

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

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ABSTRACT:

A metabolic interaction between stiripentol (STP), an anticonvulsant agent that inhibits the activity of several cytochromes P450 (P450s), 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 K_i 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 (noncompetitively) and CYP2C19 (competitively) with $K_i = 1.59 \pm 0.07$ and $0.516 \pm 0.065 \mu\text{M}$ and $IC_{50} = 1.58 \mu\text{M}$ [95% confidence interval (CI95%) = 1.20–2.08] and $3.29 \mu\text{M}$ (CI95% = 1.87–5.79), respec-

tively. STP inhibited also more strongly the 4'-hydroxylation of NCLB to 4'-hydroxy-*N*-desmethylclobazam by CYP2C19 [competitive interaction with $K_i = 0.139 \pm 0.025 \mu\text{M}$ and $IC_{50} = 0.276 \mu\text{M}$ (CI95% = 0.206–0.371)]. The inhibitory effect of STP on CLB demethylation by CYP3A4 was much weaker than that of ketoconazole [$IC_{50} = 0.023 \mu\text{M}$ (CI95% = 0.016–0.033)], whereas its effect on NCLB hydroxylation by CYP2C19 was much higher than that of omeprazole [$IC_{50} = 2.99 \mu\text{M}$ (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.

Clobazam (CLB) is a 1,5-benzodiazepine and an antiepileptic agent, frequently used as an add-on therapy in patients with refractory epilepsy (Shorvon, 1995). Stiripentol (STP) is an anticonvulsant agent whose clinical efficacy was demonstrated as an add-on treatment to clobazam and valproate in severe myoclonic epilepsy (SMEI) in infancy (Chiron et al., 2000; 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%, whereas those of CLB and norclobazam (NCLB) significantly increased on average 173%. 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). Because STP is known to be an inhibitor of several P450s (Tran et al., 1997), it might be responsible for 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 P450 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 P450s involved in CLB demethylation, whereas 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.

Materials 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/day) and valproate (30 mg/kg/day) in SMEI. After a baseline period of 1 month, placebo or stiripentol (50 mg/kg/day) 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 3rd week of the baseline period (P1) and the 7th week (P2) of the double blind period. The 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 2nd month of the double blind period compared with baseline. Patients who presented with status epilepticus during the double blind period were regarded as nonresponders. The study was approved by the local Ethics Committee (Comité de Protection des Personnes dans la Recherche Biomédicale, Paris Cochin, France).

In Vitro Study. Chemicals and Reagents. Clobazam and *N*-desmethylclobazam were obtained from Laboratoires Roussel-Uclaf/Sanofi-Synthelabo

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ABBREVIATIONS: CLB, clobazam; STP, stiripentol; SMEI, severe myoclonic epilepsy; OH-NCLB, 4'-hydroxynorclobazam; NCLB, *N*-desmethylclobazam; OH-CLB, 4'-hydroxyclobazam; P450, cytochrome P450.

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, glucose 6-phosphate dehydrogenase, 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 Baculovirus-transfected insect cell system were purchased from BD Gentest Co. (Woburn, MA). These P450s also contain cDNA-expressed human P450 reductase and human cytochrome *b*₅. P450 contents were provided by BD Gentest.

Incubations with cDNA-Expressed P450s. Incubation mixtures contained 100 mM phosphate buffer (pH 7.4), 0.5 mg/ml MgCl₂, 1 mM NADP⁺, 0.5 mg/ml glucose 6-phosphate, 0.5 U/ml glucose 6-phosphate dehydrogenase, 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 of 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 because it was shown to have the least inhibitory effect among a range of solvents (Chaufert 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 *K*_i) of STP for CLB demethylation by CYP3A4 and CYP2C19 were determined using various concentrations of CLB (2, 10, 20, 40, 60, and 100 μM) with increasing concentrations of STP (0, 0.5, 1, 2, and 5 μM). Concerning NCLB hydroxylation by CYP2C19, the apparent *K*_i was similarly determined with different concentrations of NCLB (1.5, 4, 6, 8, 12, and 14 μM) and STP (0, 0.1, 0.5, 1, and 2 μM). Because of its very limited solubility, 14 μM was the highest final concentration that could be obtained for NCLB. Each *K*_i was calculated with two repetitions of the experiment with duplicates.

*IC*₅₀ values were determined by coincubation of the substrate at concentration in the range of the therapeutic plasma concentrations (Chiron et al., 2000) (2 μM CLB or 14 μM NCLB) with increasing concentrations of STP (0.001, 0.002, 0.005, 0.01, 0.05, 0.1, 0.25, 2, 5, and 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 high-performance liquid chromatography method previously published (Giraud et al., 2004).

Data Analysis. Inhibitory constant (*K*_i) values were calculated using the nonlinear regression analysis program Sigma Plot Software (SPSS Inc., Chicago, IL). 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 values were estimated using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA).

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% confidence interval 52.1–90.7) than on placebo (5%, 95% confidence interval 0–14.6) with a high significance (*p* < 0.0001) (for details, see Chiron et al., 2000). The mean daily dose of stiripentol during the double blind period was 49 ± 2 mg/kg/day, 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/

TABLE 1

*Inhibition constants (apparent *K*_i) 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 ± S.D. or (CI95%)]*

Means correspond to two repetitions in duplicate.

	cDNA-Expressed	
	CYP3A4	CYP2C19
NCLB formation		
Apparent <i>K</i> _i (μM)	1.59 ± 0.07	0.516 ± 0.065
<i>IC</i> ₅₀ Stiripentol (μM)	1.58 (1.20–2.08)	3.29 (1.87–5.79)
<i>IC</i> ₅₀ Ketoconazole (μM)	0.023 (0.016–0.033)	
OH-NCLB formation		
Apparent <i>K</i> _i (μM)		0.139 ± 0.025
<i>IC</i> ₅₀ Stiripentol (μM)		0.276 (0.206–0.371)
<i>IC</i> ₅₀ Omeprazole (μM)		2.99 (2.11–4.24)

(mg/kg) for CLB and from 3.6 to 11.6 (mg/l)/(mg/kg) for NCLB, whereas 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%, whereas 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 1 and Fig. 1. The inhibition of CLB demethylation by STP was best described by a noncompetitive inhibition model with apparent *K*_i = 1.6 μM for the cDNA-expressed CYP3A4 (Fig. 1A) and by a competitive inhibition model with *K*_i = 0.52 for the cDNA-expressed CYP2C19 (Fig. 1B). Formation of OH-NCLB from NCLB by cDNA-expressed CYP2C19 was competitively inhibited by STP with a *K*_i = 0.14 μM (Fig. 1D). Ketoconazole inhibited the demethylation of CLB by the cDNA-expressed CYP3A4 with an *IC*₅₀ almost 70 times lower than that of STP (0.023 versus 1.58 μM for STP) (Table 1 and Fig. 1C). Omeprazole inhibited the hydroxylation of NCLB by the cDNA-expressed CYP2C19 with an *IC*₅₀ approximately 10 times higher than that of STP (2.99 versus 0.276 μM for STP) (Table 1 and Fig. 1E).

Discussion

The strong inhibitory effect of stiripentol on NCLB hydroxylation mediated by CYP2C19 (*K*_i = 0.14 μM) is consistent with the 3-fold increase of NCLB plasma concentrations *in vivo* on stiripentol therapy. Stiripentol usual steady-state plasma concentrations are in the range of 10 to 60 μM (Tran et al., 1997; Perez et al., 1999; Chiron et al., 2000) and much higher than the *K*_i. Stiripentol also inhibited the *N*-demethylation of CLB dependent on CYP3A4 (*K*_i = 1.6 μM); this result was similar to the data reported by Cazali et al. (2003), who calculated a *K*_i = 2.5 μM in a study evaluating the stiripentol inhibitory effect on the biotransformation of carbamazepine by CYP3A4. The hydroxylation of NCLB was more inhibited than the demethylation of CLB (*K*_i ratio approximately 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. Consequently, 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.,

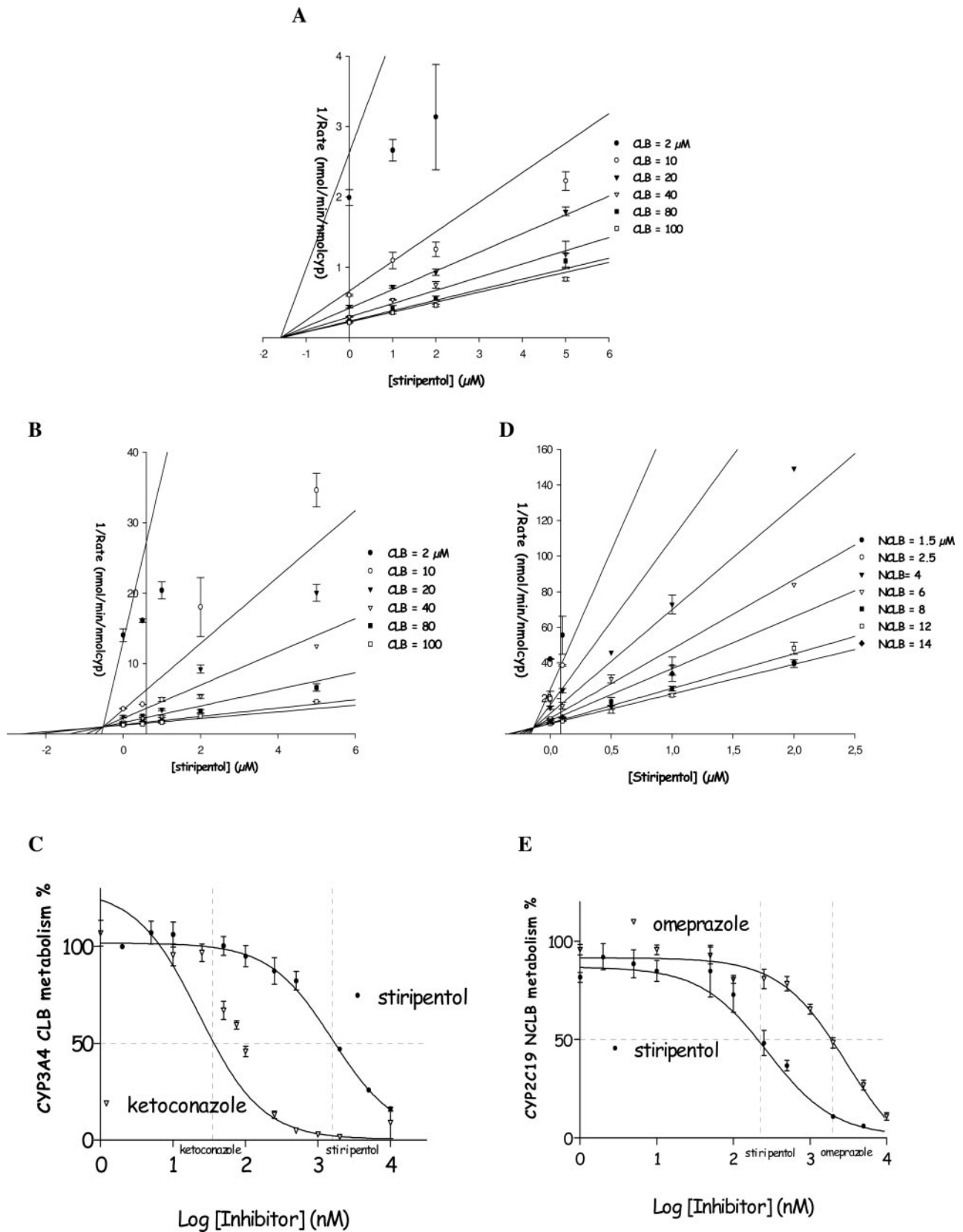


FIG. 1. A, B, and C, inhibition of CLB (2–100 μM) demethylation into NCLB by stiripentol: 1/(rate of formation of NCLB) as a function of stiripentol concentration (0–5 μM); A, with cDNA-expressed CYP3A4 (Dixon plot, noncompetitive inhibition); B, with cDNA-expressed CYP2C19 (Dixon plot, competitive inhibition); and C, percentage of CLB (2 μM) converted to NCLB by human liver microsomes in the presence of increasing concentrations of stiripentol (●) (0–10 μM) or ketoconazole (▽) (0–10 μM). D and E, inhibition of NCLB (1.5–14 μM) hydroxylation 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 (●) (0–10 μM) or omeprazole (▽) (0–10 μM). Data points represent the mean of two repetitions in duplicate.

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 (Bardy et al., 1991; Shorvon, 1995). 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: omeprazole. Its association with a CYP2C19 substrate, moclobemide, entailed no remarkable changes in the pharmacokinetic parameters of neither moclobemide nor its metabolites in poor metabolizers for the CYP2C19, whereas the inhibitory action of omeprazole was significant in the extensive metabolizers (Yu et al., 2001). Concerning the present in vivo study, three 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 three patients, one was a responder to stiripentol and the others nonresponders. 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 nonresponders to the treatment because stiripentol could no more inhibit a nonfunctional CYP2C19. On the other hand, if CYP2C19-variant allele carriers are responders, this hypothesis would favor a proper antiepileptic effect of stiripentol consistent with in vitro data showing the barbiturate-like effect of stiripentol on the GABA receptor (P. Quilichini, C. Chiron, Y. Ben-ari, and H. Gozlan, 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; Chiron et al., 2000; Thanh et al., 2002) and carbamazepine (Perez et al., 1999; Cazali et al., 2003).

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