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IN VITRO AND IN VIVO INHIBITORY EFFECT OF STIRIPENTOL ON CLOBAZAM METABOLISM

Carole Giraud, Jean-Marc Treluyer, Elisabeth Rey, Catherine Chiron, Jean Vincent, Gérard Pons and Agnès Tran

Université Paris-Descartes, Faculté de Médecine, Groupe Hospitalier Cochin St Vincent de Paul, AP–HP, Pharmacologie,(CG, JMT, ER, GP), Hôpital Necker, AP-HP, Neurologie et Métabolisme (CC), Biocodex, Gentilly (JV) and Ecole Pratique des Hautes Etudes (AT), Paris, France.

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b) Corresponding author:

Agnès Tran
Ecole Pratique des Hautes Etudes
Pharmacologie
Hôpital Saint Vincent de Paul
82 avenue Denfert-Rochereau, 75674 Paris cedex 14, France.
Phone : (33) 1 40 48 86 10, Fax : (33) 1 40 48 82 23.
E-Mail: agnes.tran@svp.aphp.fr

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ABSTRACT

A metabolic interaction between stiripentol (STP), an anticonvulsant agent that inhibits the activity of several cytochromes P450 (CYP), and clobazam (CLB), a 1,5-benzodiazepine, used in association with STP in severe myoclonic epilepsy in infancy was observed *in vivo*. This interaction was characterized *in vitro* using cDNA expressed CYP3A4 and CYP2C19 (main P450 involved in CLB metabolism) to calculate Ki and IC_{50} of stiripentol in comparison with ketoconazole (CYP3A4 inhibitor) and omeprazole (CYP2C19 inhibitor). STP inhibited N-demethylation of CLB to N-desmethylclobazam (NCLB) mediated by CYP3A4 (non-competitively) and CYP2C19 (competitively) with Ki=1.59±0.07 and 0.516±0.065µM and IC_{50}=1.58 µM [CI95%=1.20-2.08] and 3.29 µM [CI95%=1.87-5.79] respectively. STP inhibited also more strongly the 4'-hydroxylation of NCLB to 4'-hydroxy-N-desmethylclobazam by CYP2C19 (competitive- interaction with Ki=0.139±0.025 µM and IC_{50}=0.276 µM [CI95%=0.206-0.371]). The inhibitory effect of STP on CLB demethylation by CYP3A4 was much weaker than that of ketoconazole (ketoconazole IC_{50}=0.023 µM [CI95%=0.016-0.033]) while its effect on NCLB hydroxylation by CYP2C19 was much higher than that of omeprazole (omeprazole IC_{50}=2.99 µM [CI95%=2.11-4.24]). The major *in vitro* inhibitory effect of STP on CLB metabolism and mostly on NCLB biotransformation is consistent with the changes *in vivo* in CLB and NCLB plasma concentrations in children treated by the association CLB/STP.

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INTRODUCTION

Clobazam (CLB) is a 1,5-benzodiazepine and an antiepileptic agent, frequently used as an add-on therapy in patients with refractory epilepsy (Shorvon et al., 1995). Stiripentol (STP) is an anticonvulsant agent which clinical efficacy was demonstrated as an add-on treatment to clobazam and valproate in severe myoclonic epilepsy (SMEI) in infancy (Chiron et al., 2000, Nguyen Thanh et al., 2002). Changes in the plasma concentrations of CLB and its main metabolites were observed when STP was added to the treatment. Indeed, 4'-hydroxynorclobazam (OH-NCLB) mean plasma concentrations decreased on average 83% while those of CLB and norclobazam (NCLB) increased on average 173% significantly. It is known that CLB can be first either demethylated to NCLB or hydroxylated to 4'-hydroxyclobazam (OH-CLB); then, NCLB and OH-CLB can be transformed to OH-NCLB (Volz et al. 1979). As STP is known to be an inhibitor of several CYPs (Tran et al., 1997), it might be responsible the inhibition of the metabolism of CLB. Therefore, it seemed relevant to characterize the effect of STP on CLB metabolism. In a previous article, Giraud et al. (2004) identified the main CYP involved in clobazam metabolism. Hydroxylation of CLB into OH-CLB and demethylation of OH-CLB into OH-NCLB were minor pathways. CYP3A4 and CYP2C19 were found to be the major CYPs involved in CLB demethylation, while the CYP2C19 was the major P450 involved in the NCLB hydroxylation pathway. The present study provides in vivo data on stiripentol interaction with clobazam and in vitro characterization of the inhibitory effects of STP on CLB metabolism pathways mediated by CYP3A4 and CYP2C19 in comparison with specific inhibitors (ketoconazole and omeprazole respectively) are presented.

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MATERIAL AND METHODS

In vivo study

The detailed procedure of the study was described in a previous article (Chiron et al. 2000). Briefly, the epileptic patients participated to a randomized, placebo controlled, add-on trial designed to test the efficacy of stiripentol in association with clobazam (0.5 mg/kg/d) and valproate (30 mg/kg/d) in SMEI. After a baseline period of 1 month, placebo or stiripentol (50 mg/kg/d) was added to valproate and clobazam during a double blind-period of 2 months. Minimum plasma concentrations of CLB and NCLB were measured at steady–state during the third week of the baseline period (P1) and the seventh week (P2) of the double-blind period. . Primary endpoint was the percentage of responders on stiripentol and on placebo, defined as having experienced at least a 50% reduction of clonic (or tonic-clonic) seizure rate during the second month of the double-blind period compared to baseline. Patients who presented with status epilepticus during the double-blind period were regarded as non-responders. The study was approved by the local Ethics Committee (CCPPRB Paris Cochin).

In vitro study

Chemicals and reagents

Clobazam and N-desmethylclobazam were obtained from Laboratoires Roussel-Uclaf / Sanofi-Synthelabo France (Paris, France). 4'-hydroxy-N-desmethylclobazam was synthesized and kindly provided by Biocodex (Gentilly, France). Stiripentol was also provided by Laboratoires Biocodex. Glucose-6-phosphate (G6P), glucose-6-phosphate deshydrogenase (G6PDH), nicotinamide adenine dinucleotide phosphate (NADP), ketoconazole and omeprazole were purchased from Sigma-Aldrich Chimie S.a.r.l. (St. Quentin Fallavier, France). Specific human P450 enzymes (CYP2C19 and CYP3A4) expressed in the

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Baculovirus transfected insect cell system were purchased from BD GENTEST Co. (Woburn, MA, USA). These CYP also contain cDNA-expressed human P450 reductase and human cytochrome b5. P450 contents were provided by BD Gentest.

Incubations with cDNA-expressed CYP450s

Incubation mixtures contained 100 mM phosphate buffer (pH 7.4), 0.5 $mg.ml^{-1}$ $MgCl_2$, 1 mM NADP+, 0.5 $mg.ml^{-1}$ G6P, 0.5 $UI.ml^{-1}$ G6PDH, the inhibitor (stiripentol, ketoconazole or omeprazole) and the substrate (CLB or NCLB) in a final incubation volume of 0.5 ml. CLB and NCLB concentrations were chosen in the range of the therapeutic plasma concentrations (2 and 14 µM respectively). The reactions were initiated by the addition of the P450 with a final P450 concentration of 50 nM, as recommended by BD Gentest. Incubations were performed for 10 min for CYP3A4 and 30 min for CYP2C19 at 37°C and then stopped by the addition of 200 µl ice-cold acetonitrile and cooling on ice. Incubations without NADPH-generating system served as controls. All incubations were conducted in duplicate.

Acetonitrile was chosen to dissolve CLB, NCLB and the inhibitors, as it was shown to have the least inhibitory effect among a range of solvents (Chauret et al., 1998). It was present in incubation mixtures containing those compounds at a final concentration (v/v) of 0.4 %.

Inhibition studies with stiripentol, ketoconazole (CYP3A4 inhibitor) and omeprazole (CYP2C19 inhibitor)

The inhibition constants (apparent Ki) of STP for CLB demethylation by CYP3A4 and CYP2C19 were determined using various concentrations of CLB (2, 10, 20, 40, 60, 100 µM) with increasing concentrations of STP (0, 0.5, 1, 2, 5 µM). Concerning NCLB hydroxylation by CYP2C19, the apparent Ki was similarly determined with different concentrations of NCLB (1.5, 4, 6, 8, 12, 14 µM) and STP (0, 0.1, 0.5, 1, 2 µM). Because of its very limited

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solubility, 14 µM was the highest final concentration that could be obtained for NCLB. Each Ki was calculated with two repetitions of the experiment with duplicates.

IC_{50}s were determined by co-incubation of the substrate at concentration in the range of the therapeutic plasma concentrations (Chiron et al., 2000) (CLB 2 µM or NCLB 14 µM) with increasing concentrations of STP (0.001, 0.002, 0.005, 0.01, 0.05, 0.1, 0.25, 2, 5, 10 µM). For comparison, an IC_{50} was also determined for ketoconazole and omeprazole at various concentrations (0.001, 0.01, 0.05, 0.1, 0.25, 0.5, 1, 2 and, 10 µM) for the demethylation of CLB by the CYP3A4 and the hydroxylation of NCLB by the CYP2C19 respectively. Each experiment was repeated twice with duplicates for each inhibitor concentration. The concentrations of the CLB and its metabolites were determined by a validated HPLC method previously published (Giraud et al. 2004).

Data analysis

Inhibitory constant (Ki) values were calculated using the nonlinear regression analysis program Sigma Plot Software (SPSS Inc., Chicago, IL, USA) Goodness of fit was based on visual examination of the plots and by application of the Akaike's information criterion (Yamaoka et al. 1978). IC_{50} were estimated using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

The present study provides in vivo and in vitro data on stiripentol interaction with clobazam. The clinical data have already been published (Chiron et al., 2000). Briefly, it was shown that the frequency of responders was greater on stiripentol (71.4 %, 95% CI 52.1-90.7) than on placebo (5%, 95% CI 0-14.6) with a high significance ($p<0.0001$) (for details, see Chiron et

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al; 2000). The mean daily dose of stiripentol during the double-blind period was 49±2 mg/kg/d resulting in a mean minimum plasma concentration at steady state of 10.0±3.6 mg/L (42.7±15.4 µM). Mean normalized minimum plasma concentrations of CLB and NCLB increased significantly ($p<0.001$) from 0.39 to 0.84 (mg/L)/(mg/kg) for CLB and from 3.6 to 11.6 (mg/L)/(mg/kg) for NCLB while those of OH-NCLB decreased significantly ($p<0.001$) from 0.258 to 0.063 (mg/L)/(mg/kg). The ratio of NCLB/CLB minimum plasma concentration was increased significantly ($p<0.01$) by 269%, while OH-NCLB/NCLB decreased significantly ($p<0.001$) by 86%. OH-CLB was not detected in the plasma of patients. There were no significant changes in plasma concentrations in the placebo group (Chiron et al., 2000).

Mean in vitro data are presented in table I and figure 1. The inhibition of CLB demethylation by STP was best described by a non-competitive inhibition model with apparent Ki=1.6 µM for the cDNA-expressed CYP3A4 (Figure 1A) and by a competitive inhibition model with Ki=0.52 for the cDNA-expressed CYP2C19 (Figure 1B). Formation of OH-NCLB from NCLB by cDNA-expressed CYP2C19 was competitively inhibited by STP with a Ki=0.14µM (Figure 1D). Ketoconazole inhibited the demethylation of CLB by the cDNA-expressed CYP3A4 with an IC_{50} almost seventy times lower than that of STP (0.023 versus 1.58 µM for STP) (Table I, Figure 1C). Omeprazole inhibited the hydroxylation of NCLB by the cDNA-expressed CYP2C19 with an IC50 about ten times higher than that of STP (2.99 versus 0.276 µM for STP) (Table I, Figure 1E).

DISCUSSION

The strong inhibitory effect of stiripentol on NCLB hydroxylation mediated by CYP2C19 (Ki = 0.14 µM) is consistent with the three-fold increase of NCLB plasma concentrations in vivo on stiripentol therapy. Stiripentol usual steady-state plasma concentrations are in the range of

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10 to 60 µM (Tran et al., 1997, Perez et al., 1999, Chiron et al., 2000) and much higher than the Ki. Stiripentol inhibited also the N-demethylation of CLB dependent on CYP3A4 (Ki=1.6 µM), this result was similar to the data reported by Cazali et al. (2003) who calculated a Ki=2.5 µM in a study evaluating the stiripentol inhibitory effect on the biotranformation of carbamazepine by CYP3A4. The hydroxylation of NCLB was more inhibited than the demethylation of CLB (Ki ratio about 10). This led to an accumulation of NCLB explaining the higher in vivo plasma concentrations of this metabolite in the presence of STP and the lower plasma concentrations of the 4'-hydroxylated-N-demethylated metabolite In consequence, the administration of STP with CYP2C19 substrates with a narrow therapeutic range should be done cautiously.

In addition, it is important to take into account the fact that the main P450 involved in the interaction is the genetically polymorphic CYP2C19. The most common deficient alleles CYP2C19*2 (allelic frequency of 13% in Caucasians and 23 % in Japanese) and CYP2C19*3 (allelic frequency of 0% in Caucasians and 10 % in Japanese) correspond to a lack of enzyme activity (Ozawa et al. 2004). Clinical safety and efficacy of CLB treatment could be altered by CYP2C19 polymorphism. NCLB, the major metabolite of CLB is known to contribute to the therapeutic and adverse effects more than CLB in epileptic patients on long-term treatment (Shorvon et al., 1995; Bardy et al., 1991). Subjects carrying one or two copies of the defective CYP2C19*2 allele developed markedly elevated steady-state plasma concentrations of NCLB (Contin et al., 2002; Giraud et al., 2004; Kosaki et al., 2004) and are more susceptible to present adverse effects, principally sedation (Contin et al., 2002; Parmeggiani et al., 2004).

Through its strong inhibitory effect on the CYP2C19, stiripentol triggered an increase of NCLB plasma concentrations. One could hypothesize that in people carrying defective alleles, the addition of STP to an initial CLB treatment would have almost no effect on the CLB and NCLB concentrations. That has already been demonstrated for another CYP2C19 inhibitor:

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omeprazole. Its association with a CYP2C19 substrate, moclobemide, entailed no remarkable changes in the pharmacokinetic parameters of nor-moclobemide neither its metabolites in poor metabolizers for the CYP2C19, while the inhibitory action of omeprazole was significant in the extensive metabolizers (Yu et al., 2001). Concerning the present in vivo study, 3 subjects among the 20 of the stiripentol group had no rise in NCLB plasma concentration when STP was added to their initial CLB treatment. These patients perhaps carried one or two copies of CYP2C19 mutated alleles. Unfortunately, no blood sample could be collected after the study to carry out CYP2C19 genotyping and to confirm this assumption (one patient was dead and the two others were "lost-to-follow up patients"). Among these 3 patients, one was responder to stiripentol and the others non-responders. Two assumptions can be considered, on one hand, if the effectiveness of CLB and stiripentol association is mainly due to the increase in the NCLB plasma concentration, one can hypothesize that these patients were non-responders to the treatment because stiripentol could no more inhibit a non-functional CYP2C19. On the other hand, if CYP2C19 variant allele carriers are responders, it is in favour of a proper antiepileptic effect of stiripentol consistent with in vitro data showing the barbiturate-like effect of stiripentol on the GABA receptor (Quilichini et al., submitted). Indeed, high NCLB plasma concentrations in epileptic patients are not sufficient to control seizures and addition of stiripentol to clobazam treatment improves antiepileptic efficacy. A prospective study is planned to understand stiripentol effect in CYP2C19 mutated patients treated with clobazam and stiripentol.

In conclusion, Stiripentol was a potent inhibitor of CYP2C19 in vitro and in vivo. Its effect, in patients carrying variant alleles of CYP2C19, remains to be explored. In addition, this study illustrated the fact that a drug interaction should not always be regarded as an adverse effect. Stiripentol can be considered as a "booster" of clobazam. The inhibitory effect of stiripentol on cytochromes P450 is used to potentiate antiepileptic effect of clobazam (Perez et al. 1999,

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Chiron et al. 2000; Nguyen Thanh et al; 2002) and carbamazepine (Perez et al 1999; Cazali et al. 2003).

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FOOTNOTES

a) This work was supported by a grant from Biocodex (Gentilly, France)

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LEGEND FOR FIGURE

Figure 1 A,B,C:Inhibition of clobazam (CLB) (2-100 µM) demethylation into Norclobazam (NCLB) by stiripentol: 1/(rate of formation of Norclobazam (NCLB)) as a function of stiripentol concentration (0-5 µM) **A,** with cDNA-expressed CYP3A4 (Dixon plot, non-competitive inhibition); **B,** with cDNA-expressed CYP2C19 (Dixon plot, competitive inhibition); **C**, Percentage of CLB (2µM) converted to NCLB (14 µM) by human liver microsomes in the presence of increasing concentrations of stiripentol (●) STP (0-10 µM) or ketoconazole (∇) (0-10 µM).
D,E: Inhibition of Norclobazam (NCLB) (1.5-14 µM) hydroxilation into OH-NCLB by stiripentol: **D,** 1/(rate of formation of OH-NCLB) as a function of stiripentol concentration (0-5 µM) with cDNA-expressed CYP2C19 (Dixon plot, competitive inhibition); **E**, Percentage of NCLB (14 µM) converted to OH-NCLB by human liver microsomes in the presence of increasing concentrations of stiripentol (●) STP (0-10 µM) or omeprazole (∇) (0-10 µM). Data points represent the mean of two repetitions in duplicate.

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Table I: Inhibition constants (apparent Ki) and IC_{50} for the clobazam demethylation by the cDNA-expressed CYP3A4 and CYP2C19 and the N-desmethylclobazam hydroxylation by the cDNA-expressed CYP2C19 [mean ± SD or [CI95%]]. Means correspond to two repetitions in duplicate.

	cDNA-expressed	
	CYP3A4	**CYP2C19**
N-desmethyclobazam (NCLB) formation		
Apparent Ki (μM)	1.59 ± 0.07	0.516 ± 0.065
IC_{50}stiripentol (μM)	1.58 [1.20-2.08]	3.29 [1.87-5.79]
IC_{50}ketoconazole (μM)	0.023 [0.016-0.033]	
4'-hydroxy-N-desmethylclobazam (0H-NCLB) formation		
Apparent Ki (μM)		0.139 ± 0.025
IC_{50}stiripentol (μM)		0.276 [0.206-0.371]
IC_{50}omeprazole (μM)		2.99 [2.11-4.24]

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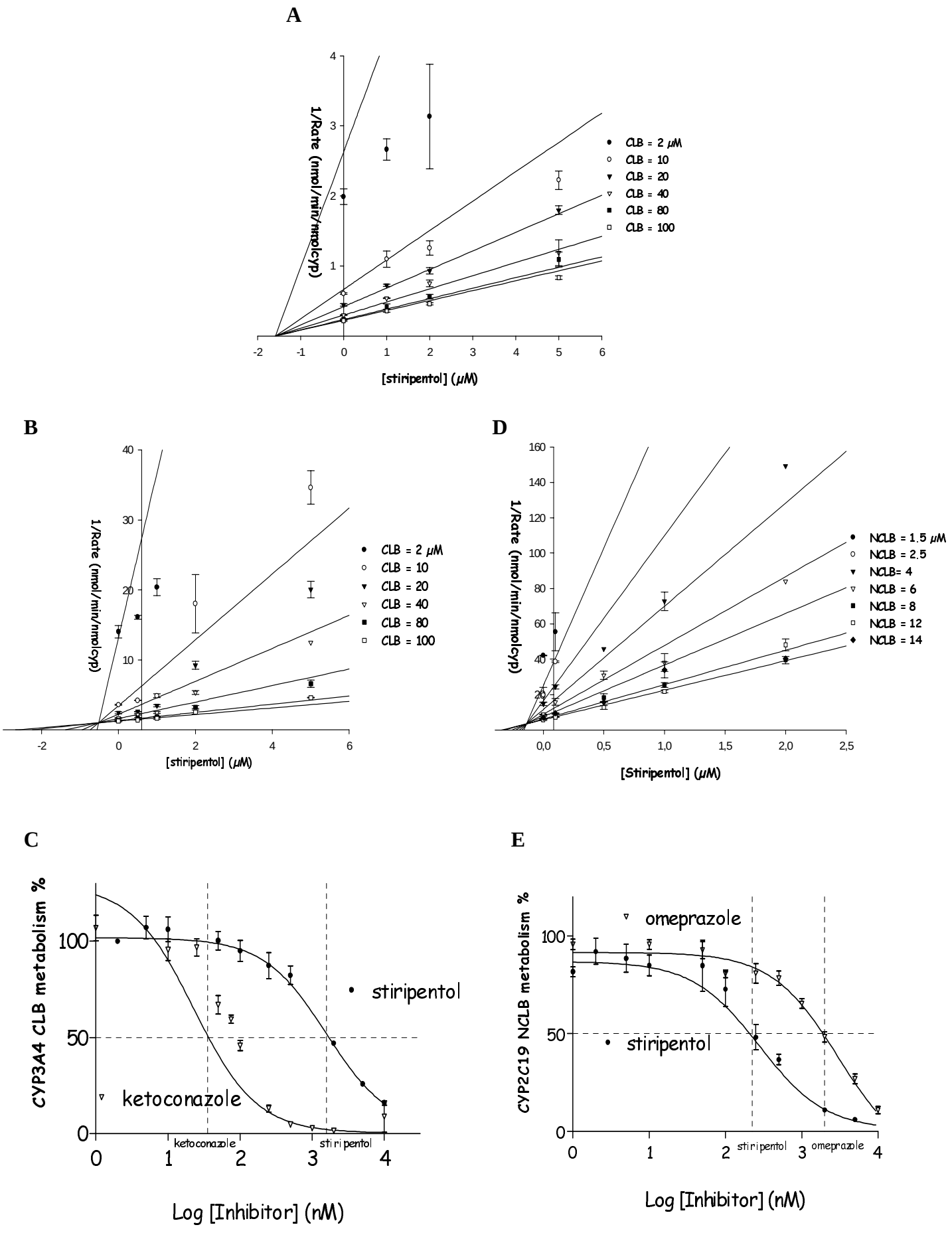

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