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A four-ligand hypercube model to quantify allosteric interactions within the $GABA_A$ receptor complex

Clementina M. van Rijn*, Elly Willems-van Bree

NICI/Department of Biological Psychology, University of Nijmegen, Post-box 9104, 6500 HE Nijmegen, The Netherlands

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Abstract

The aim of this study was to investigate the couplings between various binding sites on the $GABA_A$ receptor complex. We investigated combinations of three test compounds: (1) GABA (γ-aminobutyric acid), (2) Org 20549 [(2β3α5α)-21hydroxy-^{3}Hydroxy-2(4morpholinyl)pregnan-20one methane-sulphonate)], a neuroactive steroid and (3) retigabine (D-23129, *N*-(2-amino-4-(4-fluorobenzylamino)-phenyl) carbamic acid ethyl ester), a new antiepileptic drug. Receptor-binding assays were conducted using rat brain membranes. [^{3}H]TBOB ([^{3}H]-*t*-butyl-bicyclo-ortho-benzoate) was the tracer ligand. All three test compounds inhibited the binding of [^{3}H]TBOB with EC_{50}'s of 4.0, 98 and 23 μM, respectively. Isobolic analysis of the combination data showed that the three compounds act in synergy in displacing [^{3}H]TBOB. These interactions could be described and quantified by a hypercube model in which each of the three test compounds and [^{3}H]TBOB bind to different, allosterically coupled sites such that each of the test compounds allosterically displaces the tracer [^{3}H]TBOB and allosterically enhances the affinity of any other test compound by a factor 4.4. The simultaneous binding of any two ligands enhances the affinity of the third by a factor 9. These results may contribute to the understanding of individual variability in drug responses and to the discussion about rational polytherapy.

Keywords: $GABA_A$ receptor complex; TBOB; Neuroactive steroid; Retigabine; Synergy; Isobole method; Molecular model

1. Introduction

Epilepsy is a chronic brain disease for which the therapy of choice is treatment with antiepileptic drugs. Unfortunately, 30% of patients have refractory epilepsy. These are commonly prescribed combinations of two or more antiepileptic drugs (polytherapy) to control their seizures, but even then, few of these patients achieve seizure control. Clinical evidence to support the choice of particular combinations is only anecdotal (Deckers et al., 2000). The concept of 'rational polytherapy' aims to base the choice of combinations of antiepileptic drugs on their pharmacological characteristics (Ferrendelli, 1995). In this context, we use receptor-binding assays to investigate effects of combinations of GABAergic drugs (i.e. drugs interacting with the $GABA_A$ complex), with the aim to quantify the couplings between various binding sites on the receptor complex.

$GABA_A$ receptors are ligand-gated chloride ion channels (review Mehta and Ticku, 1999). The $GABA_A$ receptor complex comprises binding sites for a variety of compounds, such as benzodiazepines, barbiturates, neuroactive steroids and general anaesthetics. Furthermore, a picrotoxin-sensitive convulsant site is present on the complex (Mehta and Ticku, 1999). A number of these binding sites are allosterically coupled, resulting in a network of interactions, ultimately regulating the permeability of the Cl^- channel (Korpi et al., 2002; Mehta and Ticku, 1999). The functional state of the channel can be assayed by measuring the amount of ligand binding to the convulsant site (Hawkinson et al., 1994; Havoundjian et al., 1986; Im and Blakeman, 1991; Korpi et al., 2002; Maksay, 1996). Therefore, receptor-binding assays on this site can be used to measure the effects of allosteric interactions that modulate GABAergic neurotransmission.

In earlier studies, we quantified the allosteric coupling between pairs of test compounds: for example, between neurosteroids and GABA (Van Rijn et al., 1999), and between retigabine and GABA (Van Rijn and Willems-van Bree, 2003). Whereas in these studies we investigated the

* Corresponding author. Tel.: +31-24-3615612; fax: +31-24-3616066.
E-mail address: rijn@nici.kun.nl (C.M. van Rijn).

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interaction between GABA and one exogenous drug, in the present study, we investigated combinations of three test compounds: the endogenous compound GABA (γ-amino-butyric acid), and two exogenous drugs: Org 20549 [(2β3α5α)-21hydroxy-^{3}Hydroxy-2(4morpholinyl)pregnan-20one methane-sulphonate)], a neuroactive steroid (Hill Venning et al., 1996), and retigabine (D-23129, *N*-(2-amino-4-(4-fluorobenzylamino)-phenyl) carbamic acid ethyl ester), a potential new antiepileptic drug (Rostock et al., 1996). Receptor-binding assays were conducted using well-washed rat brain membranes. [^{3}H]TBOB ([^{3}H]-*t*-butyl-bicyclo-ortho-benzoate) was used as the tracer ligand for the picrotoxin-sensitive convulsant site (Lawrence et al., 1985; Van Rijn et al., 1990).

The experimental data were analysed in two different ways. First, the sigmoid-E_{max} model was fitted to the data in order to describe the results in terms of additivity, synergy and antagonism using the isobole method (Berenbaum, 1989; Greco et al., 1995; Loewe, 1953; Tallarida, 1992), which allows qualitative conclusions only (Greco et al., 1995). Next, an allosteric four-ligand molecular model, a hypercube model, was fitted to the data, which describes the observed interactions in quantitative terms as well.

2. Materials and methods

2.1. Preparation of the tissue

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. Forebrains of Wistar rats [body weight 350 ± 50 g (mean ± S.D.)] were used. The brains were homogenised in nine volumes 0.32 M sucrose at 0 °C with a Teflon-glass homogeniser. The homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C. The supernatant was decanted and centrifuged at 48 000 × *g* for 30 min at 4 °C. The pellets were washed two times by suspension in 50 mM sodium–potassium–phosphate buffer, pH 7.4, containing 500 mM NaCl (assay buffer) and centrifuged at 48 000 × *g* for 10 min at 4 °C. The pellets were frozen, thawed and washed three times in order to remove endogenous GABA. The pellets were stored at −80 °C until assay. Before assay, the pellets were washed once.

2.2. Assays

The pellets were homogenised in assay buffer: the tissue concentration in the incubation medium was 12.5 mg tissue wet weight/ml. Into glass tubes, we added consecutively 25 μl of [^{3}H]TBOB and either drugs or buffer in volumes of 25 μl until a volume of 100 μl. The final concentration of [^{3}H]TBOB during incubation was 8 nM. The incubation was started by adding 200 μl of tissue homogenate. Incubations were performed at 25 °C, lasted 90 min and were terminated by adding 3-ml ice-cold buffer to the tubes and rapid filtration of the mixture. The filters were washed two times with 3-ml cold assay buffer. Radioactivity retained in the filters was counted by liquid scintillation spectrometry. Specific [^{3}H]TBOB binding was defined as total binding minus the remaining binding in the presence of 100 μM picrotoxin. Total binding was less than 5% of the added [^{3}H]TBOB concentration. Specific binding was 65–70% of total binding at 8 nM [^{3}H]TBOB.

Dose series of [^{3}H]TBOB displacement data were collected for GABA, for retigabine and for Org 20549 and for various combinations of these three drugs.

2.3. Chemicals

GABA and picrotoxin were obtained from Sigma-Aldrich Chemie. [^{3}H]TBOB was obtained from Amersham Biosciences. The specific activity was 18 Ci/mmol (batch 28). Retigabine was a gift from Viatris, Frankfurt am Main, Germany. Retigabine was dissolved in 1N HCl and diluted 1000-fold with buffer during incubation. Org 20549 was a gift from Organon Laboratories, Newhouse, ML1 5SH, Scotland, UK. Org 20549 was dissolved in buffer.

2.4. Data analysis

2.4.1. Fitting the sigmoid-E_{max} equation to the data

The right-hand side of the sigmoid-E_{max} Eq. (1) was fitted to the data.

$$E_{drug} = \frac{E_{max}}{1 + \left[\frac{EC_{50}}{[drug]}\right]^H} \quad (1)$$

In Eq. (1), [drug] is the concentration of test drug in mol/l. E_{max} is the experimentally determined binding of [^{3}H]TBOB in the absence of test drug, representing 100% binding. E_{drug} is the experimentally determined binding of [^{3}H]TBOB in the presence of the test drug, expressed as a percentage of E_{max} binding. EC_{50} is the concentration of the drug that displaces 50% of the [^{3}H]TBOB, compared with the amount bound in the presence of the drugs with the fixed concentration, i.e. compared with the starting point of that particular dose–effect curve. H is the Hill coefficient. EC_{50} and H were estimated by nonlinear regression analysis using the software program Prism 3.02 (GraphPad Software).

2.4.2. Isobolic analysis of the data

A 3D isobologram was constructed and the normalized total dose was calculated for the $EC_{35\%\ binding\ left}$, the concentration of test drug that displaces all but 35% of the [^{3}H]TBOB, compared with the amount bound in the absence of any other drug, i.e. compared to the starting point of the single drug curves.

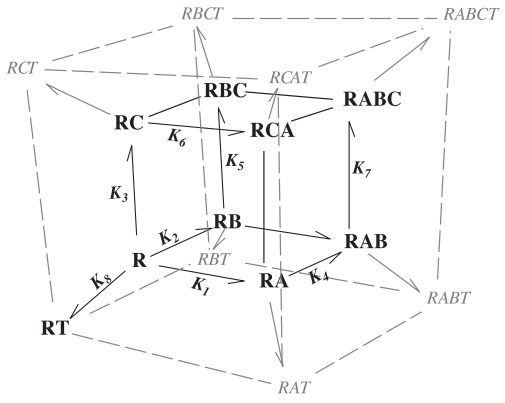

Fig. 1. The four-ligand hypercube molecular model for interactions between three different test drugs, A, B, C and a tracer T. The model reflects a set of reversible bimolecular reactions. Four binding sites for the four compounds are assumed: a site for GABA (A), for retigabine (B), for Org 20549 (C) and for [^{3}H]TBOB (T). The empty receptor complex is abbreviated as R. K_i's are dissociation constants. For clarity, the reversible reactions are abbreviated: R → RA means R + A⇆RA. All four sites are allosterically coupled. We assume that each test drug displaces [^{3}H]TBOB quasi-competitively. In that case, the concentration of receptor complexes containing one or more test drugs as well as tracer T can be neglected. These complexes are depicted in grey scale. Drugs A, B and C allosterically enhance each other's affinity, such that $K_4 = \gamma_1 K_2$, $K_5 = \gamma_2 K_3$, $K_6 = \gamma_3 K_1$ and $K_7 = \gamma_4 K_3$, with each $\gamma < 1$. The parameters K_1, K_2, K_3, γ_1, γ_2, γ_3 and γ_4 are to be estimated.

The $EC_{35\%\ \text{binding left}}$ was chosen, instead of the EC_{50}, since this point is on the steep part of all the measured dose–effect curves. $EC_{35\%\ \text{binding left}}$ values were estimated using the rewritten sigmoid-E_{max} model: Eq. (1A).

$$E_{drug} = \frac{E_{max}}{1 + \left[\frac{E_{max} - E_{35\%\_binding\_left}}{E_{35\%\_binding\_left}}\right] \times \left[\frac{EC_{35\%\_binding\_left}}{[drug]}\right]^{H}} \quad (1A)$$

In a 3D isobologram, the concentration of each drug is represented on one of the axes. Each plotted point in the graph represents a triplet of concentrations of the three drugs needed for 35% binding when added in combination. The normalized total dose was calculated according to Eq. (2).

$$\text{Normalized Total Dose} = \sum_{i=1}^{3} \frac{[drug_i]}{[DRUG_i]} \quad (2)$$

In Eq. (2), $[drug_i]$ is the concentration of drug i needed in the combination and $[DRUG_i]$ is the concentration of the pure drug i needed to reach the chosen effect point of 35% binding.

If an experimentally determined data point lies on the plane defined by the three plotted points of the pure drugs, then the normalized total dose = 1 and the drug effects are purely additive (no interaction). If the point lies below this plane, then the normalized total dose < 1 and there is supra-additivity (synergy). If it lies above this plane, then the normalized total dose>1 and there is subadditivity (antagonism) (Berenbaum, 1989; Greco, 1995; Tallarida, 1992).

2.4.3. Molecular modelling

A molecular model for allosteric interactions between the three test drugs and [^{3}H]TBOB is depicted in Fig. 1. The model reflects a set of reversible bimolecular reactions, in which R is a receptor complex. Four binding sites for the four compounds are assumed: a site for GABA (A), for retigabine (B), for Org 20549 (C) and for [^{3}H]TBOB (T). The empty receptor complex is abbreviated as R.

We use X to designate any compound A, B or C. We assume that by the binding of any combination of A, B and C, the K_d's of all reactions involving T will increase such that the equilibria of the reactions RX + T⇆RXT lie to the left, i.e. to (almost) complete dissociation. Then the ligands X and T can be interpreted as quasi-competitive and the concentrations of RXT can be neglected.

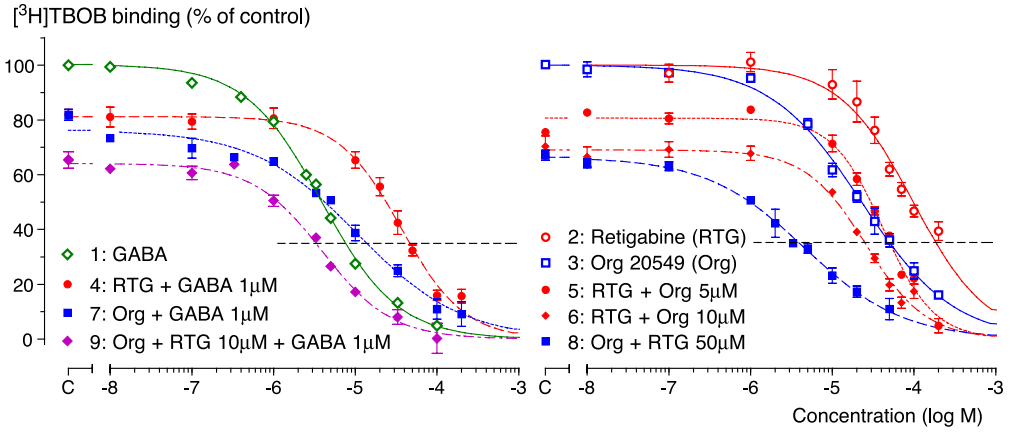

Fig. 2. The dose–effect curves of GABA, retigabine and Org 20549 and of their combinations on the binding of [^{3}H]TBOB (8 nM) (data points: mean ± S.E.M., $n = 1–6$ in triplicate). The sigmoid-E_{max} model, Eq. (1), was fitted to the data. Parameter estimates are given in Table 1. The horizontal striped line indicates the 35% binding level. The numbers of the curves in the figures and in the tables all correspond.

Table 1
Parameter estimates of the sigmoid-E_{max} curve fitted to the experimental data

No.	E_{max}		IC_{50}		Hill		N
	Percentage of control	(95% CI)	μM	(95% CI)	number	(95% CI)	
1 GABA	100	(fixed)	4.00	(3.43,4.67)	0.95	(0.80,1.10)	6
2 Retigabine	100	(fixed)	97.9	(78.9,122)	0.96	(0.70,1.22)	3
3 Org 20549	100	(fixed)	22.8	(20.0,26.2)	0.78[a]	(0.70,0.86)	3
4 Retigabine in presence of GABA, 1 μM	81.2	(78.2,84.2)	36.3	(31.1,42.3)	1.12	(0.92,1.33)	3
5 Retigabine in presence of Org 20549, 5 μM	80.6	(80.6,82.8)	42.2	(36.8,48.3)	1.37[a]	(1.11,1.62)	1
6 Retigabine in presence of Org 20549, 10 μM	69.0	(65.8,72.2)	25.8	(21.4,31.1)	1.18	(0.91,1.45)	3
7 Org 20549 in presence of GABA, 1 μM	76.2	(73.2,79.3)	10.9	(8.22,14.4)	0.68[a]	(0.56,0.81)	3
8 Org 20549 in presence of retigabine, 50 μM	66.4	(64.8,67.9)	4.61	(4.07,5.22)	0.73[a]	(0.65,0.80)	3
9 Org 20549 in presence of retigabine, 10 μM and GABA 1 μM	64.9	(62.7,67.1)	3.82	(3.17,4.60)	1.00	(0.80,1.20)	3

The sigmoid-E_{max} Eq. (1) (EC_{50} and Hill parameter) and Eq. (1A) ($EC_{35\%\ binding\ left}$) were fitted to the data. The parameter estimates and the 95% confidence intervals (CI) of the fits are given. All three compounds displaced [^{3}H]TBOB in the absence as well as in the presence of another compound. An [a] indicates Hill coefficients different from unity. The $EC_{35\%\ binding\ left}$ values were plotted in the isobologram in Fig. 3. The normalized total dose was calculated using Eq. (2). If this total dose is less than unity, then there is synergy. If the CIs of the normalized total dose do not overlap with unity, then the values are significantly different from unity (indicated by a [b]). The number of experiments in triplicate is indicated by N. The numbers of the curves in the figures and in the tables all correspond.

Table 2
Parameter estimates for isobolic analysis for 35% binding

No.	$IC_{35\%\ binding\ left}$		Normalized total dose	
	μM	(95% CI)	Fraction of the single compounds	(95% CI)
1 GABA	7.66	(6.52,9.00)	1.0	(0.85,1.17)
2 Retigabine	187	(140,250)	1.0	(0.75,1.34)
3 Org 20549	50.7	(44.8,57.5)	1.0	(0.88,1.13)
4 Retigabine in presence of GABA, 1 μM	46.5	(40.6,53.2)	0.38[b]	(0.33,0.43)
5 Retigabine in presence of Org 20549, 5 μM	51.3	(45.7,57.7)	0.37[b]	(0.33,0.42)
6 Retigabine in presence of Org 20549, 10 μM	25.2	(21.4,29.5)	0.33[b]	(0.28,0.39)
7 Org 20549 in presence of GABA, 1 μM	13.8	(10.9,17.5)	0.40[b]	(0.32,0.51)
8 Org 20549 in presence of retigabine, 50 μM	3.95	(3.59,4.37)	0.35[b]	(0.31,0.38)
9 Org 20549 in presence of retigabine 10 μM and GABA 1 μM	3.26	(2.74,3.87)	0.25[b]	(0.21,0.30)

The sigmoid-E_{max} Eq. (1) (EC_{50} and Hill parameter) and Eq. (1A) ($EC_{35\%\ binding\ left}$) were fitted to the data. The parameter estimates and the 95% confidence intervals (CI) of the fits are given. All three compounds displaced [^{3}H]TBOB in the absence as well as in the presence of another compound. An [a] indicates Hill coefficients different from unity. The $EC_{35\%\ binding\ left}$ values were plotted in the isobologram in Fig. 3. The normalized total dose was calculated using Eq. (2). If this total dose is less than unity, then there is synergy. If the CIs of the normalized total dose do not overlap with unity, then the values are significantly different from unity (indicated by a [b]). The number of experiments in triplicate is indicated by N. The numbers of the curves in the figures and in the tables all correspond.

With this assumption, the model depicted in Fig. 1 is fully characterized by the following expressions for the dissociation constants (K_d's),

$$K_1 = \frac{[R]\cdot[A]}{[RA]}$$

$$K_2 = \frac{[R]\cdot[B]}{[RB]}$$

$$K_3 = \frac{[R]\cdot[C]}{[RC]}$$

$$K_4 = \frac{[RA]\cdot[B]}{[RAB]}$$

$$K_5 = \frac{[RB]\cdot[C]}{[RBC]}$$

$$K_6 = \frac{[RC]\cdot[A]}{[RCA]}$$

$$K_7 = \frac{[RAB]\cdot[C]}{[RABC]}$$

$$K_8 = \frac{[R]\cdot[T]}{[RT]}$$

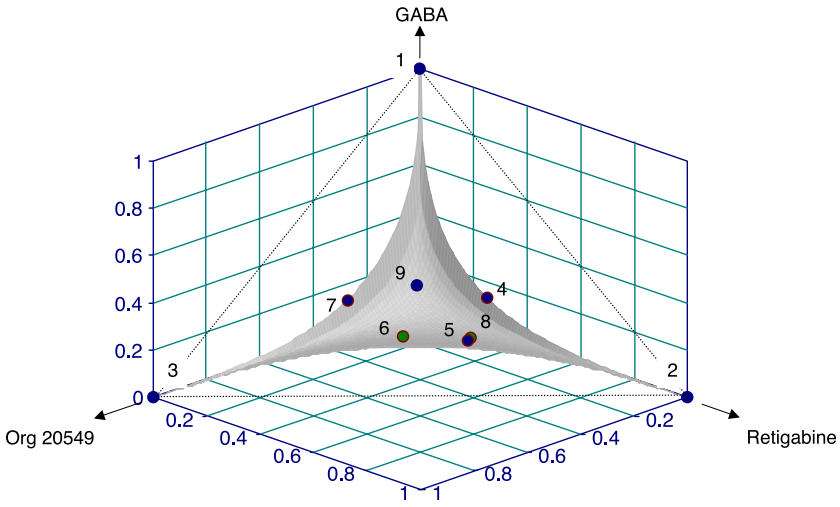

Fig. 3. 3D isobologram of combinations of the three compounds GABA, retigabine and Org 20549 for 35% binding. Plotted are the data from column '$EC_{35\%\ binding\ left}$' from Table 2. This figure demonstrates synergy between all three compounds in displacing [^{3}H]TBOB since the experimental points of the combinations all lie below the additive plane. For 95% confidence intervals, see Table 1. The numbers of the curves in the figures and in the tables all correspond.

To describe the allosteric interactions, the following factors are introduced: γ_1 describes the change in K_d of B by the binding of A: $K_4 = \gamma_1 K_2$

γ_2 describes the change in K_d of C by the binding of B: $K_5 = \gamma_2 K_3$

γ_3 describes the change in K_d of A by the binding of C: $K_6 = \gamma_3 K_1$

γ_4 describes the change in K_d of C by the binding of both A and B: $K_7 = \gamma_4 K_3$

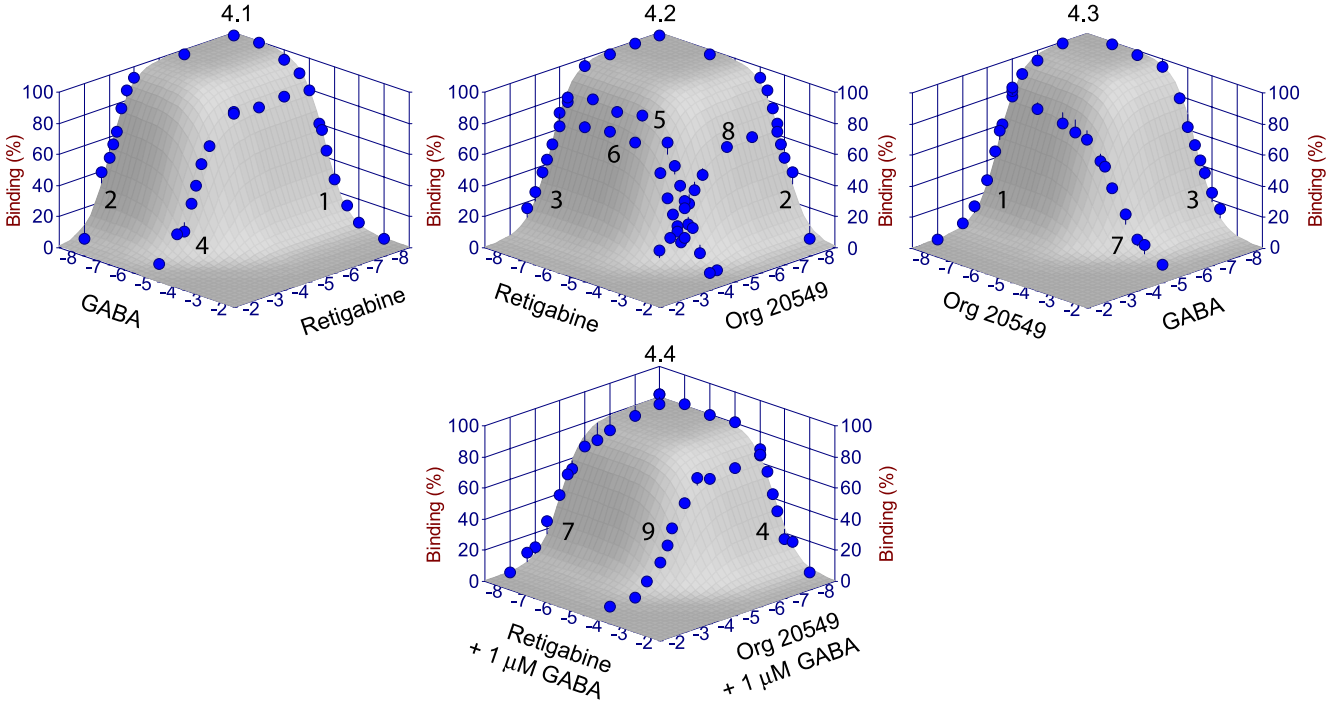

Fig. 4. Dose–effect surfaces of the hypercube molecular model, Eq. (3), fitted to the data. Same data points as in Fig. 2. The results of the four fit procedures (see Section 3.3) are shown. In 4.1–4.3, we varied the concentrations of two drugs and fixed the concentration of the third drug at zero. In a fourth fit procedure (4.4), we varied the concentrations of retigabine and Org 20459 and fixed the concentration of GABA at 1 μM. The statistics of the four fits are as follows: (1) $r^2 = 0.978$, S.E. = 5.06%; (2) $r^2 = 0.975$, S.E. = 4.97%; (3) $r^2 = 0.978$, S.E. = 5.07%; (4) $r^2 = 0.964$, S.E. = 5.25%. The parameter estimates for the K_d values and the γ values, quantifying the coupling between the three compounds, are given in Table 3. The numbers of the curves in the figures and in the tables all correspond.

Since all RXT are neglected, the concentration of [^{3}H]TBOB bound is:

$$[\mathrm{Bound_{tot}}] = [\mathrm{RT}]$$

The total receptor concentration is the sum of free receptors and occupied receptors:

$$[\mathrm{R_{tot}}] = [\mathrm{R}] + [\mathrm{RT}] + [\mathrm{RA}] + [\mathrm{RB}] + [\mathrm{RC}] + [\mathrm{RAB}] + [\mathrm{RBC}] + [\mathrm{RCA}] + [\mathrm{RABC}]$$

The displacement curves are described in terms of fractional occupancy:

$$\frac{[\mathrm{Bound_{tot}}]}{[\mathrm{R_{tot}}]}$$

Rearrangement of the above equations yields:

$$[\mathrm{Bound_{tot}}] = [\mathrm{RT}] = \frac{[\mathrm{R}]\cdot[\mathrm{T}]}{K_8}$$

$$[\mathrm{R_{tot}}] = [\mathrm{R}]\left\{1 + \frac{[\mathrm{T}]}{K_8} + \frac{[\mathrm{A}]}{K_1} + \frac{[\mathrm{B}]}{K_2} + \frac{[\mathrm{C}]}{K_3} + \frac{[\mathrm{A}]\cdot[\mathrm{B}]}{\gamma_1 K_1 K_2} + \frac{[\mathrm{B}]\cdot[\mathrm{C}]}{\gamma_2 K_2 K_3} + \frac{[\mathrm{C}]\cdot[\mathrm{A}]}{\gamma_3 K_3 K_1} + \frac{[\mathrm{A}]\cdot[\mathrm{B}]\cdot[\mathrm{C}]}{\gamma_4\gamma_1 K_1 K_2 K_3}\right\}$$

Thus, the overall expression describing the molecular model, under the assumption mentioned above, is:

$$\frac{[\mathrm{Bound_{tot}}]}{[\mathrm{R_{tot}}]} = \frac{\frac{[\mathrm{T}]}{K_8}}{\left\{1 + \frac{[\mathrm{T}]}{K_8} + \frac{[\mathrm{A}]}{K_1} + \frac{[\mathrm{B}]}{K_2} + \frac{[\mathrm{C}]}{K_3} + \frac{[\mathrm{A}]\cdot[\mathrm{B}]}{\gamma_1 K_1 K_2} + \frac{[\mathrm{B}]\cdot[\mathrm{C}]}{\gamma_2 K_2 K_3} + \frac{[\mathrm{C}]\cdot[\mathrm{A}]}{\gamma_3 K_3 K_1} + \frac{[\mathrm{A}]\cdot[\mathrm{B}]\cdot[\mathrm{C}]}{\gamma_4\gamma_1 K_1 K_2 K_3}\right\}} \quad (3)$$

Applying Eq. (3) to our experiments, [T] is the concentration of [^{3}H]TBOB, which was 8 nM. K_8 is the dissociation constant of [^{3}H]TBOB, which was assumed to be 8 nM (Van Rijn et al., 1990). [A], [B] and [C] are the concentrations of the three compounds tested (i.e. GABA, retigabine and Org 20549). The parameters K_1, K_2, K_3, γ_1, γ_2, γ_3 and γ_4 were to be estimated. Eq. (3) was fitted to the data using nonlinear regression analysis for which the software program TableCurve 3D v3.12 was used (SYSTAT Software).

3. Results

3.1. Fits using the sigmoid-E_{max} equation

The right-hand side of the sigmoid-E_{max} Eq. (1) was fitted to the data. Data points and best fits are given in Fig. 2; parameter estimates with 95% confidence intervals (CI) are given in Table 1. All three test compounds fully displaced [^{3}H]TBOB with EC_{50}'s in the micromolar range. The EC_{50}'s of all test drugs decreased in the presence of any of the other test drugs. With the exception of the curve of retigabine in presence of 5 μM retigabine ($n = 1$), the Hill coefficients of the GABA curves and the retigabine curves were not significantly different from unity, whereas the Hill coefficients of the Org 20549 curves were less than unity.

3.2. Isobolic analysis

$EC_{35\%\ \text{binding left}}$ estimates and normalized total doses, with the 95% confidence intervals (CI), are given in Table 2. The results are visualized in the 3D isobologram in Fig. 3. The experimental determined points of combinations of the drugs were all below the plane defined by the three points of the pure drugs, i.e. below the theoretical additive values. The normalized total doses of all combinations were all less than unity. Thus, this isobolic analysis showed that, at 35% binding, all three drugs interact in synergy in displacing [^{3}H]TBOB.

Table 3
Parameter estimates of the four-ligand molecular model fitted to the experimental data

Drug		See Fig. 4	K_d (μM)	(95% CI)	γ	(95% CI)
GABA	K_1	(1)	2.04	(1.88,2.21)		
		(3)	1.82	(1.68,1.96)		
		Mean	1.93	(1.78,2.09)		
Retigabine	K_2	(1)	47.2	(41.9,52.3)		
		(2)	45.9	(42.1,49.8)		
		(4)	52.5	(46.1,58.8)		
		Mean	48.5	(43.4,53.7)		
Org 20549	K_3	(2)	11.5	(10.6,12.5)		
		(3)	11.6	(10.2,12.9)		
		(4)	10.8	(8.75,12.8)		
		Mean	11.3	(9.84,12.8)		
GABA + retigabine	γ_1	(1)			0.225	(0.160,0.289)
Retigabine + Org 20549	γ_2	(2)			0.196	(0.155,0.238)
Org 20549 + GABA	γ_3	(3)			0.264	(0.172,0.356)
Retigabine + Org 20549 in presence of GABA 1 μM	γ_4	(4)			0.110[a]	(0.047,0.174)

The allosteric hypercube model Eq. (3) was fitted to the experimental data. We performed four different procedures. In three fit procedures, we varied the concentrations of two drugs and fixed the concentration of the third drug at zero (Fig. 4). These fits yielded six K_d values, two for each compound, and three interaction terms, γ_1, γ_2 and γ_3. In a fourth fit procedure, we varied the concentrations of retigabine and Org 20459 and fixed the concentration of GABA at 1 μM (Fig. 4.4). This fit yielded one more K_d value for retigabine and Org 20549 and the interaction term γ_4. The fitted K_d values and the interaction terms, with the 95% confidence intervals (CI) of the fits, are given in the table. All the interaction terms γ_{1-4} were significantly less than unity. The interaction terms γ_1, γ_2 and γ_3 were mutually not significantly different, but γ_4, which quantifies the interaction between retigabine and Org 20549 in the *presence* of GABA, was lower than γ_2, which quantifies the interaction between retigabine and Org 20549 in the *absence* of GABA (γ_4 significantly different from lower than γ_2, indicated by [a]).

3.3. Fits using the molecular model

The right-hand side of Eq. (3) from the allosteric hypercube model was fitted to the experimental data. Data points and best fits are given in Fig. 4; parameter estimates with 95% confidence intervals (CI) are given in Table 3.

Since the software accepts only two independent variables at the same time, we performed four different procedures. In three fit procedures, we varied the concentrations of two drugs and fixed the concentration of the third drug at zero (Fig. 4.1–3). These fits yielded six K_d values, two for each compound, and three interaction terms, γ_1, γ_2 and γ_3. In a fourth fit procedure, we varied the concentrations of retigabine and Org 20459 and fixed the concentration of GABA at 1 μM (Fig. 4.4). This fit yielded one more K_d value for retigabine and Org 20549 and the interaction term γ_4. The fitted K_d values and the interaction terms, with 95% confidence intervals (CI), are given in the Table 3.

All the interaction terms γ_{1-4} were significantly less than unity ($t>9.7$, $df>31$, two-tailed t-test $p<0.0001$). The interaction terms γ_1, γ_2 and γ_3 were not significantly different, but γ_4, which quantifies the interaction between retigabine and Org 20549 in the *presence* of GABA, was lower than γ_2, which quantifies the interaction between retigabine and org 20549 in the *absence* of GABA ($t=2.24$, $df=88$, two-tailed t-test $p=0.028$).

Any compound enhances the affinity of any other compound by a factor $1/\gamma$, so two drugs enhance each other's affinity for binding to the $GABA_A$ receptor complex on average by a factor 4.4, and any two drugs together enhance the affinity of the third drug by a factor 9.1.

4. Discussion

In this study, we investigated the coupling between various binding sites of the $GABA_A$ receptor complex. We investigated combinations of three test compounds: (1) GABA, (2) Org 20549, a 3α5α type neuroactive steroid, and (3) retigabine, a new antiepileptic drug. [^{3}H]TBOB was used as a tracer ligand.

All three test drugs totally displaced [^{3}H]TBOB, and each of the drugs shifted the displacement curve of any other drug to the left. Isobolic analysis of these results showed the interaction between the three test drugs to be synergistic in displacing the tracer ligand.

Although the isobole method is of great practical value, it also has a number of serious drawbacks. It does not allow extrapolation from one dose combination to other dose combinations. Neither does the method allow extrapolation to other magnitudes of effect. Extrapolations would only be allowed if all concentration–effect curves have the same Hill coefficient, which was not the case in the experiments presented here (see Table 1).

But most importantly, the isobole method cannot provide insight into which mechanism underlies the observed interactions between drugs. To describe these interactions in mechanistic terms, mathematical models that describe physical, chemical or biological states or processes are needed (Berenbaum, 1989; Greco, 1995; Motulski and Christopoulos, 2003). For this purpose, we propose a molecular model, in which four binding sites are coupled allosterically. This model can be visualized as a hypercube, depicted in Fig. 1.

Under the assumption that the interaction of each of the test drugs with [^{3}H]TBOB is quasi-competitive, the model is described mathematically by Eq. (3). This assumption was made in order to decrease the number of parameters to be estimated. We think this assumption is justified since each of the test compounds inhibits the binding of [^{3}H]TBOB completely. Our simulations show that two allosteric ligands will only displace each other almost completely if they decrease each other's affinity by a factor of at least 100, which implies that the interaction is quasi-competitive.

The proposed four-ligand model can describe the data adequately, as shown by the average r^2 of 0.97. The model quantifies the strength of the coupling between the various sites: two test compounds mutually enhance each other's affinity by a factor 4.4, and when the three ligands are present simultaneously, then the mutual enhancement of the affinities is by a factor 9.

The results of K_d values and the interaction terms between GABA and any of the other drugs are in accordance with our earlier papers (Van Rijn and Willems-van Bree, 2003; Van Rijn et al., 1999), but the absolute values do show some differences due to the presence of different concentrations of endogenous GABA. Therefore, we only compare absolute values within one tissue batch.

In the present experiment and in the one described in Van Rijn et al. (1999), we observe a Hill coefficient less than unity for Org 20459. The four-ligand hypercube model cannot account for Hill coefficients different from unity. Earlier (Van Rijn et al., 1999), we reported Hill coefficients greater than unity for thiopental and for propofol. To describe these high Hill coefficients, we constructed an extended three-ligand model (Van Rijn et al., 1999), in which a positively coupled double binding site for the ligands yielded a Hill coefficient greater than unity. In the present experiment, only the Hill coefficient of one retigabine curve (curve no. 5) was greater than unity. Since this was not the case for the other retigabine curves, and it was the only experiment conducted only once (in triplicate), it is likely to be a type 1 error, so we ignored this high Hill coefficient. However, the curves of the neurosteroid Org 20549 consistently show a Hill coefficient less than unity (Table 1; Van Rijn et al., 1999). To account for a low Hill coefficient, two independent binding sites must be hypothesized. These two independent binding sites might both be located on the same receptor complex or they might be the result of two different receptor populations. It has been reported that two independent binding sites for neuroactive steroids are present on the same $GABA_A$ receptor complex (Park-Chung et al., 1999; Pericic et al., 1998; Sousa and

Ticku, 1997). The proposed sites, however, bind neuroactive steroids with different chemical structures: one site for the 3α5α type like Org 20549 and one site for sulphated steroids (Park-Chung et al., 1999; Pericic et al., 1998; Sousa and Ticku, 1997). It is not known whether the site for sulphated steroids has any affinity for Org 20549. Nevertheless, it is not likely that two independent sites located on the same receptor complex can explain the low Hill coefficient since our simulations, according to the proposed hypercube model, show Hill coefficients of unity, with A being the GABA site, B being a steroid site with positive interaction with the GABA site (i.e. a gamma value below unity) and C being a second steroid site without an interaction with either the GABA site or the first steroid site B (i.e. gamma values of unity). The possibility of two receptor populations, such that each population can be described by the proposed four-site molecular model, but with different affinities for the neurosteroid, would be the most economical explanation for the low Hill coefficient observed for the neurosteroid. The existence of subpopulations of $GABA_A$ receptor complexes is commonly accepted (Mehta and Ticku, 1999). Moreover, it has been reported that such subpopulations do have different affinities for 3α5α types of neurosteroids (Belelli et al., 2002; Maksay et al., 2000). Our simulations show that two populations of equal size with an affinity difference by a factor of only 20 yield a Hill coefficient of 0.7. Therefore, we hypothesize that the experimental data of Org 20549 can best be described by assuming at least two receptor populations with different affinities for neurosteroids. The four-ligand hypercube model can describe each population.

The binding of tracer (e.g. [^{3}H]TBOB) to the picrotoxin-sensitive convulsant site can be seen as a physiologically relevant assay since the binding of the tracer correlates negatively with Cl^- ion flux (Hawkinson et al., 1994; Havoundjian et al., 1986; Korpi et al., 2002; Maksay, 1996; Im and Blakeman, 1991). Therefore, our findings suggest that the affinity enhancements in combinations of positive GABAergic drugs might translate into clinical effects. In vivo, a melange of endogenous and exogenous ligands modulates the function of Cl^- ion channels simultaneously and interrelatedly. The concentrations of the endogenous compounds are subject to individual variations (e.g. shown for the neurosteroids; Belelli et al., 2002; Genazzani et al., 1998; Joëls, 1997). It has even been suggested that endogenous neurosteroids actually regulate the potency of other GABAergic compounds (Pinna et al., 2000). The modelling of the allosteric couplings might help to understand the magnitudes of differences of individual responses to added drugs. The proposed molecular model quantifies the allosterical interactions between varieties of GABAergic compounds. This quantification is important because it is through these mechanistic models that predictions of the effects of drug combination might become possible. We show that the affinity enhancements induced by simultaneous binding of three drugs are greater than the enhancements induced by the binding of only two drugs. This observation would support polytherapy with a rich mixture of ligands binding to the same receptor complex.

We hope that the presented results will contribute to the identification of antiepileptic drug combinations with potential therapeutic benefit for patients with refractory epilepsy.

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