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European Journal of Pharmacology 495 (2004) 119–128

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The interaction between vigabatrin and diazepam on the electroencephalogram during active behaviour in rats: an isobolic analysis

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Received 10 May 2004; accepted 14 May 2004
Available online 20 June 2004

Abstract

To test whether polytherapy with two gamma-aminobutyric acid (GABA) -ergic drugs might be clinically relevant for epilepsy treatment, effects on spike and wave discharges, the fraction of time spent being behaviourally active, and the background electroencephalogram (EEG) during behavioural activity of vigabatrin (15–500 mg/kg i.p.) and diazepam (1.25–10 mg/kg i.p.) were compared with their combination (dose ratio 1:25) in rats. Isobolic analyses were performed to describe the interactions. Unfortunately, no conclusions can be drawn concerning the interaction of both drugs on the spike and wave discharge activity because the effect of diazepam was shown to be dominant. Only vigabatrin decreased the behavioural activity, whereas there was a trend towards a decrease after diazepam. All treatments dose dependently increased the power in the beta frequency band. Unfortunately, the dose ratio was not optimal to describe the interaction. The theta peak frequency was dose dependently decreased after all treatments. There was a synergistic interaction between the two drugs on this variable. These data support both the idea that an increase in power in the beta frequency band can serve as a biomarker for GABAergic inhibition and the suggestion that clinically effective anxiolytics decrease the theta peak frequency. Furthermore, we show that on different variables, there might be different optimal dosage combinations, which might complicate the clinical application of polytherapy.

Keywords: Polytherapy; GABA; Isobolic analysis; EEG; (Rat)

1. Introduction

Most patients with epilepsy can be effectively treated with a single antiepileptic drug. Unfortunately, some patients do not respond to a single antiepileptic agent, even when the drug is prescribed at the maximally tolerated dose. Many physicians will try to treat these patients with combinations of antiepileptic drugs. A combination of drugs would be considered advantageous if the two drugs show additive therapeutic (antiepileptic) effects with infraadditivity (antagonistic) for the other (unwanted) effects or supra-additive (synergistic) therapeutic effects with additivity for the other effects. The question is which drugs to combine to achieve this goal (Deckers et al., 2000).

In rational polytherapy, drugs are combined based on knowledge of their pharmacological modes of action. There is still some discussion about whether using drugs that target the same receptor complex is better than using drugs that target different receptor complexes (Leach, 1997). Gamma-aminobutyric acid (GABA) is generally believed to be implicated in the mechanisms of initiation and propagation of epileptic seizures. The $GABA_A$ receptor complex has separate, allosterically coupled binding sites for several different antiepileptic drugs (e.g., benzodiazepines and barbiturates; Mehta and Ticku, 1999). Therefore, this receptor seems to be an ideal target receptor for investigating whether rational polytherapy can be achieved by targeting one receptor complex.

It is known since long that benzodiazepines and GABA allosterically enhance each other's binding (Haefely, 1989; Olsen, 1981). Thus, the combination of diazepam and GABA is likely to increase the GABAergic neurotransmission in vivo. Therefore, the combination of diazepam and a drug that increases endogenous GABA concentrations might be a clinically useful candidate for polytherapy. Endogenous GABA can be increased by use of vigabatrin, an irreversible GABA transaminase inhibitor (Hammond and Wilder, 1985).

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doi:10.1016/j.ejphar.2004.05.027

Both vigabatrin and diazepam are clinically effective in epileptic patients. In general, increases in GABAergic neurotransmission decrease convulsive types of epilepsy whereas they increase absence epileptic spike and wave discharges in the electroencephalogram (EEG). Exceptions to this rule are the benzodiazepines, which are effective against both types of epilepsy.

In addition to the therapeutic antiepileptic (anticonvulsive) effect, both vigabatrin and diazepam have been shown to affect the electroencephalogram (EEG) of rats. Vigabatrin increases beta and theta frequency spectral power in rats during mobility (Halonen et al., 1992; Riekkinen et al., 1991). Diazepam increases beta frequency and decreases theta frequency spectral power in rats (Coenen and van Luijtelaar, 1989; Van Rijn and Jongsma, 1995; Visser et al., 2003). Furthermore, Mandema et al. (1991) showed that for the benzodiazepines, there is a close correlation between an increase on the beta frequency spectral power and anticonvulsant activity. So, some effects of the two drugs are equally directed whereas others are oppositely directed. For polytherapy research, it would be very interesting to investigate the effects of polytherapy with a drug that decreases (diazepam) and a drug that increases (vigabatrin) absence epileptic spike and wave discharge activity while both drugs increase GABAergic neurotransmission.

In the present study, the effects on the total spike and wave discharge duration, the fraction of time spent being behaviourally active, and the background EEG during this active behaviour of several doses of vigabatrin and diazepam monotherapy and those of the combination of these drugs were measured. Where applicable, the results are analysed using the isobolic method (Greco et al., 1995). We tested the effects of these treatments in WAG/Rij rats, an inbred strain of rats which is recognised as an animal model of human absence epilepsy (Van Luijtelaar and Coenen, 1986).

2. Materials and methods

This study was performed in accordance with the guidelines of the European Community for the use of experimental animals. Approval of the local ethical committee for animal studies was obtained.

2.1. Drugs

Vigabatrin (Yamanouchi Pharma, The Netherlands) was dissolved in saline (0.9%). Diazepam (Dumex, The Netherlands) was dissolved in intralipid (15%) (Fresenius Kabi, The Netherlands). Saline was used as the control for vigabatrin and intralipid was used as the control for diazepam. Both drugs were administered intraperitoneally (i.p.), vigabatrin and saline in a volume of 2 ml/kg and diazepam and intralipid in a volume of 1 ml/kg except for the 10-mg/kg dose which was also in a volume of 2 ml/kg, to avoid effects of great osmolarity differences (Bouwman et al., 2003a). For vigabatrin, the following doses were used: 0 (saline), 15, 31, 62, 125, 250, or 500 mg/kg. The diazepam treatment consisted of the doses: 0 (intralipid), 1.25, 2.5, 5, or 10 mg/kg. For the polytherapy, the following combinations (vigabatrin/diazepam) were used: 15/0.62, 31/1.25, 62/2.5, and 125/5 mg/kg (dose ratio = 25:1). A fixed dose ratio was used because these, according to Tallarida (1992), permit easy dose adjustments, have direct application to drug development, and are amenable to statistical analysis.

2.2. Animals and experimental conditions

Experiments were performed in 36 1-year-old male WAG/Rij rats with an average weight of 337 g (S.E. = 3 g). The rats were maintained on a reversed 12-h light–dark cycle with lights on at 2000 h. The rats had ad libitum access to food and water.

Each dose or combination was tested in at least eight animals; a total of 116 observations was made. To limit the number of animals needed, each rat participated in up to four experimental sessions (four treatments) with at least a 14-day interval between each session. Rats were semirandomly assigned to up to four treatments, in a way that none of the rats received only control or high dose treatments. The selected treatments per rat were randomised over the four sessions. Experimental sessions were performed between 900 and 1630 h. The experiment was performed on 29 separate experimental days, testing four rats per day. For the experimental design, see Table 1. Per experimental day, each rat received three injections: at 900 h, saline or vigabatrin; at 1430 h, saline; and at 1530 h, diazepam or intralipid. The saline injection at 1430 h was administered to control for the injection effect directly prior to observation period 2. In order to minimize the number of rats needed for this experiment, one vigabatrin dose yielded two data points: one for the monotherapy at 1430 h, i.e., 5 1/2 h postinjection and one for polytherapy by adding diazepam at 1530 h, i.e., 6 1/2 h postinjection (see last row in Table 1). These observation periods (5 1/2 and 6 1/2 h postinjection) were chosen because GABA transaminase levels reach a minimum and GABA

Table 1
The experimental schedule

900 h injection	5 1/2 h postinjection		6 1/2 h postinjection	
	1430 h injection	Observation period 1	1530 h injection	Observation period 2
Saline	Saline	Control data, for vigabatrin group	Intralipid	Control data, for diazepam and polytherapy groups
Vigabatrin	Saline	Vigabatrin monotherapy data	Intralipid	Vigabatrin 500 mg/kg data
Saline	Saline	Control data, for vigabatrin group	Diazepam	Diazepam monotherapy data
Vigabatrin	Saline	Vigabatrin monotherapy data	Diazepam	Polytherapy data

levels are maximally increased 4 h after vigabatrin injection, and these levels are maintained for 24 h (Hammond and Wilder, 1985). However, there might be a slight time effect between these observation periods, which were 1 h apart. Therefore, the 500 mg/kg vigabatrin data of observation period 2 were included as a time control (see row two in Table 1).

2.3. Surgery and EEG recording

Isoflurane anaesthesia was used during the implantation of a tripolar EEG electrode (Plastics One MS-332/2-A) on the cortical surface: one electrode on the frontal region (coordinates with skull surface flat and bregma zero–zero: A2.0 L3.5) and a second one in the parietal region (A-6.0 L4.0). The reference electrode was placed over the cerebellum. The rats were allowed to recover from surgery for 28 days before experimentation began. The rats were habituated to the experimental setting during 30 min per day on 2 successive days before experimentation. On experimental days, four rats were placed in separate recording cages (19 × 19 × 40 cm) and connected with leads through swivels to an amplifier and a computer-based data acquisition system (Dataq Instruments, Ohio, USA). The EEG signals in a bandwidth between 0.1 and 100 Hz were sampled with a frequency of 512 Hz.

The behaviour of the rats was observed by two observers, continuously for two 30-min periods, beginning directly after the injection at 1430 h and at 1530 h. The active behaviour of the rats was scored and saved along with the EEG. Offline, the total duration of the spike and wave discharge activity was determined by visual inspection based on the criteria for standard spike and wave discharges as proposed by Van Luijtelaar and Coenen (1986). The background EEG data during active behaviour (walking, rearing, and sniffing were scored as active behaviour) were analysed (in windows of 4 s) using the Fast Fourier Transformation with a Hanning window. The peak frequency in the theta frequency band (4–9 Hz), the absolute theta peak power, and the average absolute EEG spectral power in the beta (13–29.75 Hz) and fast (30–44.75 Hz) frequency bands were calculated.

2.4. Statistics

The data of the total spike and wave discharge duration, time spent being behaviourally active, and the theta peak frequency, the absolute theta peak power, and the average absolute power in the beta and fast frequency bands during active behaviour were analysed. First, the two control treatments, i.e., the second saline injection 5 1/2 h after the initial saline injection and intralipid 6 1/2 h after the initial saline injection, and the data of these two observation periods after the 500-mg/kg vigabatrin treatment were compared using independent (saline vs. intralipid) or paired (500-mg/kg vigabatrin period 1 vs. period 2) samples *t*-tests ($\alpha = 0.05$) in SPSS version 11.5 for Windows (SPSS, Chicago, IL, USA).

Second, the data of the six mentioned variables per drug were analysed using univariate analyses of variance in SPSS version 11.5 for Windows (SPSS) with dose as a between factor. Followed by Bonferroni's multiple comparison when appropriate ($\alpha = 0.05$).

To describe the dose–response relationship, a sigmoid E_{max} model was fitted to the data, using Graphpad Prism 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Because most of the presented data sets here do not have measured data points in the full range of the curve, any interpretation of the E_{max} is hazardous. Therefore, effect points within the measured range were chosen; the $ED_{(2 \times baseline)}$, the $ED_{(0.75 \times baseline)}$, $ED_{(0.5\text{-Hz decrease})}$, $ED_{(1\text{-Hz decrease})}$, or the $ED_{(1.5\text{-Hz decrease})}$ were estimated when appropriate. The $ED_{(2 \times baseline)}$ was defined as the dose necessary to obtain twice the baseline value. The $ED_{(0.75 \times baseline)}$ was defined as the dose necessary to obtain 75% of the baseline value. The $ED_{(0.5\text{-Hz decrease})}$, $ED_{(1\text{-Hz decrease})}$, and the $ED_{(1.5\text{-Hz decrease})}$ were defined as the doses necessary to obtain 0.5 Hz, 1 Hz, and 1.5 Hz decreases of the baseline frequency, respectively. When the obtained curve fits well on the data, these EDs are practically independent of the E_{max}.

When equal directed effect curves were obtained, the type of interaction between the drugs was analysed using the isobolic method (Greco et al., 1995): visually by plotting the $ED_{(2 \times baseline)}$, $ED_{(0.5\text{-Hz decrease})}$, $ED_{(1\text{-Hz decrease})}$, or the $ED_{(1.5\text{-Hz decrease})}$ values on an isobologram and numerically by comparing the drug load, needed to reach the chosen effect point, of the observed polytherapy with the drug load of the theoretical additive combination.

The drug load defined as $gvg/GVG + dzp/DZP$ was determined. *gvg* is the dose of vigabatrin in the polytherapy, *GVG* is the dose of vigabatrin in the monotherapy, *dzp* is the dose of diazepam in the polytherapy, and *DZP* is the dose of diazepam in the monotherapy needed to reach the chosen effect point (Berenbaum, 1989). If an estimated drug load = 1, then the drug effects are purely additive (no interaction). If the drug load < 1, then there is supraadditivity (synergy). If the drug load>1, then there is subadditivity (antagonism; Greco et al., 1995). *T*-tests were performed to test for differences between the theoretical additive and the experimental polytherapy combinations (Motulsky, 1995).

3. Results

3.1. Effects on the total spike and wave discharge duration

There was no significant difference between the 500-mg/kg vigabatrin treatment of observation periods 1 and 2. Therefore, the vigabatrin monotherapy data (observation period 1) may be compared with the polytherapy data

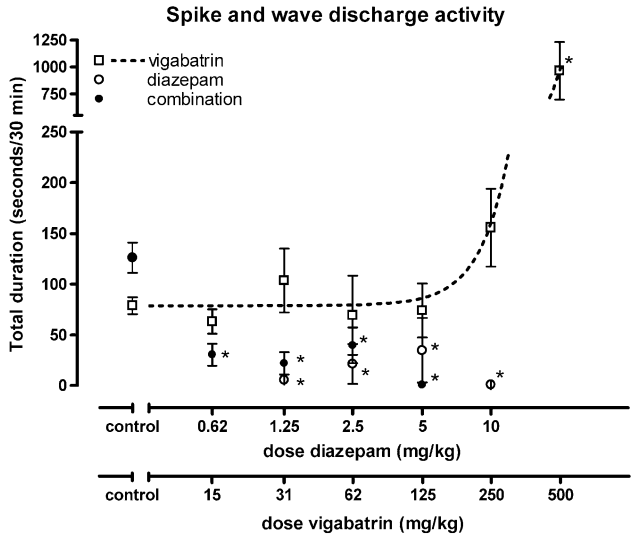

Fig. 1. The means with the standard errors of the total spike and wave discharge duration per dose of vigabatrin, diazepam, and their combination. Significances compared to the control are indicated by asterisks.

(observation period 2). However, there was a significant difference between the two control groups [i.e., the saline (observation period 1) and the intralipid treatments (observation period 2)] on the total spike and wave discharge duration [$t(76) = 2.943$; $P = 0.004$].

There was a significant effect of vigabatrin on the total spike and wave discharge duration [$F(6,102) = 21.592$; $P > 0.001$]. Post hoc analyses showed a significant increase in the total spike and wave discharge duration after 500 mg/kg compared to saline ($P < 0.001$), 15 mg/kg ($P < 0.001$), 31 mg/kg ($P < 0.001$), 62 mg/kg ($P < 0.001$), 125 mg/kg ($P < 0.001$), and 250 mg/kg ($P < 0.001$). To describe the dose–response relationship, a sigmoid E_{max} model was fitted through the data ($ED_{(2 \times baseline)} = 254$ mg/kg, S.E. = 3.03 mg/kg; see Fig. 1).

There was a significant effect of diazepam on the total spike and wave discharge duration [$F(4,51) = 11.626$; $P > 0.001$]. Post hoc analyses showed a significant decrease in the total spike and wave discharge duration after 1.25 mg/kg ($P < 0.001$), 2.5 mg/kg ($P < 0.01$), 5 mg/kg ($P < 0.01$), and 10 mg/kg ($P < 0.001$) as compared to intralipid. However, no dose–response relationship could be described because the maximal effect seems to be already obtained by the lowest dose tested (see Fig. 1).

There was a significant effect of polytherapy on the total spike and wave discharge duration [$F(4,56) = 14.394$; $P < 0.001$]. Post hoc analyses showed a significant decrease in the total spike and wave discharge duration after 15/0.62 mg/kg ($P < 0.001$), 31/1.25 mg/kg ($P < 0.001$), 62/2.5 mg/kg ($P < 0.01$), and 125/5 mg/kg ($P < 0.001$) as compared to intralipid. However, no dose–response relationship could be described because the maximal effect seems to be already obtained by the lowest dose tested (see Fig. 1).

Because for two of the treatments opposite effects were observed and no dose–response curve could be constructed, the isobolic analysis of the interaction could not be performed.

3.2. Effects on the behavioural activity

There was no significant difference between the two control groups [i.e., the saline (observation period 1) and the intralipid treatments (observation period 2)] on the fraction of time spent being behaviourally active. Moreover, there was no significant difference between the 500-mg/kg vigabatrin treatment of observation periods 1 and 2. Therefore, the vigabatrin monotherapy data (observation period 1) may be compared with the polytherapy data (observation period 2).

There was a significant effect of vigabatrin on the fraction of time spent being behaviourally active [$F(6,109) = 6.476$; $P > 0.001$]. Post hoc analyses showed a significant decrease in the fraction of time spent being behaviourally active after 500 mg/kg compared to saline ($P < 0.001$) and 250 mg/kg compared to saline ($P < 0.01$). To describe the dose–response relationship, a sigmoid E_{max} model was fitted through the data ($ED_{(0.75 \times baseline)} = 53$ mg/kg, S.E. = 4.4 mg/kg; see Fig. 2). There was a nonsignificant trend towards a decrease after diazepam ($P = 0.06$) and no significant effect of the polytherapy on the fraction of time spent being behaviourally active (see Fig. 2).

Because the three treatments differed in their effect, i.e., two of the treatments were shown to have no significant effect, the isobolic analysis of the interaction could not be performed.

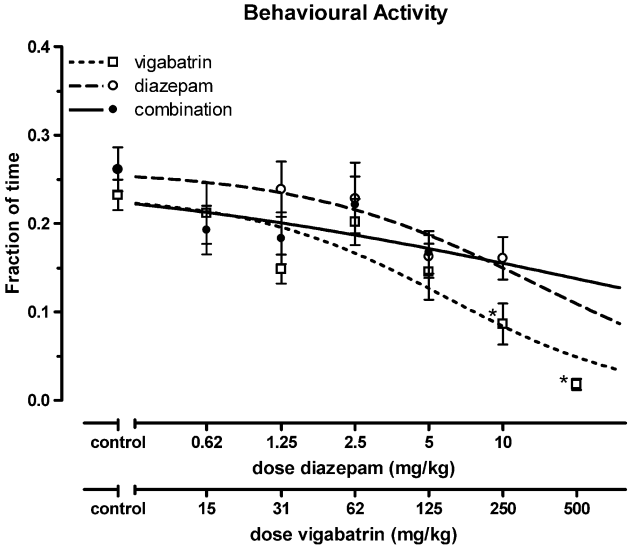

Fig. 2. The means with the standard errors of the fraction of time spent being behaviourally active per dose of vigabatrin, diazepam, and their combination. Significances compared to the control are indicated by asterisks.

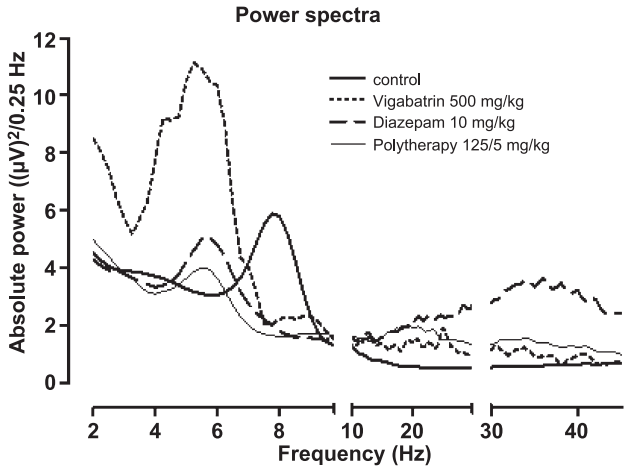

Fig. 3. The absolute power as a function of frequency for the highest doses of each treatment and the control group during active behaviour (smoothed).

3.3. Effects on the background EEG

Based on visual inspection of the power spectra (see Fig. 3), four variables were extracted: the theta (4–9 Hz) peak frequency, the absolute theta peak power, and the average absolute power in the beta frequency band (13–29.75 Hz) and the fast frequency band (30–44.75 Hz).

There were no significant differences between the two control groups [i.e., the saline (observation period 1) and the intralipid treatments (observation period 2)] on any of these variables. Moreover, there were no significant differences between the results of the 500-mg/kg vigabatrin treatment of observation periods 1 and 2 on any of the variables. Therefore, the vigabatrin monotherapy data (observation period 1) may be compared with the polytherapy data (observation period 2).

3.3.1. Effects on the average absolute power in the beta frequency band

There was a significant effect of vigabatrin on the average absolute power in the beta frequency band during active behaviour [$F(6,89)=3.411$; $P=0.004$]. Post hoc analyses showed a significant increase in the average absolute power in the beta frequency band after 500 mg/kg compared to saline ($P<0.01$), 15 mg/kg ($P<0.05$), 31 mg/kg ($P<0.05$), and 125 mg/kg ($P<0.05$). To describe the dose–response relationship, a sigmoid E_{max} model was fitted through the data ($ED_{(2\times baseline)}=499$ mg/kg, S.E. = 14 mg/kg; see Fig. 4A).

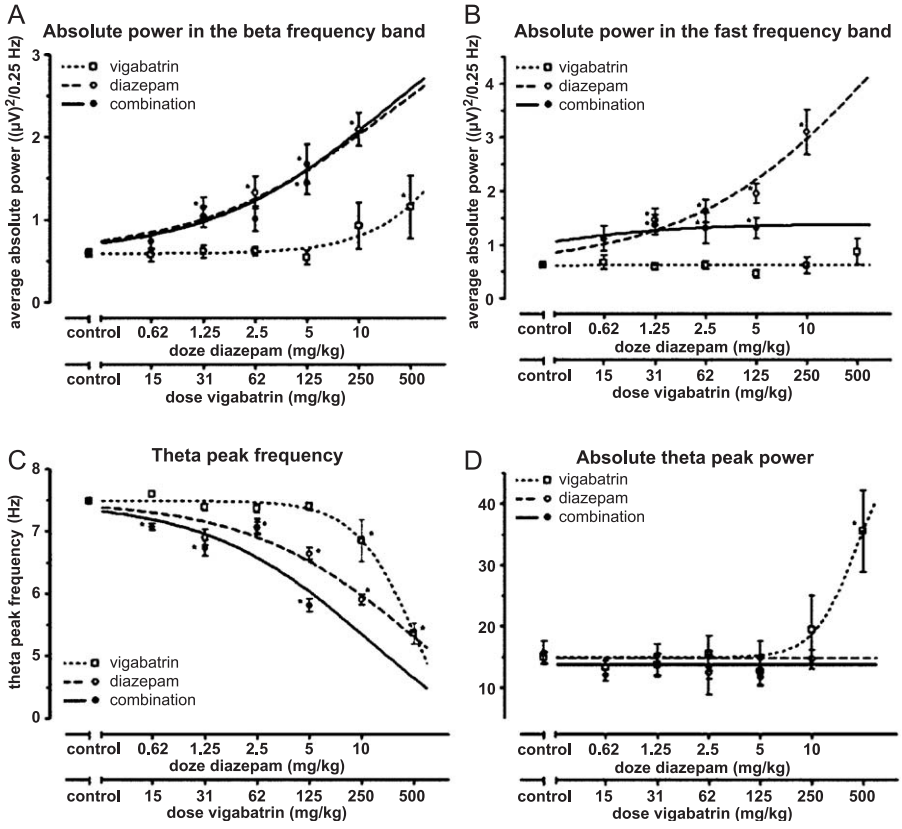

Fig. 4. The means with the standard errors of the average absolute power on the beta frequency band (A) and the fast frequency band (B), the average theta peak frequency (C) and the absolute power on the theta peak frequency (D) during active behaviour per dose of vigabatrin, diazepam, and their combination. Significances compared to the control are indicated by asterisks.

There was a significant effect of diazepam on the power in the beta frequency band [$F(4,52) = 24.147$; $P = 0.001$]. Post hoc analyses showed a significant increase in the average absolute power in the beta frequency band after 10 mg/kg compared to intralipid ($P < 0.001$), 1.25 mg/kg ($P < 0.001$), 2.5 mg/kg ($P < 0.01$), and 5 mg/kg ($P < 0.05$), and after 5 mg/kg ($P < 0.01$) and 2.5 mg/kg ($P < 0.001$) compared to intralipid. To describe the dose–response relationship, a sigmoid E_{max} model was fitted through the data ($ED_{(2 \times baseline)} = 2.13$ mg/kg, S.E. = 0.08 mg/kg; see Fig. 4A).

There was a significant effect of polytherapy on the power in the beta frequency band [$F(4,54) = 12.951$; $P = 0.001$]. Post hoc analyses showed a significant increase in the average absolute power in the beta frequency band after 125/5 mg/kg compared to intralipid ($P < 0.001$), 15/0.62 mg/kg ($P < 0.001$), and 62/2.5 mg/kg ($P < 0.05$), and after 31/1.25 mg/kg ($P < 0.01$) compared to intralipid. To describe the dose–response relationship, a sigmoid E_{max} model was fitted through the data ($ED_{(2 \times baseline)} = 56.4$ mg/kg of vigabatrin + 2.25 mg/kg of diazepam, S.E. = 3.03 mg/kg of vigabatrin + 0.12 mg/kg of diazepam; see Fig. 4A).

3.3.1.1. Isobolic analysis of the average absolute power in the beta frequency band. The chosen effect point for the isobolic analyses of the effects of the monotherapy and polytherapy treatments on the beta frequency band during active behaviour was the $ED_{(2 \times baseline)}$. Both Fig. 5A and Table 2 show the $ED_{(2 \times baseline)}$ with the 95% confidence intervals (95% CIs) of vigabatrin, diazepam, and their experimental combination for increasing the total power on the beta frequency band during active behaviour. Furthermore, they show the theoretical additive combination and the 95% CIs needed to obtain twice the baseline.

The normalised $ED_{(2 \times baseline)}$, i.e., drug load, for the experimental combination and that of the theoretical additive combination are not significantly different [$t(8) = 2.24$; $P = 0.055$]. Thus, there was no evidence for other than an additive interaction between vigabatrin and diazepam on this variable.

3.3.2. Effects on the average absolute power in the fast frequency band

There was no significant effect of vigabatrin on the average absolute power in the fast frequency band during active behaviour (see Fig. 4B).

There was a significant effect of diazepam monotherapy on the power in the fast frequency band [$F(4,52) = 25.986$; $P = 0.0001$]. Post hoc analyses showed a significant increase in the power in the fast frequency band after 10 mg/kg ($P < 0.001$), 5 mg/kg ($P < 0.001$), 2.5 mg/kg ($P < 0.01$), and 1.25 mg/kg ($P < 0.05$) compared to intralipid. Further post hoc analyses showed a significant increase in the power in the fast frequency band after 10 mg/kg compared to 5 mg/kg ($P < 0.01$), 2.5 mg/kg ($P < 0.001$), and 1.25 mg/kg ($P < 0.001$). To describe the dose–response relationship, a sigmoid E_{max} model was fitted through the data ($ED_{(2 \times baseline)} = 1.15$ mg/kg, S.E. = 0.08 mg/kg; see Fig. 4B).

There was a significant effect of polytherapy on the power in the fast frequency band [$F(4,54) = 5.914$; $P = 0.001$]. Post hoc analyses showed a significant increase in the power in the fast frequency band after 125/5 mg/kg ($P < 0.05$), 62/2.5 mg/kg ($P < 0.05$), and 31/1.25 mg/kg ($P < 0.01$) compared to control. To describe the dose–response relationship, a sigmoid E_{max} model was fitted through the data ($ED_{(2 \times baseline)} = 20.61$ mg/kg of vigabatrin + 0.82 mg/kg of diazepam, S.E. = 2.07 mg/kg of vigabatrin + 0.08 mg/kg of diazepam; see Fig. 4B).

Because the vigabatrin had no effect on the power in the fast frequency band, no equal effect curves could be obtained. Therefore, the isobolic analysis of the interaction could not be performed.

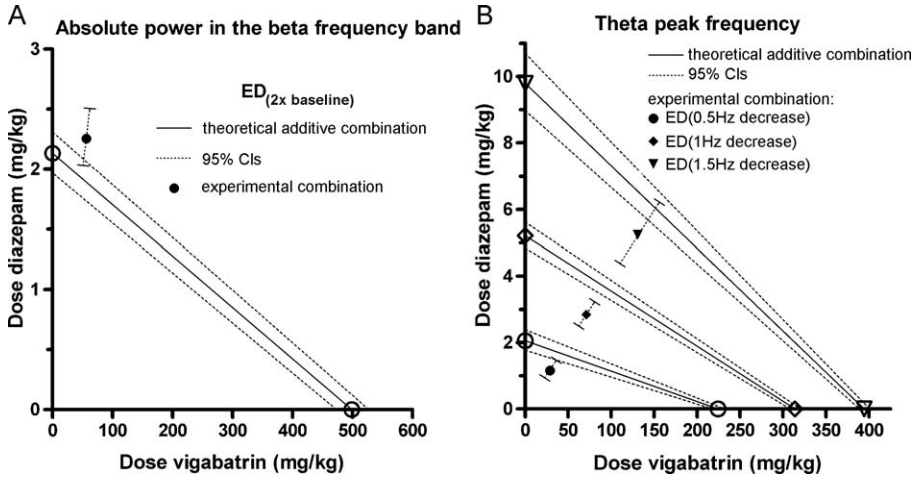

Fig. 5. The isoboles of the $ED_{(2 \times baseline)}$ on the average absolute power in the beta frequency band (A) and the $ED_{(0.5\text{-Hz decrease})}$, $ED_{(1\text{-Hz decrease})}$, and the $ED_{(1.5\text{-Hz decrease})}$ on the theta peak frequency (B) for the combination of vigabatrin and diazepam, showing the theoretical additive combination and the experimental combination with the 95% confidence intervals.

Table 2
Isobolic analysis on the beta frequency spectral power and the theta peak frequency

		Vigabatrin (GVG)	Diazepam (DZP)	Theoretical additive (GVG/DZP)	Experimental combination (GVG/DZP)
Beta frequency power	Top (E_{max})[a]	4.0	4.0		4.0
	Bottom[a]	0.59	0.59		0.59
	Hill[b]	1.66	0.80		0.87
	95% CIs	1.43–1.90	0.75–0.86		0.73–1.00
	$ED_{(2 \times baseline)}$[b] (mg/kg)	499	2.13	48.2/1.93	56.4/2.25
	95% CIs (mg/kg)	473–527	1.97–2.31	45.7–50.9/1.78–2.09	50.6–62.8/2.02–2.51
	Drug load	1.0	1.0	1.0	1.17
	95% CIs	0.95–1.06	0.92–1.27	0.93–1.08	1.05–1.30
Theta peak frequency	Top[a]	7.49	7.49		7.49
	Bottom (E_{max})[a]	3.0	3.0		3.0
	Hill[b]	−2.46	−0.89		−0.92
	95% CIs	−2.57–2.35	−0.99– −0.78		−1.12– −0.72
	$ED_{(0.5\text{-Hz decrease})}$[b] (mg/kg)	224	2.05	41.8/1.67	29.0/1.16
	95% CIs (mg/kg)	218–232	1.77–2.39	40.5–43.1/1.44–1.94	22.7–36.9/0.91–1.47
	Drug load	1.0	1.0	1.0	0.69
	95% CIs	0.97–1.03	0.86–1.16	0.88–1.14	0.54–0.88
	$ED_{(1\text{-Hz decrease})}$[b] (mg/kg)	314	5.21	92.1/3.68	71.2/2.85
	95% CIs (mg/kg)	308–321	4.84–5.62	90.4–94.0/3.42–3.97	62.4–81.2/2.50–3.25
	Drug load	1.0	1.0	1.0	0.77
	95% CIs	0.98–1.02	0.93–1.08	0.94–1.06	0.68–0.88
	$ED_{(1.5\text{-Hz decrease})}$[b] (mg/kg)	394	9.79	151/6.04	131/5.23
	95% CIs (mg/kg)	389–401	8.97–10.7	149–153/5.54–6.60	110–156/4.38–6.26
	Drug load	1.0	1.0	1.0	0.87
	95% CIs	0.99–1.02	0.92–1.09	0.94–1.06	0.73–1.04

The curve fit parameters and the corresponding 95% confidence intervals for the vigabatrin and diazepam monotherapies, the theoretical additive combination, and the experimental combination.

[a] Indicating fixed parameters.

[b] Indicating fitted parameters.

3.3.3. Effects on the theta peak frequency

There was a significant decrease of the theta peak frequency after vigabatrin during active behaviour [$F(6,99) = 44.55$; $P = 0.001$]. Post hoc analyses showed significant differences between the saline and 250-mg/kg ($P < 0.001$) and the 500-mg/kg ($P < 0.001$) groups. To describe the dose–response relationship, a sigmoid E_{max} model was fitted through the data ($ED_{(0.5\text{-Hz decrease})} = 224$ mg/kg, S.E. = 3.54 mg/kg; $ED_{(1\text{-Hz decrease})} = 314$ mg/kg, S.E. = 3.09 mg/kg; $ED_{(1.5\text{-Hz decrease})} = 394$ mg/kg, S.E. = 3.05 mg/kg; see Fig. 4C).

There was a significant decrease of the theta peak frequency after diazepam [$F(4,50) = 69.29$; $P = 0.001$]. Post hoc analyses showed significant differences between intralipid and the 1.25-mg/kg ($P < 0.001$), 2.5-mg/kg ($P < 0.01$), 5.0-mg/kg ($P < 0.001$), and the 10-mg/kg ($P < 0.001$) groups. Further post hoc analyses showed significant differences between the 10-mg/kg and the 1.25-mg/kg ($P < 0.001$), 2.5-mg/kg ($P < 0.001$) and 5.0-mg/kg ($P < 0.001$) groups, and between the 2.5-mg/kg and the 5.0-mg/kg ($P < 0.05$) groups. To describe the dose–response relationship, a sigmoid E_{max} model was fitted through the data ($ED_{(0.5\text{-Hz decrease})} = 2.05$ mg/kg, S.E. = 0.15 mg/kg; $ED_{(1\text{-Hz decrease})} = 5.21$ mg/kg, S.E. = 0.19 mg/kg; $ED_{(1.5\text{-Hz decrease})} = 9.79$ mg/kg, S.E. = 0.43 mg/kg; see Fig. 4C).

There was a significant decrease of the theta peak frequency after polytherapy [$F(4,56) = 56.92$; $P = 0.001$]. Post hoc analyses showed significant differences between control and the 15/0.62-mg/kg ($P < 0.01$), 31/1.25-mg/kg ($P < 0.001$), 62/2.5-mg/kg ($P < 0.01$), and the 125/5-mg/kg ($P < 0.001$) groups. Further post hoc analyses showed significant differences between the 125/5-mg/kg and the 15/0.62-mg/kg ($P < 0.001$), 31/1.25-mg/kg ($P < 0.001$) and 62/2.5-mg/kg ($P < 0.001$) groups. To describe the dose–response relationship, a sigmoid E_{max} model was fitted through the data ($ED_{(0.5\text{-Hz decrease})} = 29.0$ mg/kg of vigabatrin + 1.16 mg/kg of diazepam, S.E. = 3.50 mg/kg of vigabatrin + 0.14 mg/kg of diazepam; $ED_{(1\text{-Hz decrease})} = 71.2$ mg/kg of vigabatrin + 2.85 mg/kg of diazepam, S.E. = 4.67 mg/kg of vigabatrin + 0.19 mg/kg of diazepam; $ED_{(1.5\text{-Hz decrease})} = 131$ mg/kg of vigabatrin + 5.23 mg/kg of diazepam, S.E. = 11.7 mg/kg of vigabatrin + 0.47 mg/kg of diazepam; see Fig. 4C).

3.3.3.1. Isobolic analysis of the theta peak frequency. The chosen effect points for the isobolic analyses of the effects of the monotherapy and polytherapy treatments on the theta peak frequency during active behaviour were the $ED_{(0.5\text{-Hz decrease})}$, $ED_{(1\text{-Hz decrease})}$, and $ED_{(1.5\text{-Hz decrease})}$. Both Fig. 5B and Table 2 show the $ED_{(0.5\text{-Hz decrease})}$, $ED_{(1\text{-Hz decrease})}$, and the $ED_{(1.5\text{-Hz decrease})}$ with the 95% CIs of vigabatrin, diazepam, and their experimental combination for decreasing the theta peak frequency during active behaviour. Furthermore, they show the theoretical additive combination and the 95% CIs needed to obtain

these ED values. The normalised $ED_{(0.5\text{-Hz decrease})}$ [$t(8)=2.84$; $P=0.022$] and the $ED_{(1\text{-Hz decrease})}$ [$t(8)=3.79$; $P=0.0053$], i.e., drug load, for the experimental combination are significantly lower than that of the theoretical additive combination, whereas for the normalised $ED_{(1.5\text{-Hz decrease})}$ [$t(8)=1.57$; $P=0.155$], there is no significant difference . Thus, there was a synergistic interaction between vigabatrin and diazepam on this variable for the effect range up to 1-Hz decrease as compared to control, whereas for the effect of 1.5-Hz decrease, there was an additive interaction between the drugs.

3.3.4. Effects on the absolute theta peak power

There was a significant increase in the absolute power on the theta peak frequency after vigabatrin during active behaviour [$F(6,99)=5.951$; $P=0.001$]. Post hoc analyses showed significant differences between 500-mg/kg and the saline ($P<0.001$), 15-mg/kg ($P<0.001$), 31-mg/kg ($P<0.001$), 62-mg/kg ($P<0.001$), 125-mg/kg ($P<0.001$), and the 250-mg/kg ($P<0.05$) groups. To describe the dose–response relationship, a sigmoid E_{max} model was fitted through the data ($ED_{(2\times\text{baseline})}=415$ mg/kg, S.E. = 5.74 mg/kg; see Fig. 4D).

There was no significant effect of diazepam or the polytherapy on the absolute power on the theta peak frequency during active behaviour (see Fig. 4D).

Because diazepam and the polytherapy had no effect on the absolute power on the theta peak frequency, no equal effect curves could be obtained. Therefore, the isobolic analysis of the interaction could not be performed.

4. Discussion

We investigated the effects of the interaction between vigabatrin and diazepam on variables that change in the same as well as in the opposite direction (i.e., the total spike and wave discharge duration, the fraction of time spent being behaviourally active, and the power spectrum of the EEG during active behaviour). Only vigabatrin dose dependently increased the total spike and wave discharge duration, whereas both diazepam and the polytherapy combination decreased the total spike and wave discharge duration. Only vigabatrin dose dependently decreased the fraction of time spent being behaviourally active, whereas there was a trend towards a decrease after diazepam for the doses tested (up to 10 mg/kg). All treatments dose dependently increased the spectral power of the beta frequency band. Both diazepam and the polytherapy combination dose dependently increased the spectral power of the fast frequency band as well. All treatments were also shown to dose dependently decrease the frequency of the theta peak, whereas only vigabatrin dose dependently increased the power of the theta peak.

The increased total spike and wave discharge duration after vigabatrin agrees with our earlier findings (Bouwman et al., 2003b) and with findings of Vergnes et al. (1984). The decreased total spike and wave discharge duration after diazepam agrees with findings of Coenen and van Luijtelaar (1989). Despite the fact that no isobolic analysis could be performed, the decrease after the polytherapy combination shows that in the chosen combination, the effect of diazepam is dominant. To further investigate the interaction on this variable, in the future, another dose combination should be chosen to prevent possible dominance of diazepam over vigabatrin.

The decrease in the fraction of time spent being behaviourally active after vigabatrin agrees with earlier findings (Bouwman et al., 2003a) and with reports of Huot and Palfreyman (1982); Sayin et al. (1992), and Sherif et al. (1994). The trend towards a decrease after diazepam (up to 10 mg/kg) agrees with earlier findings in WAG/Rij rats (Coenen and van Luijtelaar, 1989). Therefore, it is remarkable that our polytherapy combination did not have an effect on the behavioural activity of the rats.

The increase in the beta frequency spectral power during active behaviour after both GABAergic drugs vigabatrin and diazepam agrees with earlier reports stating that GABAergic compounds such as benzodiazepines, neurosteroids, and tiagabine increase the spectral power of the beta frequency band (Cleton et al., 2000; Coenen et al., 1995; Visser et al., 2002a,b, 2003). These present data support the idea that the beta frequency spectral power (during active behaviour) can serve as a biomarker for enhanced GABAergic inhibition (Cleton et al., 2000; Jonker et al., 2003; Lopes da Silva, 2002). Increasing the GABA conductances in the brain is a common method of antiepileptic treatment (Löscher, 1998). Mandema et al. (1991) showed that for the benzodiazepines, there is a close correlation between an increase on the beta frequency spectral power and anticonvulsant activity, suggesting that at least for the benzodiazepines, an increase in beta frequency spectral power might be a biomarker, a functional correlate, for the antiepileptic activity of a drug. However, besides these benzodiazepines, this was not shown for any other type of antiepileptic drug.

Because the different treatments all increased the beta frequency spectral power, an isobolic analysis could be performed. Isobolic analysis showed that there was no evidence for other than an additive interaction between vigabatrin and diazepam on the beta frequency spectral power. Unfortunately, the $ED_{(2\times\text{baseline})}$ on the beta frequency spectral power of the diazepam monotherapy was equal to the diazepam dose in the $ED_{(2\times\text{baseline})}$ of the polytherapy combination. Thus, for changes in the beta frequency spectral power, the chosen dose ratio in the combination of doses in the polytherapy was not optimal, which makes it improper to base any strong conclusions on these data. The used dose ratio was diazepam/vigabatrin = 1:25, whereas the optimal combination for this variable turned out to be 1:234 (see Table 2). However, for

changes in the theta peak frequency, the used combination turned out to be adequate.

The decrease in theta peak frequency after diazepam agrees with earlier reports stating that benzodiazepines decrease the theta peak frequency in rodents (Caudarella et al., 1987; Zhu and McNaughton, 1994; Yoshimoto et al., 1999), whereas there have not been any reports about this effect after vigabatrin. However, Ylinen et al. (1991) presented raw EEG signals in their report which suggest the same decrease in theta peak frequency after vigabatrin as in the present study, although they did not describe this effect. A decrease in theta peak frequency is commonly used as a biomarker for anxiolytic effects because all the tested clinically effective anxiolytics were shown to produce this effect (McNaughton and Coop, 1991). This biomarker not only holds for GABAergic anxiolytics but also for other non-GABAergic anxiolytics. Therefore, the decrease in theta peak frequency after vigabatrin possibly suggests that vigabatrin might have an anxiolytic action as well. Indeed, Sayin et al. (1992) and Sherif et al. (1994) reported that vigabatrin has anxiolytic effects in rats. Furthermore, there have been some reports about possible anxiolytic effects of vigabatrin in humans (Zwanzger et al., 2001a,b).

Because on the theta peak frequency similarly directed curves were obtained, the isobolic analysis could be performed. As mentioned, the chosen combination was adequate for the measurement of the effect on the theta peak frequency, therefore allowing conclusions about the interaction. This isobolic analysis showed that vigabatrin and diazepam combined in a synergistic way on the theta peak frequency in the effect range up to 1-Hz decrease. These data suggest that interaction within the $GABA_A$ receptor complex might underlie the synergistic decrease in theta peak frequency. Furthermore, these data agree with the enhanced binding of GABA to the $GABA_A$ receptor by benzodiazepines and vice versa (Haefely, 1989; Olsen, 1981). This synergistic interaction, in this effect range, between vigabatrin and diazepam on the theta peak frequency suggests that a combination of a GABA transaminase inhibitor and a benzodiazepine might have a higher clinical potential than would be expected based on their anxiolytic potential as monotherapies. However, because it has not yet been shown that theta peak frequency decreases are a direct reflection of anxiolytic potency, extrapolations towards clinical practice might be premature.

In conclusion, for the variables that change in the same direction, the results of this study further support the idea that the beta frequency spectral power, during active behaviour, can serve as a reliable biomarker for enhanced GABAergic inhibition. Unfortunately, the dose combination in the polytherapy was not optimal for analysing the interaction between the two drugs on this variable; therefore, no conclusions about this interaction can be drawn. Furthermore, these results also support the suggestion that clinically effective anxiolytics produce a decrease in theta peak frequency. The interaction between vigabatrin and diazepam on the theta peak frequency was shown to be synergistic, in a certain effect range, as was expected based on their interaction on the $GABA_A$ receptor (Haefely, 1989; Olsen, 1981).

Unfortunately, for the variable that changes in the opposite direction (i.e., the total spike and wave discharge duration), based on the results from this study, no conclusions can be drawn concerning the possible clinical usefulness of polytherapy with vigabatrin and diazepam in epilepsy treatment. Furthermore, in the present study, the possible pharmacokinetic interaction between the two drugs was not investigated. A pharmacokinetic interaction might, however, be a confounding factor underlying the obtained results. Therefore, generalisation towards clinical practice is premature. It was seen that on the beta frequency spectral power, the combination of doses in the polytherapy was not optimal, whereas it was adequate for the theta peak frequency. This indicates that on different variables, there might be different optimal dose combinations for the polytherapy. Which, in turn, implies that for different disorders/illnesses, the optimal combination might differ. This makes the clinical application of rational polytherapy even more complicated.

Acknowledgements

The authors wish to thank the Dutch National Epilepsy Fund for their financial support (NEF 20-08), Yamanouchi Europe for the kind gift of vigabatrin, and Hans Krijnen, Elly Willems-van Bree, and Martine Bouwman for their assistance.

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