to conjugate etamicastat albeit at different rates. NAT1 4 is a low affinity enzyme with a $K_m$ of 3399 ± 312.0 µM for etamicastat N-acetylation and NAT2 4 is a high affinity enzyme with a $K_m$ of 17.14± 3.577 µM. In addition, the N-acetylation by cytosolic fraction from individual donors with different NAT1 and NAT2 activity correlates well
with sulfamethazine \(N\)-acetylation, a selective substrate to NAT2, but not to \(p\)-aminosalicylic acid, a selective substrate to NAT1. The results, therefore, suggest that etamicastat \(N\)-acetylation is primarily produced through NAT2. In fact, it was shown that the high inter-individual variability in etamicastat \(N\)-acetylation observed in subjects included in clinical trials was attributed to different NAT2 phenotype evaluated by PCR/RFPLC (Rocha et al., 2012). Slow acetylator status may predispose patients to excessive pharmacological effects of etamicastat by allowing more parent drug to be available.

\(N\)-acetylation has been suggested to be the primary route of etamicastat biotransformation and the results obtained herein extend our understanding of etamicastat metabolism. NAT2 is possibly the major NAT involved in etamicastat \(N\)-acetylation; however, the contribution of NAT1 should not be excluded, because the enzymes involved will be dependent not only on the kinetics of the reaction, but also on the amount of compound that reaches the respective tissue and most significantly on the enzyme levels present in the tissues. NAT2 is expressed predominantly in liver whereas NAT1 is ubiquitously expressed (Winter and Unadkat, 2005). Therefore, it cannot be excluded that NAT1-mediated acetylation may have a role in the metabolism of etamicastat in non-hepatic tissues.

In conclusion results herein indicate that NAT2 and to a lesser extent, NAT1, contribute to etamicastat \(N\)-acetylation and the high interspecies and intra-species differences in \(N\)-acetylation should be taken into account in what concerns the bioavailability of etamicasat.
Authorship Contributions

Participated in research design: Loureiro, Wright, Soares-da-Silva

Conducted experiments: Loureiro, Fernandes-Lopes

Contributed new reagents or analytic tools:

Performed data analysis: Loureiro, Bonifácio

Wrote or contributed to the writing of the manuscript: Loureiro, Bonifácio, Soares-da-Silva
References


Footnotes

This study was supported by BIAL – Portela & Cª, S.A..
Legends to figures

**Figure 1.** Structural formula of etamicastat and its N-acetylated metabolite BIA 5-961.

**Figure 2.** Mean plasma etamicastat (closed bars) and etamicastat N-acetylated (open bars) in the dog, rabbit, mouse, minipig, hamster, monkey, rat and young healthy humans following *p.o.* administration of etamicastat (mice, hamsters and rats =100 mg/kg; dog = 20 mg/kg; rabbit = 60 mg/kg; minipig = 120 mg/kg; monkey = 75 mg/kg; humans = 1200 mg). Columns represent mean values and vertical lines indicate S.E.M. of n=3-14 subjects per group.

**Figure 3.** Etamicastat N-acetylation by liver S9 from rat, dog, monkey and by human S9 and cytosol pools. Etamicastat concentration was 500 µM. Values represent means±S.E.M. of duplicates.

**Figure 4.** Kinetics of etamicastat N-acetylation by human liver cytosol. Etamicastat concentrations ranged 1-1000 µM. Values represent means±S.E.M. of duplicates. Lines represent the fitting curves to Michaelis-Menten equation as described under Materials and Methods.

**Figure 5.** Etamicastat N-acetylation by cytosolic fractions from different donors. Liver cytosol from 4-5 donors (HH18, HH31, HH35, HG42, and HH47) were incubated with 500 µM of etamicastat for 15 min at 37°C. Values represent means±S.E.M. of duplicates.
**Figure 6.** Relationship between etamicastat N-acetylated and sulfamethazine NAT activity, a selective substrate to NAT2, and p-aminosalicylic acid, a selective substrate for NAT1. Liver cytosol from 4 donors was incubated with 500 µM of etamicastat for 15 min at 37°C. Values represent means±S.E.M. of duplicates.

**Figure 7.** Kinetics of etamicastat N-acetylation by recombinant human NAT enzymes. etamicastat concentrations ranged 5-2000 µM for A) NAT1 and 5-500 µM for B) NAT2. Values represent means±S.E.M. of duplicates. Lines represent the fitting curves as described under Materials and Methods.
TABLE 1

Apparent kinetic parameters of etamicastat N-acetylation in human liver cytosol and recombinant NAT enzymes

<table>
<thead>
<tr>
<th></th>
<th>$K_m$</th>
<th>$V_{max}$</th>
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<tbody>
<tr>
<td></td>
<td>$\mu M$</td>
<td>pmol mg prot$^{-1}$ min$^{-1}$</td>
</tr>
<tr>
<td>Human Cytosol$^a$</td>
<td>124.8 ± 9.031</td>
<td>15.8 ± 0.432</td>
</tr>
<tr>
<td>NAT1$^b$</td>
<td>3399 ± 312.0</td>
<td>1.50 ± 0.0995</td>
</tr>
<tr>
<td>NAT2$^b$</td>
<td>17.14 ± 3.577</td>
<td>0.810 ± 0.0874</td>
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$^a$ 0.25 mM AcCoA; $^b$ 0.1 mM AcCoA. Rates were fitted to Michaelis-Menten equation (human cytosol, NAT1) and substrate inhibition equation (NAT2; $K_{si}$ value obtained was 161.7±36.3). Values represent best fit values ± S.E.M..
TABLE 2

Activities of NATs in single donor human liver cytosol. Enzyme activity is expressed as pmol product per mg protein per min. Data is from BD Biosciences.

<table>
<thead>
<tr>
<th>Assay</th>
<th>HH18</th>
<th>HH31</th>
<th>HH35</th>
<th>HG42</th>
<th>HH47</th>
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<tbody>
<tr>
<td>NAT1 p-aminosalicylic acid</td>
<td>60</td>
<td>23</td>
<td>220</td>
<td>430</td>
<td>120/700</td>
</tr>
<tr>
<td>NAT2 sulfamethazine</td>
<td>270</td>
<td>520</td>
<td>500</td>
<td>200</td>
<td>50/10</td>
</tr>
</tbody>
</table>
Figure 1

Etamicastat

BIA 5-961
Figure 2

- **Dog**
  - 3h: 0, 1000, 2000 ng/ml
  - 9h: 3000, 4000 ng/ml

- **Rabbit**
  - 2h: 0, 800 ng/ml
  - 24h: 0, 2400 ng/ml

- **Mouse**
  - 3h: 0, 800 ng/ml
  - 9h: 1600, 2400 ng/ml

- **Minipig**
  - 2h: 0, 800 ng/ml
  - 24h: 0, 2400 ng/ml

- **Hamster**
  - 3h: 0, 1000 ng/ml
  - 9h: 1500, 2400 ng/ml

- **Monkey**
  - 2h: 0, 800 ng/ml
  - 24h: 0, 2400 ng/ml

- **Rat**
  - 3h: 0, 1000 ng/ml
  - 9h: 2000, 4000 ng/ml

- **Human**
  - 2h: 0, 800 ng/ml
  - 24h: 0, 2400 ng/ml
Figure 3.
Figure 4

![Graph showing the relationship between Etamicast (µM) and BIA 5-961 activity (nmol mg prot⁻¹ min⁻¹).]
Figure 5

BIA 5-961 (pmol mg protein⁻¹ min⁻¹)

- Cyt HH18
- Cyt HH31
- Cyt HH35
- Cyt HG42
- Cyt HH47
Figure 6

**NAT1 activity**

- BIA 5-453 N-acetylated (pmol/mg protein.min⁻¹)

**NAT2 activity**

- BIA 5-453 N-acetylated (pmol/mg protein.min⁻¹)
Figure 7

A

BIA 5-961 (nmol mg prot⁻¹ min⁻¹)

0.0 0.1 0.2 0.3 0.4 0.5 0.6

0 500 1000 1500 2000 2500

Etamicastat (µM)

B

BIA 5-961 (nmol mg prot⁻¹ min⁻¹)

0.0 0.1 0.2 0.3 0.4 0.5 0.6

0 100 200 300

Etamicastat (µM)