IVAX Research, Inc., Miami[1], FL, USA, Dept. of Clinical Pharmacology, Medical and Health Science Center, University of Debrecen[2], IVAX Drug Research Institute, Budapest[3], Hungary, College of Pharmacy, University of Florida, Gainesville[4], FL, USA

Influence of the *N*-acetylation polymorphism on the metabolism of talampanel: An investigation in fasted and fed subjects genotyped for NAT2 variants

P. Buchwald,[1] A. Juhász,[1,2] C. Bell,[1] M. Pátfalusi,[3] P. Kovács,[2] G. Hochhaus,[4] J. Howes,[1] N. Bodor[1,3,4]

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Peter Buchwald, IVAX Research, Inc., 4400 Biscayne Boulevard, Miami, FL 33137
Peter_Buchwald@ivax.com

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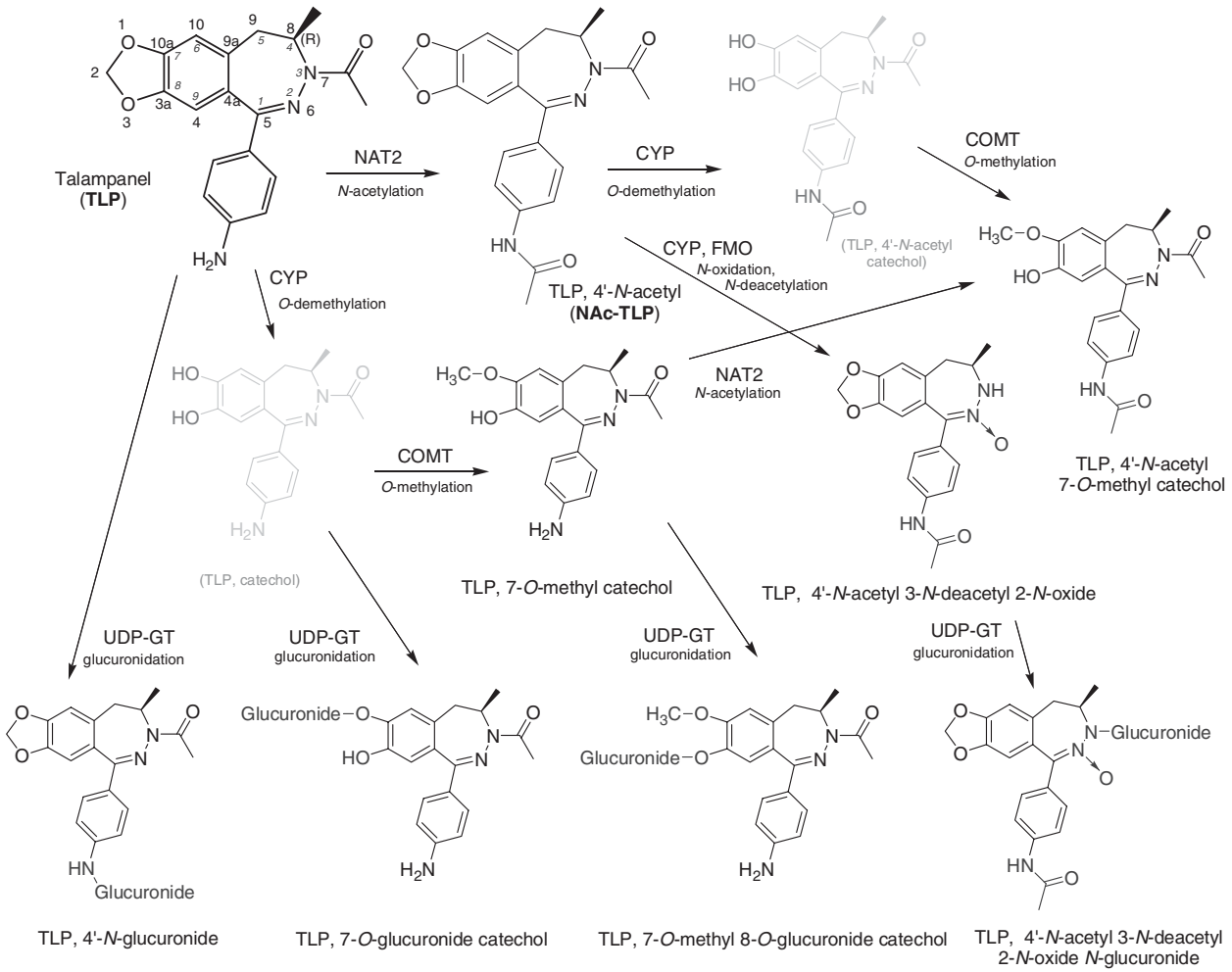
Talampanel is a 2,3-benzodiazepine-type allosteric (noncompetitive) AMPA-antagonist currently being developed as an orally active, broad-spectrum anticonvulsant. Here, a detailed study of its *N*-acetylation in humans is presented using plasma concentration data of both TLP and its *N*-acetyl metabolite obtained from healthy volunteers (n = 28) genotyped for *N*-acetyltansferase NAT2 isozymes. Plasma samples were obtained for up to 48 h after a single oral dose of 75 mg TLP both in fasted and in fed subjects. A perfect correspondence could be established between the phenotype inferred before the study from genotyping and that determined after the study by using plasma metabolite-to-parent molar ratios confirming that this route of metabolism is indeed mediated by NAT2. Analysis of the data has been performed using both noncompartmental analysis and a custom-built, unified parent-metabolite PK model, which incorporates three different acetylation rates according to the genotype-based classification of each subject as slow, intermediate, or fast acetylator to simultaneously fit plasma levels for both TLP and its metabolite. This suggest that for TLP in humans, (i) *N*-acetylation represents only a relatively small fraction of its total elimination (about one-fourth in fast acetylators and much less in slow acetylators), (ii) acetylation is about eight-twelve times faster in fast and three-six times faster in intermediate acetylators than in slow acetylators, and (iii) the *N*-acetyl metabolite is eliminated faster than the parent TLP.

1. Introduction

Even with the introduction of a number of new antiepileptic drugs (AEDs) in the past decade, there still is a considerable unmet medical need as 30–40% of epilepsy patients continue to have seizures despite the use of AEDs either alone or in combination (LaRoche and Helmers 2004). Talampanel (GYKI-53773, LY-300164) is an orally active, broad-spectrum anticonvulsant with a novel mechanism of action and very promising results obtained in various human clinical trials (Bialer et al. 2001; Bialer et al. 2002; Bialer et al. 2004; Chappell et al. 2002; Langan et al. 2003). Talampanel (TLP; 7-acetyl-5-(4-aminophenyl)-8,9-dihydro-8(*R*)-methyl-7*H*-1,3-dioxolo[4,5-*h*][2,3]-benzodiazepine; Scheme) is a selective, allosteric (noncompetitive) antagonist of the AMPA subtype of glutamate excitatory amino acid receptors (Bleakman et al. 1996; Bond et al. 1999; Lodge et al. 1996; May et al. 1999; Vizi et al. 1996). It was selected as a lead for clinical development from a family of 2,3-benzodiazepine compounds with no classical, 1,4-benzodiazepine activity at the γ-aminobutyric acid (GABA) receptor complex that was originally identified at the Institute for Drug Research (IDR or GYKI in Hungarian), Budapest, Hungary (now IVAX Drug Research Institute) (Ábrahám et al. 2000; Andrási 2001; Horváth 2001; Sólyom and Tarnawa 2002; Tarnawa et al. 1993; Tarnawa et al. 1989; Vizi et al. 1996). Its structure and that of its precursor GYKI-52466, the first noncompetitive AMPA antagonist identified, still serve as the standard of all related research (Buchwald et al. 2005; Sólyom and Tarnawa 2002).

TLP is a single *R*(−) stereoisomer (the corresponding *S*(+) isomer is inactive) (Bleakman et al. 1996; Lodge et al. 1996; May et al. 1999) that is thought to act at a novel allosteric site, often referred to as the "GYKI site" (Sólyom and Tarnawa 2002), on the AMPA receptor complex (Menniti et al. 2000; Vizi et al. 1996). It is essentially inactive (> 100 mM K_i) in α_1- and β-adrenergic, D_1, D_2, H_1, GABA, $5HT_2$, and muscarinic receptor binding assays. It is, however, orally effective in blocking AMPA receptor-mediated responses in multiple species. AMPA receptors are one of the three major subtypes of ionotropic glutamate receptors (*N*-methyl-D-aspartate – NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid – AMPA, and kainic acid – KA). Activation of these ionotropic glutamate receptors causes the entry of cations (Na^+ and/or Ca^{2+}) into the neuron, which results in depolarization (excitation). Glutamate activation of AMPA receptors is thought to mediate most fast synaptic neurotransmission in the brain, and the underlying molecular

Scheme: Possible metabolism scheme for talampanel (TLP; shown with two commonly used numbering systems) in humans on the basis of structures identified in plasma and urine in preliminary metabolism studies in two normal subjects following a single oral dose of 100 mg TLP



mechanisms are beginning to unravel (Armstrong and Gouaux 2000; Sun et al. 2002). Because antagonists can reduce neuronal death caused by excessive glutamatergic activity, which is present in many acute and chronic neurodegenerative diseases, they are of considerable therapeutic interest (Lees 2000; Madsen et al. 2001). AMPA antagonists are potential anticonvulsants, and they are expected to exert antiseizure activity by limiting neuronal hyperexcitability and by preventing glutamate-driven neuronal damage, a novel (and dual) mechanism compared to traditional antiepileptic drugs (AEDs), which act through GABA agonism or Na^+ channel blockade (Nicolson and Leach 2001). Hence, TLP not only promises antiseizure efficacy via a unique mechanism, but also the prospect of much needed neuroprotection in epileptic patients. Unlike NMDA antagonists, AMPA antagonists do not block the induction of long-term potentiation (LTP), a cellular mechanism underlying memory formation (Kapus et al. 2000). Furthermore, noncompetitive (allosteric) antagonists, such as TLP itself, that interact with binding sites on the receptor that are topographically distinct from the classic (so-called orthosteric) site have shown therapeutic advantages over competitive (orthosteric) antagonists, especially in ligand-gated ion channels (LGIC), but also at G-protein-coupled receptors (GPCR). There are a number of distinct advantages in using allosteric (noncompetitive) modulators (Christopoulos 2002): (i) the effect is saturable (hence, there is a "ceiling" of the effect even with excessive doses) contrary to competitive modulation; (ii) the effect cannot be overcome by the presence of excessive endogenous ligands, because they do not compete for the same binding site (especially important for glutamate antagonists as glutamate can be released in high local concentrations); (iii) better selectivity to produce responses only in tissues in which the endogenous agent is present, because they are expected to produce an effect only when the endogenous agent is present; and (iv) potential for greater receptor subtype selectivity.

Confirming the pharmaceutical potential of AMPA/KA receptor ligands, TLP seems to be not only a broad-spectrum anticonvulsant, which suppresses chemically and electrically kindled seizures (Borowicz et al. 2001) and synergistically potentiates the anticonvulsant activity of other AEDs administered concomitantly (Borowicz et al. 2000; Czuczwar et al. 1998), but also an agent that shows promising activity in various neuroprotection (Belayev et al. 2001; Erdő et al. 2005; Matucz et al. 2004; Világi et al. 2002), dyskinesia (Chase et al. 2000; Konitsiotis et al. 2000), autoimmune encephalomyelitis (Groom et al. 2003; Smith et al. 2000), and possibly even cancer (Rzeski et al. 2001) animal models. It showed synergistic activity when added to standard anticonvulsants (e.g., phenytoin and carbamazepine): in preclinical models, it increased their therapeutic window by rendering them efficacious at pre-

viously ineffective doses without impacting their toxicity (Borowicz et al. 2000; Czuczwar et al. 1998). There is preclinical evidence from studies in Parkinsonian monkeys that TLP can be useful in the symptomatic treatment of Parkinson's patients in combination with L-DOPA (Chase et al. 2000; Konitsiotis et al. 2000), and TLP has been shown to potentiate the stimulatory effect of L-DOPA on dopamine release in dopamine-defficient corticostriatal slice preparations (Juranyi et al. 2004). It also has the advantage of being orally effective in all species tested, including humans.

In humans, TLP is well absorbed from the gastrointestinal tract. Mean time to reach maximum plasma concentration, t_{max}, is around 2 h, and mean terminal half-life at steady state, $t_{1/2ss}$ is approximately 6–7 h after chronic administration (Bialer et al. 2001, 2002; Langan et al. 2003). Following single-dose administration (30–35 mg), mean TLP $t_{1/2}$ values are somewhat shorter: around 4 h in healthy volunteers and 3 h in patients on enzyme-inducing AEDs, such as carbamazepine (CBZ), phenytoin (PHT), or phenobarbital (PBA) (Langan et al. 2003). The same study also suggested that, in induced patients, apparent plasma clearance (CL/F) values are considerably increased compared to healthy volunteers following single-dose administration (81.4 L/h vs. 26.2 L/h), and they decrease somewhat after repeated oral administration (CL/F_{ss} of 39.5 L/h) (Langan et al. 2003). A lovastatin interaction study found no effect on lovastatin plasma levels after 10 days of talampanel dosing (60 mg, t.i.d.) indicating no CYP3A4 inhibition or induction (Bialer et al. 2002). Plasma protein binding was estimated to be in the 67–88% range.

TLP has different metabolite profile across species. In humans, preliminary studies identified six metabolites including the 7-*O*-methyl catechol TLP, the 4′-*N*-acetyl TLP (NAc-TLP), and *O*- or *N*-glucuronidated compounds in plasma (Bialer et al. 2001, 2002, 2004) and another two including the 4′-*N*-acetyl-7-*O*-methyl catechol TLP and a putative *N*-oxide metabolite in urine. On the basis of these data, a possible metabolic scheme for TLP in humans is presented in the Scheme. In agreement with the known metabolic pathway of methylenedioxyphenyl compounds (Anders et al. 1984; Kaye et al. 1989; Maurer 1996), oxidation to an unstable catechol intermediate was assumed, which is then methylated or glucuronidated. These intermediates (denoted in gray and with their names in parenthesis) were not detected in humans, but the 4′-*N*-acetyl catechol metabolite was detected in monkeys. The 7-*O*-methylation is in agreement with the usual regioselectivity for the *meta* position in catechols.

Preclinical studies indicated that *N*-acetylation represents a route of metabolism of varying significance in various species: it is extensive in monkeys, moderate in rats, poor in mice, and negligible in dogs (Bialer et al. 2001, 2002). Interestingly, the *N*-acetyl metabolite is essentially inactive *in vitro*, but it was found about one-third as active as TLP in some preclinical *in vivo* studies, possibly because it can be deacetylated to TLP. Because of its extensive presence in monkeys, and because of its possible polymorphic nature, which might cause variability in human kinetics (Dorne et al. 2003; Lin and Lu 1997), the *N*-acetylation of TLP received considerable interest in early clinical development, but recent Phase I and II studies indicate that this route is of relatively small significance in humans. Consequently, acetylator status seems to have only a relatively minor influence on TLP concentrations and pharmacokinetics. The main goal of the present investigation was to clarify this issue in details.

The *N*-acetyltansferase (EC 2.3.1.5) isozymes NAT1 and NAT2 that catalyze the *N*-acetylation (usually deactivation) and *O*-acetylation (usually activation) of aromatic and heterocyclic amine compounds are polymorphic (Brockton et al. 2000; Hein et al. 2000). These isozymes use acetyl coenzyme A as a cofactor, are classified as Phase II conjugating enzymes, and differ in their substrate specificities: isoniazid and sulfamethazine are NAT2-specific substrates, whereas *p*-aminobenzoic acid and *p*-aminosalicylic acid are NAT1-specific substrates. Whereas NAT2 is primarily expressed in the liver, NAT1 is primarily expressed at other sites, including the colon (Brockton et al. 2000). In fact, the discovery in 1953 of the acetylation polymorphism (NAT2) in tuberculosis patients undergoing isoniazid therapy (Bönicke and Reif 1953; Hughes et al. 1954) was the first evidence of polymorphic metabolism. Three NAT2 phenotypes have been described: fast, intermediate, and slow acetylators, but many early studies did not distinguish between fast and intermediate acetylators and categorized both as fast acetylators.

In humans, the two expressed genes for NAT1 and NAT2 are 870 base-pair intron-less protein coding regions located on chromosome 8 that encode 290 amino acid proteins (Brockton et al. 2000). Specific single base-pair substitutions responsible for altered enzyme activity were first identified in 1990 (Blum et al. 1990), and they now allow genotypic classification. Fast acetylators possess two copies of the wild type allele (NAT2*4), intermediate acetylators possess one wild-type and one mutated allele (most commonly, especially in Caucasian populations, NAT2*5, NAT2*6, or NAT2*7), and slow acetylators possess two mutated alleles. The three major variants identified were originally designated as M1, M2, and M3 and are now termed NAT2*5A, NAT2*6A, and NAT2*7A, respectively. For example, NAT2*5A corresponds to two nucleotide transitions: $C^{481}T$ and $T^{341}C$. A study on the relative (*in vitro*) capacity of recombinant human NAT2 allozymes to catalyze the *N*-acetylation of 2-aminofluorene found that allozymes expressed with NAT2*5 alleles had the greatest reductions in metabolic activation, those expressed with NAT2*6 (and NAT2*14) alleles had more moderate reductions, and those expressed with NAT2*7 alleles had the smallest but yet significant reductions (Hein et al. 1995). The frequency of NAT2 slow acetylators varies markedly with ethnicity: it is lowest in Asian populations (e.g., close to only 10% in Japanese) and is around 50–60% in European populations. It was highest in two small African studies (~80%), but, it seems to be lower in US studies of African-Americans (Brockton et al. 2000). Discrepancies between genotype and phenotype have been observed (in particular for African Americans and US Hispanics, but also for Europeans), some of them might result from the fact that the assumptions related to the presence or absence of the selected indicator mutation(s) are not tenable in all populations.

2. Investigations, results, and discussion

2.1. Phenotype-genotype correspondence

A total of fifty prospective subjects have been NAT2-genotyped to ensure that adequate numbers of enrolled subjects will be present within each acetylator group. Prescreening of these subjects (all healthy Caucasian men recruited in Debrecen, Hungary) found frequencies of 32%, 34%, 32%, and 2% for NAT2 alleles *4, *5, *6, and *7, respectively. This resulted in inferred phenotype fre-

quencies of 12%, 40%, and 48% for fast (*4/*4 homozygous), intermediate (*4/[*5/*6/*7] heterozygous), and slow ([*5/*6/*7]/[*5/*6/*7]) acetylators, respectively. The frequency of the NAT*4 wild-type allele in this population (32%) and consequently the frequency of true (*4/*4 homozygous) fast acetylators (12%) was slightly higher than that usually found in European populations. For example, the NAT*4 allele in healthy male controls in a UK population had a frequency of only 18% (n = 99) (Nakago et al. 2001), and the frequency of true fast acetylators in European populations is usually in the 4–9% range (http://www.cdc.gov/genomics/hugenet/reviews/tables/print/N-ACET-tbls.pdf) (Brockton et al. 2000). There is a possibility that this slightly increased frequency found for fast acetylators might be related to the assumed (somewhat mythology-based) Asian origin of the Hungarian population (the frequency of fast acetylators being much higher in Oriental populations), but because of the relatively small sample size (n = 50), no conclusions can be drawn. Genetic analyses of Hungarian populations found some, but only very little, connection to Asian populations and found results that are mostly typical of other Europeans (Guglielmino and Béres 1996; Guglielmino et al. 2000; Semino et al. 2000), despite Hungarian being one of the six non-Indo-European languages spoken in Europe and evidence for at least some connections between genetic changes and linguistic borders (Barbujani and Sokal 1990), suggesting that Magyars imposed their language on Hungarians but seem not to have significantly affected their genetic structure (Semino et al. 2000).

Considerable variability was observed both in individual TLP and in its *N*-acetyl metabolite (NAc-TLP) plasma levels as there seems to be some variability in the absorption rate of TLP: in about 20% of the subjects, TLP absorption is faster than in the rest (Fig. 1a). In these subjects, the observed t_{max} corresponds to the first sampling point (0.5 h). Fast gastric emptying could be an explanation, especially since the slightly basic TLP is un-

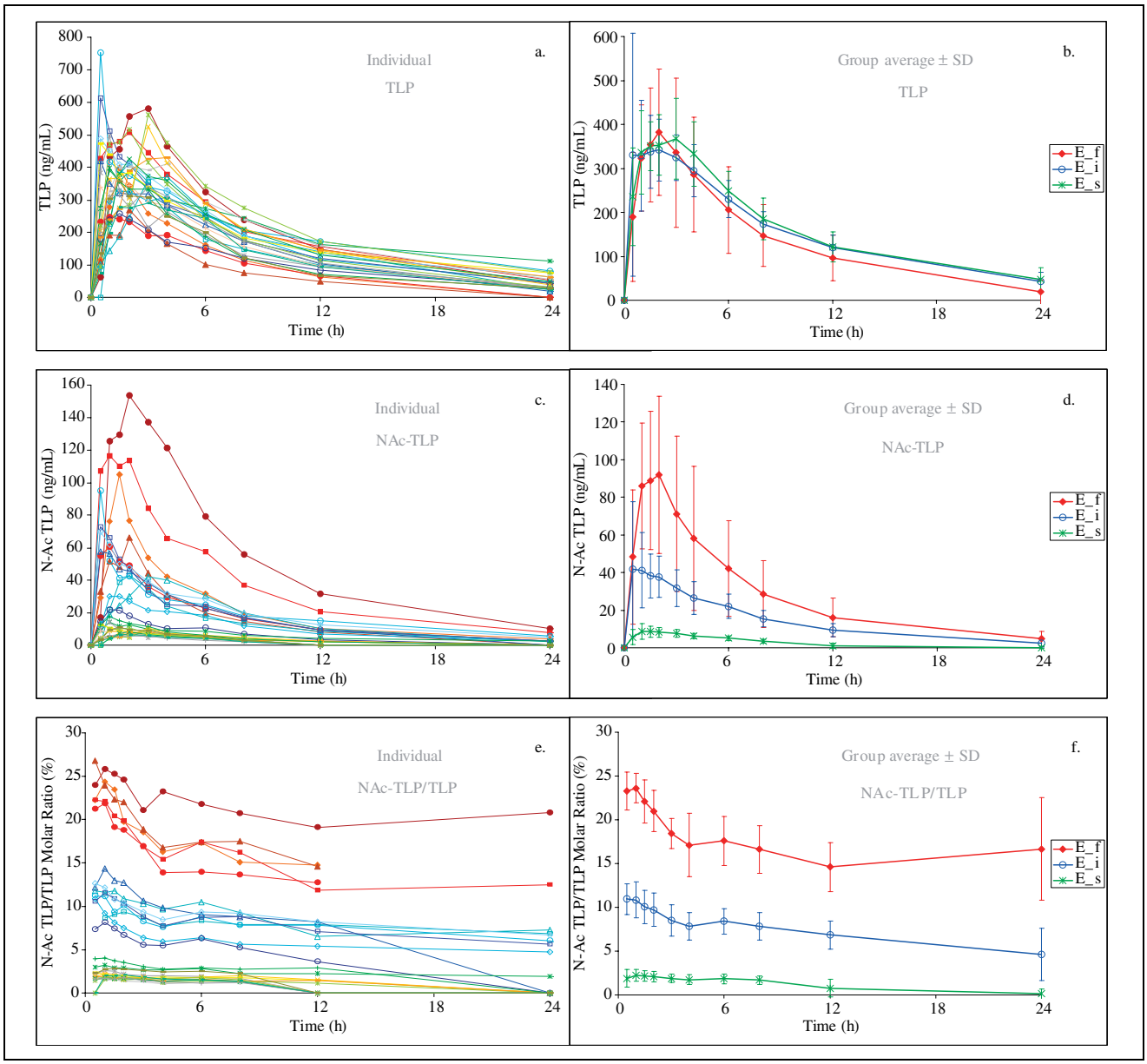


Fig. 1: Individual (left column) and average by acetylator status ± SD (right column) values for TLP plasma concentrations (TLP, ng/mL; a, b), *N*-acetyl TLP plasma concentrations (NAc-TLP, ng/mL; c, d), and the molar ratio of NAc-TLP to TLP (%; e, f) in 27 fasted healthy volunteers following a single oral dose of 75 mg. Values are shown as a function of time up to 24 h and are color-coded by acetylator status as determined by genotyping for NAT2 (fast: red, closed symbols n = 5; intermediate: blue, open symbols n = 8; slow: green, other symbols n = 14)

likely to be absorbed under the acidic conditions of the stomach. As the color coding in Fig. 1a reveals, no separation in plasma TLP levels is obvious if subjects are grouped according to their acetylator status (fast, intermediate, and slow). A separation in the NAc-TLP metabolite levels is much more clearly present, but there are some overlaps even here (Fig. 1c). Nevertheless, a clear separation of the acetylator groups could be obtained by calculating the NAc-TLP/TLP metabolite-to-parent (molar) ratios at any given time-point (Fig. 1e, f). With this method, a perfect correspondence was found between the phenotype inferred before the study from genotyping for *N*-acetyltansferase NAT2 isozymes (alleles NAT2*4, *5, *6, and *7) and the phenotype determined after the study by using the metabolite-to-parent plasma ratios. Therefore, even if there are overlaps in the plasma levels of the NAc-TLP metabolite among the different phenotypes (most likely due to interindividual variability in TLP absorption rates), a clear separation of the acetylator groups can be obtained by calculating the NAc-TLP/TLP metabolite-to-parent (molar) ratios. The perfect correspondence found between the phenotype determined this way and the phenotype inferred from genotyping for NAT2 isozymes is a clear indication that this route of TLP-metabolism is indeed mediated by NAT2.

The NAc-TLP/TLP metabolite-to parent ratio had a value of <5% in slow, 5–15% in intermediate, and 15–25% in fast acetylators (Fig. 1e, f). The relatively low value of this ratio for TLP (<25% even in fast acetylators) has to be noted: for isoniazid, the standard NAT2 substrate with polymorphic acetylation, a plasma metabolite-to-parent molar ratio (MR, usually measured at 3 h following administration) of approximately 50% is used to separate slow (MR < 50%) and fast acetylators (MR > 50%) (Ait Moussa et al. 2002; Rey et al. 2001). Corresponding values are even much higher (e.g., >250% in fast acetylators) for sulfasalazine (Kita et al. 2001; Kumagai et al. 2004). Interestingly (and as a possible indication of a relatively rapid elimination of the metabolite), this metabolite-to-parent ratio decreases in its early phase for TLP (1–4 h; see Fig. 1e, f) as, the NAc-TLP metabolite consistently tends to reach its C_{max} somewhat earlier than the parent TLP (see t_{max} values of Table 1 vs. Table 2).

Table 2: PK parameters obtained for NAc-TLP by noncompartmental analysis for the various acetylator groups (average of individual values with SD shown in row below and smaller font) both in fasted and in fed subjects

NAc-TLP, fasted subjects						
Group	t_{max} (h)	C_{max} (ng/mL)	AUC_t (ng · h/mL)	AUC_∞ (ng · h/mL)	$t_{1/2}$ (h)	λ_z (h^{-1})
Fast, n = 5	1.50	100.4	664	695	6.18	0.115
SD	0.50	38.3	400	391	1.01	0.022
Interm., n = 8	1.13	54.1	324	360	6.09	0.122
SD	0.92	24.4	109	124	1.65	0.036
Slow, n = 14	1.71	10.4	57	74	4.80	0.155
SD	0.83	3.4	26	29	1.56	0.036
All, n = 27	1.50	40.0	248	274	5.44	0.138
SD	0.82	40.3	288	293	1.60	0.037
NAc-TLP, fed subjects						
Fast, n = 5	2.80	80.5	589	633	5.64	0.129
SD	1.30	26.3	279	290	1.30	0.032
Interm., n = 8	1.94	44.5	306	349	6.53	0.113
SD	1.21	14.5	116	116	1.72	0.028
Slow, n = 14	2.29	10.2	58	76	4.69	0.161
SD	1.03	3.2	36	42	1.72	0.042
All, n = 27	2.28	33.4	230	260	5.41	0.141
SD	1.13	30.4	242	255	1.79	0.042

2.2. Noncompartmental PK

PK parameters for TLP and NAc-TLP derived from noncompartmental analyses (C_{max}, t_{max}, AUC_t, AUC_∞, V/F, CL/F, $t_{1/2}$, λ_z) are presented in Table 1 and Table 2 for fasted and fed subjects, respectively, grouped according to acetylator status. Overall, PK parameters obtained in fasted and fed subjects are very similar (Fig. 2): the only parameters affected in statistically significant manner (paired t-test) by having a high-calorie, high-fat meal are the times to reach maximum plasma concentration, t_{max}, which increased for both TLP and NAc-TLP (Table 1, Table 2).

Fast acetylators tend to have somewhat reduced TLP peak plasma concentration (C_{max}), area under the (concentration) curve (AUC_t), and elimination half-life ($t_{1/2}$) values

Table 1: PK parameters obtained for TLP by noncompartmental analysis for the various acetylator groups (average of individual values with SD shown in row below and smaller font) both in fasted and in fed subjects

TLP, fasted subjects								
Group	t_{max} (h)	C_{max} (ng/mL)	AUC_t (ng · h/mL)	AUC_∞ (ng · h/mL)	V/F (L)	CL/F (L/h)	$t_{1/2}$ (h)	λ_z (h^{-1})
Fast, n = 5	1.90	400	2948	3414	217	26.9	6.00	0.121
SD	0.74	146	1688	1744	81	12.2	1.51	0.029
Interm., n = 8	1.38	455	3687	4086	204	19.3	7.50	0.097
SD	1.22	161	879	967	52	4.5	1.70	0.024
Slow, n = 14	2.18	427	3980	4367	195	18.3	7.70	0.094
SD	1.15	79	1019	1156	32	4.7	1.93	0.019
All, n = 27	1.89	430	3702	4107	202	20.2	7.33	0.100
SD	1.13	117	1147	1232	48	7.1	1.85	0.024
TLP, fed subjects								
Fast, n = 5	2.70	342	2707	3341	219	25.0	6.30	0.113
SD	1.40	111	1219	1228	61	8.8	1.28	0.021
Interm., n = 8	2.13	387	3432	3914	219	20.4	7.80	0.098
SD	1.09	101	1018	1042	66	5.4	2.45	0.034
Slow, n = 14	2.46	422	3825	4292	190	19.1	7.20	0.100
SD	0.95	80	1252	1381	42	5.9	1.51	0.020
All, n = 27	2.41	397	3501	4004	204	20.6	7.21	0.102
SD	1.06	94	1213	1269	53	6.5	1.81	0.025

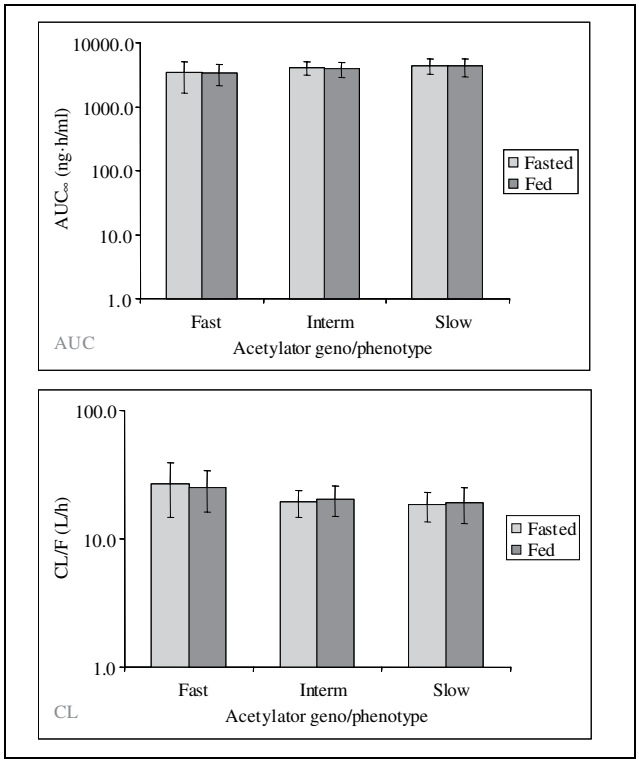


Fig. 2: Comparison of the AUC_∞ and CL values (average ± SD) obtained for TLP in various acetylator groups both in fasted and fed subjects

and somewhat increased plasma clearance (CL/F) values, but none of these differences were statistically significant at the $p < 0.01$ level even if compared to slow acetylators. For TLP, no other differences among acetylator-groups (fast vs. slow, fast vs. intermediate, or intermediate vs. slow acetylators) seemed obvious. Age, height, weight, or body mass index (BMI) showed little correlation with C_{max}, $t_{1/2}$, or λ_z ($r^2 < 0.10$); weight showing some effect on AUC, V/F, and CL/F (r^2 of 0.15, 0.31, and 0.21, respectively). Because even this was mainly due to data from the three subjects with the largest weights, and because the relatively limited weight-range, 78.2(±10.9) kg, in this set of healthy male volunteers, we focused on the modeling of *N*-acetylation made possible by genotyping and detailed metabolite monitoring.

Comparison of the corresponding PK parameters among various acetylator groups (Table 1) indicates that *N*-acetylation is not a highly significant metabolic route for TLP in humans. The difference in CL/F values following a single 75 mg dose obtained in the present study between (true) fast and slow acetylators, 26.9(±12.2) vs. 18.3(±4.7) L/h in fasted and 25.0(±8.8) vs. 19.1(±5.9) L/h in fed subjects, is considerably smaller than those obtained earlier in the exploratory H4H-EW-LGAB study, which used dapsone-phenotyped acetylators and a much smaller number of subjects (n = 3/group): 32.2(±9.4) vs. 19.9(±5.7) L/h for a 20 mg dose and 36.7(±9.5) vs. 15.8(±3.0) L/h for a 30 mg dose. Interestingly, these previous larger differences were obtained despite distinguishing only fast and slow acetylators and, hence, incorporating intermediate acetylators in the fast group. The more recent studies, which are also more accurate from design- (geno- and phenotyping), statistical- (larger number of subjects), and analytical- (lower LLQ) perspectives, suggest a considerably smaller difference in the PK of TLP among different acetylator groups. At present, there seems to be no indication supporting earlier observations made on the basis of the exploratory H4H-EW-LGAB study that plasma TLP levels are affected by acetylator status with a potential twofold difference in drug concentration (Bialer et al. 2001). Polymorphic differences in *N*-acetylation do not seem to be significant enough to cause special clinical concern, and they are likely to be even smaller in patients taking other AEDs (especially enzyme-inducing AEDs) because, in such patients, TLP clearance is somewhat increased, and, therefore, the relative role of *N*-acetylation is further reduced. These results also seem to confirm the observation that mice, which were poor TLP-acetylators, provide a good model for human NAT (Sim et al. 2003).

The AUC-ratios of the NAc-TLP metabolite obtained directly from the noncompartmental analysis of the metabolite data (Table 2) can be used as a first estimate of the ratios of rates of formation of the metabolite in the various acetylator groups. The corresponding average NAc-TLP AUC_t ratios are 11.6 and 10.1 for the fast/slow and 5.7 and 5.3 for the intermediate/slow comparisons in fasted and fed subjects, respectively. For comparison, Antila and co-workers obtained recently an AUC ratio of approximately 3.5 for OR-1896, an *N*-acetylated metabolite of levosimendan, when comparing "rapid" (according to genotype, in fact intermediate) and slow acetylators (Antila et al. 2004). By using a different approach, Kita and co-workers estimated a 2.7-fold difference between fast and intermediate acetylators in the acetylation of sulfapyridine (Kita et al. 2001), similar to the 2–3-fold difference obtained here for TLP.

2.3. Parent-metabolite PK model with acetylation polymorphism

Analysis of the data from fasted subjects has also been carried out with a custom-built, pharmacogenetics-based, unified parent-metabolite PK model that incorporated three different rates of acetylation to simultaneously fit plasma levels for both TLP and NAc-TLP following genotype-based classification of each subject as slow, intermediate, or fast acetylator (Buchwald et al. 2005). Because a compartmental analysis performed for TLP with a one compartment (V), first-order input (k_{01}), first-order elimination (k_{10}) model indicated that a one compartmental model fit the data well, and the obtained parameters were in general agreement with the noncompartmental analysis, a parent-metabolite model consisting of one parent and one metabolite compartment was used (Fig. 3). In order to fit the decreasing time-profile of the NAc-TLP/TLP ratio and to obtain realistic V_M values (Buchwald et al. 2005), early formation of the metabolite (i.e., during first-pass of the parent; hence, even before the parent enters the main compartment) was allowed ($k_{ma} > 0$), a known possibility for orally administered drugs undergoing hepatic extraction (Rowland and Tozer 1995). As the metabolite input rate (k_{ma}) of this model is related to the rate of acetylation during first-pass of the parent, it was allowed to have three possible values through the same multiplying factors used for the rate of metabolite formation (acetylation) $k_f^{f/i/s}$ (Fig. 3). Because of the relatively large number of parameters in this model, pooling of data was necessary to obtain meaningful and consistent results; therefore, simultaneous fitting of all the $2 \times 27 = 54$ individual concentration-time profiles was performed with the same model using k_m^f, k_m^i, or k_m^s for each subject as adequate (naïve pooled data approach). Optimization using unweighted data resulted in somewhat low and ill-defined V_M values, but use of a weighting schemes corresponding

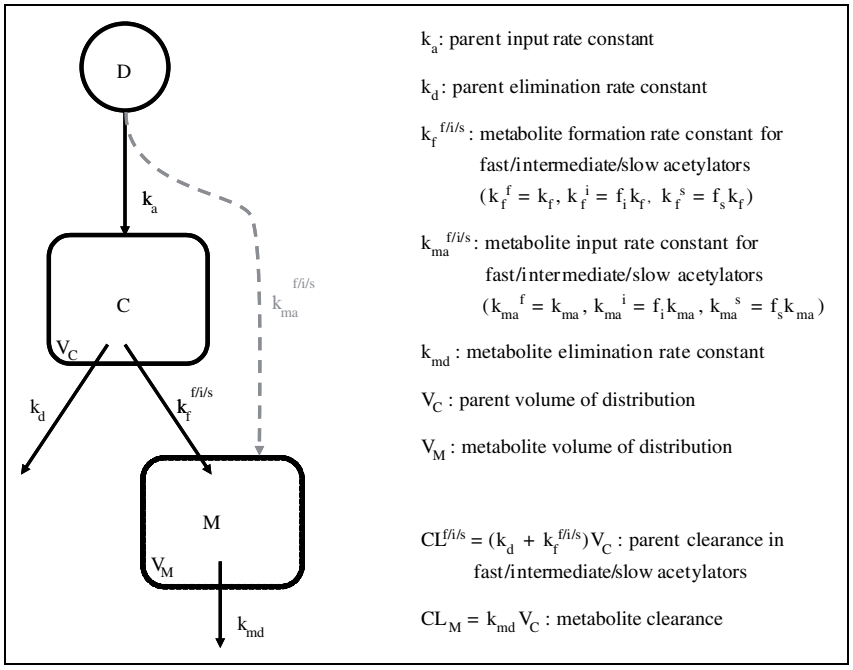


Fig. 3:
The unified parent-metabolite PK model incorporating polymorphic acetylation used to simultaneously fit both parent (TLP) and its *N*-acetyl metabolite (NAc-TLP) plasma levels (C and M, respectively) in slow, intermediate, and fast acetylators

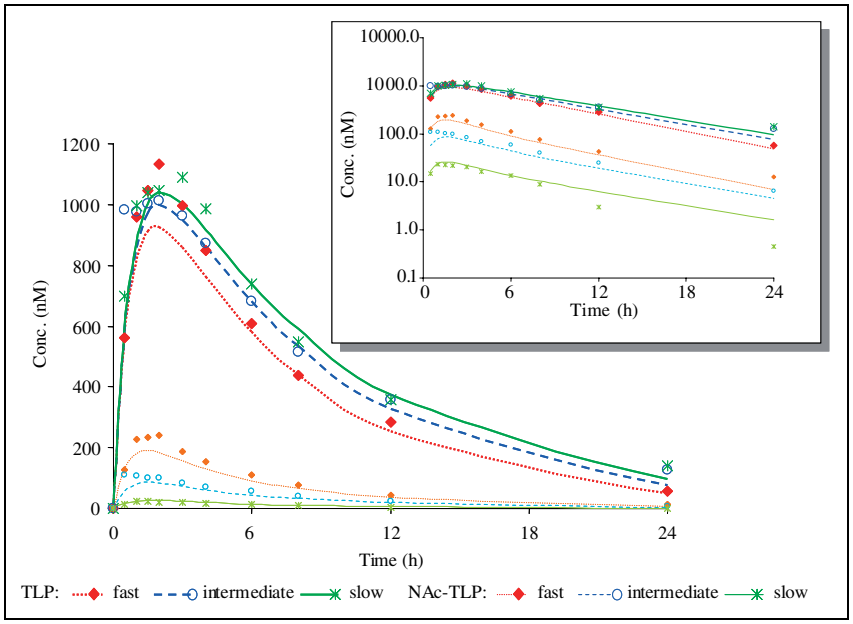


Fig. 4:
Plasma concentrations for TLP and NAc-TLP by acetylator status following an oral dose of 75 mg TLP in fasted, healthy volunteers. Values shown for measured (TLP: darker symbols, NAc-TLP: smaller, lighter symbols) and predicted data (TLP: darker colored lines, NAc-TLP: lighter colored lines) grouped by acetylator status (fast, n = 5: closed diamonds; intermediate, n = 8; open circles; slow, n = 14: other). The smaller inset presents the same data on a semilogarithmic plot. Measured values shown are averages for each acetylator group; predicted values shown were obtained with the unified PK model (Fig. 3, Table 3)

Table 3: Parameters obtained for the unified parent-metabolite PK model (Fig. 3) incorporating polymorphic acetylation (fasted subjects)

Parameter	Value (±SE)
k_a (h^{-1})	1.22(±0.10)
k_{ma} (h^{-1})	0.146(±0.115)
k_d (h^{-1})	0.109(±0.009)
k_f (h^{-1})	0.029(±0.019)
f_i	0.426(±0.131)
f_s	0.125(±0.092)
k_{md} (h^{-1})	0.538(±0.566)
V_C/F (L)	165.5(±7.5)
V_M/F (L)	82.7(±79.0)
s (nM)	133.3
CL^f/F (L/h)	22.8
CL^i/F (L/h)	20.1
CL^s/F (L/h)	18.6

Identical parameters are used for all groups except for the rate constant of acetylation $k_f^{f/i/s}$ and metabolite input $k_{ma}^{f/i/s}$, which have three different values for the three different acetylator phenotypes. Total TLP clearance values ($CL^{f/i/s}$) calculated from these values are shown in the last rows to allow comparisons with other PK models

to Poisson errors ($w_i = 1/C_i$), which gives more emphasis to the metabolite data, resulted in good fit of the concentration data of fasted subjects (Fig. 4) with realistic and sufficiently well-defined parameters (Table 3). In this Figure that shows group averages, the data of fast acetylators are somewhat less well fit because fast acetylators represented the smallest fraction of all subjects.

Total TLP plasma clearance values obtained with this approach, $CL = (k_d + k_f)V_C$, are similar to those obtained in the previous models (Table 3 vs. Table 1), and again they show only a relatively modest difference between fast and slow acetylators (22.8 vs. 18.6 L/h). All tested models indicated the clearance of the metabolite NAc-TLP (CL_M) to be faster than that of the parent TLP. All obtained clearances, including those of CL_M/F, have realistic values, well below a blood-flow limited value (~90 L/h for liver in humans (Gabrielsson and Weiner 2000; Gibaldi and Perrier 1982; Jolivette and Ward 2005)) even with a 100% oral bioavailability (F = 1.0). Considering the mean weight of subjects in this study, the volume of distribution

obtained for TLP corresponds to a value of 2.1 L/kg. According to the obtained parameters, only a relatively small portion of the drug is lost to first-pass acetylation even in fast acetylators:

$$k_{ma}/(k_a + k_{ma}) = 11\% \quad (1)$$

One weakness of the model is that the V_M/F (and the related k_{md}) values are somewhat ill-defined and sensitive to the structure of the model and the fitting procedure used. Hence, the V_M/F and k_{md} values of Table 3 have to be considered only as first, rough estimates. This uncertainty is a result of the apparent rapid elimination of the metabolite and the possibility of reversible metabolism (acetylation-deacetylation), which could also result in parallel terminal slopes for parent and metabolite (Cheng and Jusko 1993). Definitive clarification of these issues could be obtained only by administration of NAc-TLP itself.

An analysis of the rate constants obtained (Table 3) suggest that for TLP, *N*-acetylation represents only about one fifth to one fourth of the total elimination even in true ($^*4/^*4$ homozygous) fast acetylators: k_f^f/k_{d+f} is $0.029/0.138 = 0.21$, and much less in slow acetylators. The ratios of the acetylation rate constants (k_f^f, k_f^i, and k_f^s) suggest that the acetylation is about eight – ten times faster in fast and three – five times faster in intermediate acetylators than in slow acetylators: the fast/slow ratio being $k_f^f/k_f^s = 1/f_s = 8.0$, and the intermediate/slow ratio being $k_f^i/k_f^s = f_i/f_s = 3.4$ with the final parameter set of Table 3. Because individual data of all patients had been used in the fitting procedure and fast acetylator represent only a relatively small proportion (5/27), k_f^f (and to some degree k_f^i also) and, therefore, these corresponding ratios as well are slight underestimates (Fig. 4).

The quantitative estimates of the TLP acetylation rates obtained from this unified, pharmacogenetics-based parent-metabolite PK model (8–12 times faster in fast and 3–6 times faster in intermediate acetylators than in slow acetylators) have to be regarded as tentative only until further confirmation because they could be model-dependent. Nevertheless, they are in good agreement with the AUC-ratios of the NAc-TLP metabolite obtained directly from the noncompartmental analysis of the metabolite data. Such detailed PK modeling of *in vivo* human data with unified parent-metabolite models that incorporates polymorphic rates of metabolism can also provide quantitative estimates of relative metabolism rates for other routes catalyzed by functionally polymorphic enzymes.

3. Experimental

3.1. Study design and subjects

TLP and NAc-TLP concentrations following single oral doses of 75 mg of TLP administered were obtained from a single-center, randomized, single-dose, two-treatment, two-period, two-sequence, fed vs. fasting crossover food effect study (IXR-102-1-189) in which 28 non-smoking healthy men (18–45 years old, all Caucasians) were enrolled following genotyping for *N*-acetyltransferase (NAT2) of 50 prospective subjects. In the morning of the dosing day, a single dose of TLP (3 capsules, 25 mg each) was administered with 240 ml water either after 10 h fasting or after a test-meal. Subjects randomized to the fed state consumed a standardized high-calorie, high-fat meal within 30 min before taking the medication. In agreement with FDA guidance (U.S. 2002), the test-meal consisted of 2 eggs fried in butter, 2 stripes of bacon, 2 slices of toast with butter, 110 g of hash brown potatoes, and 240 ml of whole milk; in total, approximately 600 calories from fat, 260 calories from carbohydrates, and 140 calories from proteins, respectively. Scheduled, standardized meals were served starting 4 h post-dose in both treatment groups. A crossover sequence was repeated after a one-week wash-out. The study was conducted in accordance with the clinical research guidelines established by the US Code of Federal Regulations (CFR) Title 21, Part 312.20; the ethical guidelines of the Declaration of Helsinki (as modified in 2000, Edinburgh); the Hungarian Laws and Regulations; and the policies and the standards defined by the International Conference on Harmonization (ICH) Harmonized Tripartite Guideline for Good Clinical Practice approved January 1997 by the United States, European Union, and Japan. The study protocol was reviewed and approved by the National Institute of Pharmacy (NIP) in Budapest, Hungary, and by the Clinical Pharmacology Ethics Committee of the Health Scientific Council (Central Ethics Committee), the appropriate Ethical Review Board for this study. A total of 5 fast, 9 intermediate, and 14 slow acetylators were enrolled; all but one (an intermediate acetylator) completed the study.

3.2. Bioanalytical methods and genotyping

Serial blood samples were collected at 0 (baseline), 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, and 48 h after dosing into lithium-heparin tubes for determination of plasma TLP and NAc-TLP concentrations. The tubes were centrifuged at 3000 rpm for 10 min to obtain plasma. Harvested plasma samples were frozen at −70 °C and sent for analysis. Plasma concentrations were determined at the IVAX Drug Research Institute, Ltd. (IDRI) Budapest, Hungary using a fully validated HPLC/MS/MS method. Thermo Finnigan (San Jose, CA) Surveyor MS pump and Surveyor autosampler TSQ Quantum Ultra AM system were used with Xcalibur TSQ Quantum 1.3 and LCQuan 2.0 software. Deuterated derivatives of TLP and NAc-TLP synthesized at IDRI served as internal standards. Plasma samples were thawed at room temperature, and compounds were extracted using solid phase extraction method. 200 µl of plasma was loaded onto a pre-activated Strata C8 cartridge, washed, and then the analytes were eluted with 70% ethyl acetate in *n*-heptane. After evaporation in a vacuum centrifuge, the residue was reconstituted in 400 µl methanol/water (50 : 50 v/v). HPLC separation was carried out on Hichrom RPB C8/C18 multialkyl phase reverse phase column (Berkshire, UK) with a ChromTech Whatman pellicular ODS (20 × 2 mm, 30/40 µm) guard column. Isocratic elution with a flow rate of 1.0 ml/min and a mobile phase of 43% methanol, 9% acetonitrile, 0.05% formic acid, and 0.05% ammonium hydroxide in water was used (injected volume: 20 µl, column temperature: 28 °C). For the MS/MS detection, atmospheric pressure ionization source in positive mode was used, and detection was performed by multiple reaction monitoring (MRM). Retention times were in the 4.77–4.82 and 5.94–6.02 min ranges for TLP and NAc-TLP, respectively. Lower limits of quantification (LLQ) were 20 ng/ml for TLP and 2 ng/ml for NAc-TLP. Before the analysis of the clinical samples, a short pre-study validation was carried out on two analytical batches to evaluate the accuracy, precision, sensitivity, selectivity, and linearity of the method. The coefficients of variation were calculated for the 20–1800 and 2–180 ng/ml concentration ranges for TLP and NAc-TLP. The coefficients of variation of TLP and NAc-TLP were less than 8% and 7% for the intra-batch and less than 6% and 9% for the inter-batch comparisons. All plasma samples from a single subject obtained in both periods were analyzed in a single run, and the analysis of samples was done with single determination. Plasma samples were analyzed in nine batches, and for in-study validation, besides the clinical samples, each analytical batch contained system suitability, blank, zero, eight calibration, quality control (low, medium, and high), and matrix blank samples. Good linearity was achieved in each run ($r^2 > 0.99$), and none of the 72 calibration points had to be excluded as each accuracy value was within a ±15% limit of the respective nominal value. Improved analytical techniques allowed monitoring of NAc-TLP levels for much longer time periods, especially in slow acetylators, than in previous studies, which had LLQ of 10–20 ng/ml (Langan et al. 2003).

Genotyping has been carried out by DxS, Ltd (Manchester, UK). DNA was extracted from SAFEspot blood sample cards and NAT2 alleles (*4wt, *5, *6, and *7) were determined for each sample using real-time monitoring of ARMS PCR with a Hot start Taq polymerase method. Monitoring for nucleotide transitions $T^{341}C$, $G^{590}A$, and $G^{857}A$ was done to identify NAT2*5, *6, and *7 alleles, respectively, and inferred phenotype was assigned based on these results (fast : $^*4/^*4$ wild-type homozygous, intermediate: $^*4/[^*5, ^*6, ^*7]$ heterozygous, and slow: $[^*5, ^*6, ^*7]/[^*5, ^*6, ^*7]$).

3.3. Pharmacokinetic and statistical analyses

Pharmacokinetic analyses were performed using WinNonlin Professional 4.0.1 (Pharsight Corp., Mountain View, CA). Noncompartmental analyses were performed with the standard method implemented in WinNonlin. The values for maximum plasma concentration, C_{max}, and the time to reach maximum concentration, t_{max}, were obtained directly from the concentration-time data for TLP and NAc-TLP. Areas under the curve (AUCs) were calculated using the linear up/log down method. Terminal elimination rate constants (λ_z) were obtained using log-linear regression on the terminal phase, which was selected by the default WinNonlin procedure that uses the largest number of last points with non-zero concentrations that maximize the adjusted r^2 of the corresponding regression. Elimination half-lives were calculated as $t_{1/2} = \ln(2)/\lambda_z$. Exploratory compartmental analyses were performed for TLP with standard one- and two-compartment, first-order input, first-order elimination models implemented in WinNonlin. Each individual was fitted separately, and averages ± standard deviations were calculated for each acetylator group and for all subjects separately.

Software provided initial parameter estimates and bounds were used except for a few cases (4 out of 27) when errors were reported during the minimization or convergence could not be achieved and user-supplied bounds had to be used. The Gauss-Newton (Levenberg and Hartley) minimization algorithm (Gabrielsson and Weiner 2000) was used with the convergence criteria set to 0.0001, the increment for partial derivatives set to 0.001, and the number of iterations set to 50. Estimated residual standard deviations were obtained as the square root of the sum of squared residuals divided by the degrees of freedom, $s = (SSR/DF)^{1/2}$. Concentration data below the LLQ were considered as zero, and unweighted data were used in all models. For the compartmental models, weighting schemes corresponding to Poisson errors ($w_i = 1/C_i$) have also been explored. Statistical analyses were performed using a standard spreadsheet program (Microsoft Excel 2002; Microsoft Corp., Redmond, WA). Statistical comparisons were made on log-transformed data using (as adequate) either paired t-test or t-tests assuming equal variances.

Analysis of the data from fasted subjects has also been carried out using a pharmacogenetics-based PK model (Fig. 3) that allowed simultaneous fitting of both parent and metabolite plasma levels. Because of the different molecular weights of TLP and NAc-TLP, this unified parent-metabolite model had to be fitted on concentrations converted to molar scale. Details of the model have been described elsewhere (Buchwald et al. 2005). Briefly, the PK parameters (Rowland and Tozer 1995; Wagner 1975) of this model (Fig. 3) were assumed to have identical values across the different acetylator groups except for the rate constant of metabolite formation (acetylation) ($k_f^{f/i/s}$), which was assumed to have three different values for the three different acetylator phenotypes (f/i/s) through multiplying factors $f_{i/s}$ ($k_f^f = k_f$, $k_f^i = k_f f_i$, $k_f^s = k_f f_s$). To obtain an improved fit, it also was assumed that a portion of the absorbed parent drug is already metabolized by the liver during first-pass, hence even before the parent enters the main compartment, and this appears directly as the metabolite (Rowland and Tozer 1995). The corresponding metabolite input rate ($k_{ma}^{f/i/s}$), which is also related to the rate of acetylation, was allowed to have three possible values through the same multiplying factors $f_{i/s}$ ($k_{ma}^f = k_{ma}$, $k_{ma}^i = k_{ma} f_i$, $k_{ma}^s = k_{ma} f_s$). The corresponding differential equations are:

$$\begin{cases} \dfrac{dC}{dt} = \dfrac{D}{V_C/F} k_a e^{-(k_a + k_{ma}^{f/i/s})\,t} - (k_d + k_f^{f/i/s})\,C \\ \dfrac{dM}{dt} = \dfrac{D}{V_C/F} k_{ma}^{f/i/s} e^{-(k_a + k_f^{f/i/s})\,t} + k_f^{f/i/s} C \dfrac{V_C/F}{V_M/F} - k_{md} M \end{cases} \quad (2)$$

Analytic solution by the Laplace transform method resulted in (Buchwald et al. 2005)

$$\begin{aligned} C &= \frac{D}{V_C/F} \frac{k_a}{k_a + k_{ma}^{f/i/s} - k_d - k_f^{f/i/s}} \left[e^{-(k_d + k_f^{f/i/s})\,t} - e^{-(k_a + k_{ma}^{f/i/s})\,t}\right] \\ M &= \frac{D}{V_M/F} \frac{k_{ma}^{f/i/s}}{k_a + k_{ma}^{f/i/s} - k_{md}} \left[e^{-k_{md} t} - e^{-(k_a + k_{ma}^{f/i/s})\,t}\right] \\ &\quad + \frac{D}{V_M/F} k_a k_f^{f/i/s} \left[\frac{e^{-(k_d + k_f^{f/i/s})\,t}}{(k_a + k_{ma}^{f/i/s} - k_d - k_f^{f/i/s})\,(k_{md} - k_d - k_f^{f/i/s})} \right. \\ &\quad + \frac{e^{-(k_a + k_{ma}^{f/i/s})\,t}}{(k_d + k_f^{f/i/s} - k_a - k_{ma}^{f/i/s})\,(k_{md} - k_a - k_{ma}^{f/i/s})} \\ &\quad \left. + \frac{e^{-k_{md} t}}{(k_d + k_f^{f/i/s} - k_{md})\,(k_a + k_{ma}^{f/i/s} - k_{md})} \right] \end{aligned} \quad (3)$$

The model was implemented in WinNonlin Professional 4.0.1. User-supplied initial parameter values and bounds were used; if needed, bounds were modified until they did not interfere with full minimization. The same Gauss-Newton (Levenberg and Hartley) minimization algorithm was used as for the other models, but the convergence criteria had to be decreased to 10^{-6} to achieve consistent full minimization in all cases. Both unweighted data and weighting schemes corresponding to Poisson errors were tested. Results presented here were obtained by simultaneously fitting all the $2 \times 27 = 54$ individual concentration-time profiles (naïve pooled data approach) (Gabrielsson and Weiner 2000) by using eq. 2 with k_m^f, k_m^i, or k_m^s for each subject as adequate.

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