

RESEARCH PAPER

Binding site location on GABA_A receptors determines whether mixtures of intravenous general anaesthetics interact synergistically or additively in vivo

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Background and Purpose: General anaesthetics can act on synaptic GABA_A receptors by binding to one of three classes of general anaesthetic sites. Canonical drugs that bind selectively to only one class of site are etomidate, alphaxalone, and the mephobarbital derivative, R-mTFD-MPAB. We tested the hypothesis that the general anaesthetic potencies of mixtures of such site-selective agents binding to the same or to different sites would combine additively or synergistically respectively.

Experimental Approach: The potency of general anaesthetics individually or in combinations to cause loss of righting reflexes in tadpoles was determined, and the results were analysed using isobolographic methods.

Key Results: The potencies of combinations of two or three site-selective anaesthetics that all acted on a single class of site were strictly additive, regardless of which single site was involved. Combinations of two or three site-selective anaesthetics that all bound selectively to different sites always interacted synergistically. The strength of the synergy increased with the number of separate sites involved such that the percentage of each agent's EC₅₀ required to cause anaesthesia was just 35% and 14% for two or three sites respectively. Propofol, which binds non-selectively to the etomidate and R-mTFD-MPAB sites, interacted synergistically with each of these agents.

Conclusions and Implications: The established pharmacology of the three anaesthetic binding sites on synaptic GABA_A receptors was sufficient to predict whether a mixture of anaesthetics interacted additively or synergistically to cause loss of righting reflexes in vivo. The principles established here have implications for clinical practice.

1 | INTRODUCTION

Although early work linked the action of general anaesthetics with the GABA_A receptors (Leeb-Lundberg, Snowman, & Olsen, 1980; Nicoll,

Eccles, Oshima, & Rubia, 1975), the definitive study was based on the N265M mutation in the second transmembrane helix of the β₃ subunit. This mutation both attenuated the action of **etomidate** on heterologously expressed GABA_A receptors in vitro (Belelli, Lambert, Peters, Wafford, & Whiting, 1997) and attenuated etomidate's anaesthetic potency in the β₃ N265M knock-in mouse (Jurd et al., 2003). However, **alphaxalone** and the mephobarbital derivative, R-mTFD-MPAB, had the same general anaesthetic potency in wild-type and the N265M knock-in mouse (Amlong, Perkins, Houle, Miller, & Pearce,

Abbreviations: 3α5βP, 5β-pregnan-3α-ol-20-one (pregnanolone); ECD, extracellular domain; LoRR, loss of righting reflexes; R-mCF₃-MPAB, 5-allyl-1-methyl-5-(3-trifluoromethylphenyl)-pyrimidine-2,4,6-trione; R-miPr-MPAB, 5-allyl-5-(3-isopropylphenyl)-1-methyl-pyrimidine-2,4,6-trione; R-mTFD-MPAB, allyl m-trifluoromethyl-diazirine mephobarbital; THDOC, tetrahydrodeoxycorticosterone; TMD, transmembrane domain

2016), but the N265M mutation did not ablate their ability to enhance GABA currents at anaesthetic concentrations in vitro (Siegwart, Krahenbuhl, Lambert, & Rudolph, 2003; Szabo, Nourmahnad, Halpin, & Forman, 2019), suggesting, but not proving, that their in vivo actions are mediated by GABA_A receptors.

GABA_A receptors belong to the superfamily of pentameric Cys-loop ligand-gated ion channels. Synaptic GABA_A receptors have the stoichiometry ($\alpha_2\beta_2\gamma$) arranged as shown in Figure 1a. There is a high degree of homology between subunits, each being composed of a ~200 residue extracellular domain (ECD) containing the GABA sites, a transmembrane domain (TMD) consisting of a bundle of four transmembrane helices, and an unstructured intracellular domain between the third and fourth transmembrane helices (Figure 1b; Laverty et al., 2019). Photolabelling, site-directed cysteine accessibility mutagenesis experiments (reviewed in Forman & Miller, 2016) and more recently structural studies (Chen et al., 2018; Laverty et al., 2017; Miller et al., 2017) have identified three distinct general anaesthetic binding sites located at subunit interfaces in the TMD (Figure 1c). Each distinct site consists of two homologous binding pockets. In the extracellular third of the TMD, etomidate binds at the two β^+/α^- interfaces (Site 1), whereas R-mTFD-MPAB binds at the γ^+/β^- and the α^+/β^- interfaces (Site 2; Chiara et al., 2013). Neurosteroids also bind at the two β^+/α^- interfaces but at a separate sites in the intracellular third of the TMD (Site 3; Figure 1a,b; Hosie, Wilkins, da Silva, & Smart, 2006; Ziemba et al., 2018).

Site-selective agents all stabilize the open state of the GABA_A receptor because their binding sites have higher affinity for that state

What is already known

- Mixtures of general anaesthetics are known to sometimes interact additively and other times synergistically.
- There are three distinct transmembrane-domain intra-subunit binding sites for general anaesthetics on synaptic GABA_A receptors.

What this study adds

- Combinations of anaesthetics that act on just one of the three known sites interact additively.
- Combinations of anaesthetics that each act on a different site interact synergistically.

What is the clinical significance

- Combining agents at 15% of their EC₅₀s that selectively target three different sites causes anaesthesia.
- Such a strategy may be generalizable to other ligand-gated ion channels and other multi-subunit targets.

than for the resting state (reviewed in Forman & Miller, 2011). Consequently, binding to one site will allosterically enhance the affinity of the other sites, leading to the hypothesis that combinations of general anaesthetics should act synergistically if they bind selectively to distinct sites. Pioneering studies have shown that steroids interact

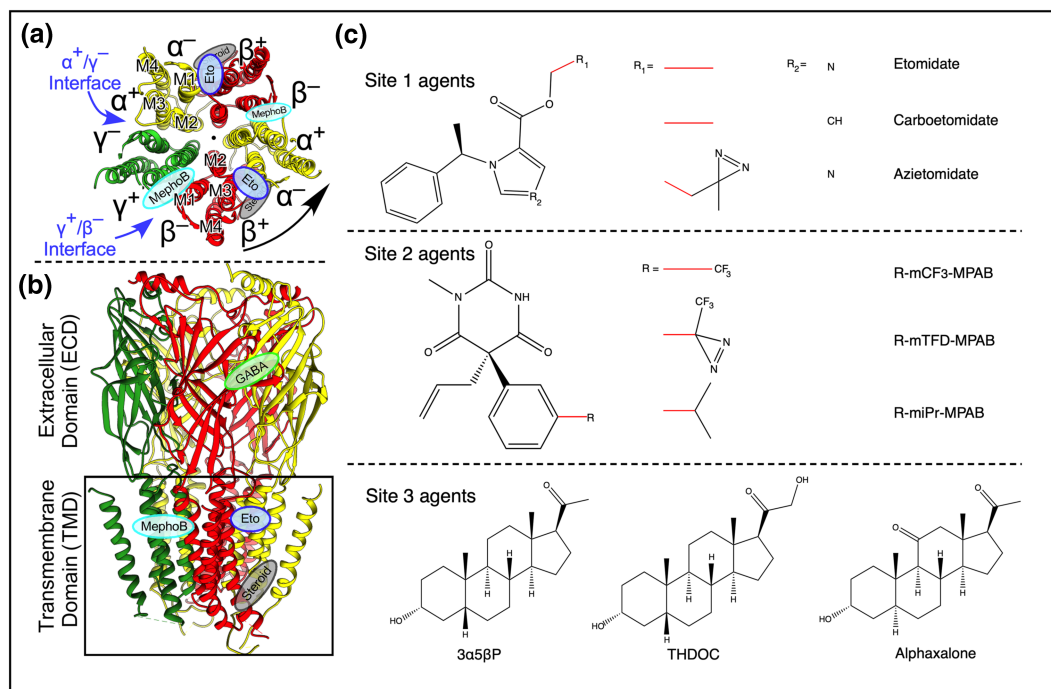


FIGURE 1 The structure, binding sites, and agonists of the $\alpha_1\beta_3\gamma_2$ GABA_A receptor. (a) Cross section through the TMD viewed from the extracellular side showing previously established drug binding sites and the arrangement of the five subunits around a central pore. (b) Side view; the black box indicates the plasma membrane. Lozenges indicate the binding sites for GABA (green), etomidate (blue), mephobarbital derivatives (cyan), and steroids (grey). The protein backbone is in ribbon notation (Forman & Miller, 2016; Laverty et al., 2019). (c) The chemical structures of agents used in this study

allosterically with etomidate and propofol both in vivo and in vitro (Cao et al., 2018; Chen et al., 2014; Drexler, Balk, & Antkowiak, 2016). Here, using three sets of general anaesthetics that bind with high selectivity to one of the three sites shown in Figure 1c, we find that the potencies of combinations of site-selective anaesthetics that bind to the same site are additive, whereas those that bind to two or three distinct sites are increasingly synergistic.

2 | METHODS

2.1 | Tadpole behavioural assay

Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, Altman, & Group, 2010) and with the recommendations made by the *British Journal of Pharmacology*. All animal studies were conducted according to animal protocols preapproved by the Massachusetts General Hospital (MGH) Subcommittee on Research Animal Care (Protocols #2006N000124 and 2015N000012) with the approval of the MGH Institutional Animal Care and Use Committee.

Early pre-limb bud stage *Xenopus laevis* tadpoles (XEP Cat#: Xla100, RRID:XEP_Xla100, approximately 2–2.5 cm in length) were purchased from Xenopus One (Ann Arbor, MI) and were housed in the MGH Center for Comparative Medicine Facilities. Fifty tadpoles were housed in 4-L tanks with circulating dechlorinated water under a 12-hr light/dark cycle. They were allowed 24 hr after arrival before experiments were conducted. Loss of righting reflexes (LoRR) assays were conducted in 100 ml of Tris buffered water (2.5 mM, pH = 7.4) in vessels containing five tadpoles. LoRR was assessed by inverting a tadpole with a bent Pasteur pipette. Tadpoles that then righted themselves within 5 s were scored 0, whereas those that failed to do so were scored 1. LoRR was assessed after 10, 30, and 60 min. Responses stabilized after 30 min. Values recorded after 1 hr of exposure were used in the analysis. After 1 hr, tadpoles were transferred to recovery beakers filled with 200 ml of dechlorinated tap water and were assessed 24 hr later to ensure recovery from anaesthesia. Once recovered, tadpoles were killed with 5% tricaine or a lethal concentration of pentobarbital. Tadpoles that did not recover (<0.01% of animals tested) were excluded from the analysis. All data were obtained by the first author and were not blinded.

Because the tadpole extracellular fluid readily equilibrates with the anaesthetic concentration in which they swim, the data obtained represent concentration–response rather than dose–response relationships. Data were fitted to a logistic curve to yield the EC_{50} (Waud, 1972). The average slope of site-selective anaesthetics (3.3) was used in fitting all experimental concentration–response curves.

2.2 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). Statistical analysis was performed

using independent values. For control experiments with one drug alone, a varying number of tadpoles was required to accurately determine each compound's EC_{50} . This was necessary because the EC_{50} s were often unknown, so choosing concentrations that gave sufficient points on the steep part of the curve was by trial and error. In no case were less than five animals used at a given concentration. For combination experiments, one drug's concentration was held constant in solution while the other's concentration was varied to determine its new EC_{50} . Forty tadpoles were normally sufficient to define each concentration–response curve, except when propofol was fixed at 0.158 and 0.315 μ M and etomidate was varied ($n = 50$ and 55, respectively) because additional concentrations were needed for the reasons stated above. These results were analysed using isobolographic analysis.

An isobologram plots the mixtures of drugs eliciting the desired response (EC_{50} , in our case). For simplicity, we normalized the concentrations of each drug relative to the EC_{50} , creating fractional values rather than concentrations. The sum of the coordinates for each point gives the total effective fractional sum of drug, which has been characterized by Tallarida as the interaction index, or simply α , according to Equation (1). The line for additivity spans across the graph ($\alpha = 1$). Points below this line ($\alpha < 1$) indicate synergy, and points above this line ($\alpha > 1$) indicate antagonism (Tallarida, 2002; Tallarida, 2016).

$$\frac{[\text{Drug A}]}{EC_{50A}} + \frac{[\text{Drug B}]}{EC_{50B}} = \alpha. \quad (1)$$

The determined α values for groups of data were averaged together, and the SDs were calculated based on Equation (2) (σ_{total} is the combined SD, σ_i = the SD from an α value).

$$\sigma_{\text{total}} = \sqrt{\sum_{i=1}^n \sigma_i^2}. \quad (2)$$

The GraphPad one sample t test and unpaired t test were used to compare average α values of experiments to the expected value of one or to one another respectively (RRID:SCR_000306). The one-way ANOVA was used to compare groups of three sets of experiments with one another (RRID:SCR_016762). For all analyses, significance was set at 1% ($P < .01$).

2.3 | Materials

R-miPr-MPAB and R-mCF₃-MPAB were synthesized by methods similar to R-mTFD-MPAB as previously described (Savechenkov et al., 2012). See Supporting Information for details on the full synthesis. The chemicals 3 α 5 β P, THDOC, propofol, methanol, DMSO, and Tris were bought from Sigma-Aldrich (St. Louis, MO). Alphaxalone was bought from Tocris Bioscience (Bristol, UK). Etomidate was purchased from Bachem (Bubendorf, Switzerland).

Sites 1 and 2 are subtly different homologous sites, causing some overlap in binding selectivity. The anaesthetics used in this study had at least a 20-fold binding selectivity for their respective site and are considered site-selective. The etomidate derivatives are site-selective for Site 1 while the mephobarbital derivatives are site-selective for Site 2 (Chiara et al., 2013; Cohen, J. 2018, unpublished data).

Neurosteroids have only been shown to act on Site 3, due to their unique structure, and are considered site-selective.

2.5-mM Tris buffer was used for all assays and was made from a 1-M Tris stock. Stocks of 16 L of dechlorinated tap water (AmQuel Plus; from Kordon, Hayward, CA) were used to fill the recovery beakers for the tadpoles post anaesthesia. Stock solutions of anaesthetics were dissolved in either DMSO or methanol. The final DMSO concentration did not exceed 0.04%. Control experiments showed that 0.2% DMSO caused no LoRR (0/10 animals). For drug stocks in methanol, the methanol was evaporated in glass containers using argon gas before buffer was added. All stocks of photo-sensitive drugs were stored in amber glassware and covered in aluminium foil while in use.

2.4 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common

portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Fabbro et al., 2017; Alexander, Peters et al., 2017).

3 | RESULTS

3.1 | Potencies of individual anaesthetics

The concentration dependence of the LoRR was determined for the 10 anaesthetics in Table 1 as described in Section 2. For example, in the case of alphaxalone, groups of tadpoles were equilibrated in one of seven concentrations of alphaxalone and the fraction of tadpoles experiencing LoRR is shown in Figure 2a. The EC_{50} s of individual anaesthetics are reported in Table 1 and are consistent with the previously published literature (alphaxalone: 1.12 ± 0.14 , Stastna et al., 2011;

TABLE 1 Tadpole LoRR EC_{50} s of individual anaesthetics

Drug	Site	EC_{50} (μ M)	n^a
Etomidate	1	2.22 ± 0.18	95
Azietomidate		1.22 ± 0.11	50
Carboetomidate		5.43 ± 0.59	50
R-mTFD-MPAB	2	2.34 ± 0.14	130
R-mCF ₃ -MPAB		7.33 ± 0.81	55
R-miPr-MPAB		1.14 ± 0.20	40
Propofol	1 and 2	0.63 ± 0.09	85
Alphaxalone	3	1.42 ± 0.13	55
3 α 5 β P		0.31 ± 0.03	50
THDOC		2.50 ± 0.16	85

^an is the number of tadpoles used to determine the concentration–response curve.

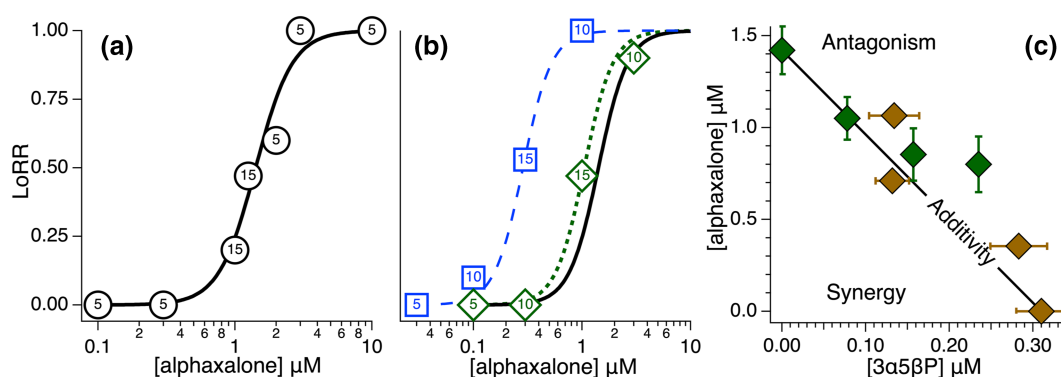


FIGURE 2 The EC_{50} of alphaxalone (Site 3) is shifted differently by the presence of 25% of the EC_{50} of either 3 α 5 β P (Site 3) or etomidate (Site 1). In (a), the loss of righting reflexes (LoRR) is plotted against the free concentration of alphaxalone in the aqueous phase surrounding the tadpoles. The fraction of animals experiencing LoRR is represented by the vertical placement of the circles, with the number of animals tested displayed within them (total $n = 55$). The quantal data was fit as described in Methods and the EC_{50} is displayed in Table 1. (b): Alphaxalone concentration response curves (C.R.C.) are shown in the presence of 25% of either 3 α 5 β P's or etomidate's EC_{50} . (c): The concentrations of mixtures of alphaxalone and 3 α 5 β P that together produce an EC_{50} response are plotted. The concentration of either alphaxalone or 3 α 5 β P was varied while the concentration of the other anaesthetic was fixed. In these and succeeding figures, the thick black line represents the theoretical line for additivity where $\alpha = 1$ (Equation 1; Tallarida, 2016). The error bars shown are the SDs of the EC_{50} s.

etomidate: 2.3 μM [95% confidence limits, 1.9–2.7 μM], Belelli et al., 2003; azietomidate: 2.2 \pm 0.14 μM , Husain et al., 2003; R-mTFD-MPAB: 3.7 \pm 0.25 μM , Savechenkov et al., 2012; propofol: 0.52 \pm 0.08 μM , Germann et al., 2016; carboetomidate: 5.4 \pm 0.5 μM , Cotten et al., 2010; and 3 α 5 β P: 63 \pm 3 nM, Chen et al., 2014). Additionally, the primary binding site of these anaesthetics are listed in Table 1.

25% of either 3 α 5 β P's (Site 3) or etomidate's (Site 1) EC₅₀ was held fixed in solution and alphaxalone's new concentration–response curve was determined and is shown in Figure 2b. The concentration–response curves both shifted left, but the alphaxalone + etomidate mixture shifted

much more so, indicating synergy. The α values for alphaxalone + 3 α 5 β P and alphaxalone + etomidate are reported in Tables 2 and 3, respectively. The principles established here will guide our comparison of synergy and additivity among different anaesthetic mixtures.

3.2 | Two site-selective anaesthetics acting on the same site interact additively

The Site 3 anaesthetics, alphaxalone and 3 α 5 β P, were tested in six different combinations. In three cases, alphaxalone was fixed in solution

TABLE 2 Fixed concentrations, EC₅₀s, and α s of all same-site anaesthetic mixtures

	Drug A	[Drug A] μM	Drug B	[Drug B] μM	Drug C	[Drug C] μM	α	Average α
Site 1	Etomidate	0.60	Azietomidate	0.77 \pm 0.12	–	–	0.90 \pm 0.10	1.01 \pm 0.14
		1.00		0.86 \pm 0.11	–	–	1.15 \pm 0.09	
		1.78		0.34 \pm 0.07	–	–	1.08 \pm 0.06	
		1.46 \pm 0.16		0.305	–	–	0.91 \pm 0.07	
		1.05 \pm 0.12		0.53	–	–	0.90 \pm 0.05	
		0.42 \pm 0.09		1.20	–	–	1.12 \pm 0.04	
		0.111		1.04 \pm 0.15	Carboetomidate	0.27	0.95 \pm 0.12	
	0.222	0.96 \pm 0.54	0.54	0.98 \pm 0.12				
	0.555	0.34 \pm 0.06	1.36	0.78 \pm 0.05				
	0.111	0.06	4.22 \pm 0.55	0.88 \pm 0.10				
	0.222	0.12	3.51 \pm 0.49	0.85 \pm 0.09				
	0.555	0.305	1.44 \pm 0.39	0.77 \pm 0.07				
	1.93 \pm 0.26	0.06	0.27	0.97 \pm 0.12				
	1.67 \pm 0.22	0.12	0.54	0.95 \pm 0.10				
	1.00 \pm 0.13	0.305	1.36	0.95 \pm 0.06				
	Site 2	R-mTFD-MPAB	0.585	R-mCF ₃ -MPAB	6.93 \pm 0.85	–	–	1.20 \pm 0.12
1.17			2.75 \pm 0.39		–	–	0.88 \pm 0.05	
1.755			1.12 \pm 0.21		–	–	0.90 \pm 0.03	
1.78 \pm 0.20			1.82		–	–	1.01 \pm 0.09	
1.23 \pm 0.12			3.64		–	–	1.03 \pm 0.05	
1.20 \pm 0.12			5.46		–	–	1.26 \pm 0.05	
R-miPr-MPAB		0.12	4.42 \pm 0.67	0.06	0.70 \pm 0.09			
		0.234	3.39 \pm 0.54	0.11	0.66 \pm 0.07			
		0.585	3.07 \pm 0.48	0.29	0.92 \pm 0.07			
		0.12	0.37	0.97 \pm 0.15	0.95 \pm 0.13			
		0.23	0.73	0.83 \pm 0.15	0.93 \pm 0.13			
		0.585	1.83	0.30 \pm 0.04	0.77 \pm 0.04			
		1.09 \pm 0.17	0.37	0.06	0.56 \pm 0.07			
		1.06 \pm 0.13	0.73	0.11	0.65 \pm 0.07			
		0.82 \pm 0.17	1.83	0.285	0.85 \pm 0.07			
		Site 3	Alphaxalone	0.355	3 α 5 β P	0.28 \pm 0.03	–	–
0.71	0.13 \pm 0.02			–		–	0.93 \pm 0.06	
1.065	0.13 \pm 0.03			–		–	1.18 \pm 0.10	
1.05 \pm 0.12	0.078			–		–	0.99 \pm 0.08	
0.85 \pm 0.14	0.157			–		–	1.10 \pm 0.10	
0.80 \pm 0.15	0.235			–		–	1.31 \pm 0.11	
0.07	0.26 \pm 0.04			THDOC		0.125	0.94 \pm 0.13	
0.14	0.20 \pm 0.03		0.25		0.85 \pm 0.10			
0.355	0.13 \pm 0.02		0.625		0.92 \pm 0.06			
0.07	0.016		1.84 \pm 0.35		0.83 \pm 0.14			
0.14	0.031		1.34 \pm 0.20		0.74 \pm 0.08			
0.355	0.078		0.93 \pm 0.13		0.87 \pm 0.05			
0.86 \pm 0.11	0.016		0.125		0.71 \pm 0.08			
0.95 \pm 0.11	0.031		0.25		0.87 \pm 0.08			
0.45 \pm 0.08	0.078		0.625		0.82 \pm 0.05			

Note. α values are reported as \pm SD. SD of average α s are calculated according to Equation (2). Each concentration–response curve had 40 animals.

^aDiffers significantly ($P < .01$) from the value of one expected for additivity.

TABLE 3 Fixed concentrations, EC₅₀s, and α s for all combinations of Site 1, 2, and 3 anaesthetics

Site 1	Site 2	Site 3	α	Average α
[Etomidate] μ M	[R-mTFD-MPAB] μ M	[Alphaxalone] μ M		
0.555	–	0.29 \pm 0.03	0.45 \pm 0.02	
1.11	–	0.10 \pm 0.02	0.57 \pm 0.01	
1.665	–	0.03 \pm 0.01	0.77 \pm 0.00	
0.74 \pm .08	–	0.355	0.58 \pm 0.04	
0.26 \pm 0.03	–	0.71	0.62 \pm 0.01	
0.31 \pm .04	–	1.065	0.89 \pm 0.02	0.65 \pm 0.16^a
0.22	1.33 \pm 0.20	–	0.67 \pm 0.08	
0.55	1.16 \pm 0.22	–	0.75 \pm 0.09	
1.2	0.25 \pm 0.05	–	0.65 \pm 0.02	
1.32 \pm 0.17	0.25	–	0.70 \pm 0.08	
0.76 \pm 0.10	0.625	–	0.61 \pm 0.04	
0.23 \pm 0.04	1	–	0.53 \pm 0.02	0.69 \pm 0.09^a
–	0.585	0.79 \pm 0.10	0.80 \pm 0.07	
–	1.17	0.31 \pm 0.05	0.72 \pm 0.03	
–	1.755	0.21 \pm 0.03	0.90 \pm 0.02	
–	1.35 \pm 0.15	0.355	0.83 \pm 0.06	
–	0.31 \pm 0.56	0.71	0.63 \pm 0.04	
–	0.09 \pm 0.01	1.065	0.79 \pm 0.01	0.78 \pm 0.10^a
0.111	0.30 \pm 0.03	0.07	0.23 \pm 0.01	
0.222	0.12 \pm 0.02	0.14	0.25 \pm 0.10	
0.555	0.01 \pm 0.01	0.355	0.51 \pm 0.01	
0.111	0.12	0.26 \pm 0.01	0.28 \pm 0.02	
0.222	0.23	0.34 \pm 0.01	0.44 \pm 0.03	
0.555	0.585	0.03 \pm 0.01	0.52 \pm 0.01	
0.43 \pm 0.06	0.12	0.07	0.29 \pm 0.03	
0.96 \pm 0.10	0.23	0.14	0.63 \pm 0.04	
0.29 \pm 0.04	0.585	0.355	0.63 \pm 0.02	0.42 \pm 0.16^a

Note. α values are reported as \pm SD. SD of average α s are calculated according to Equation (2). Each concentration–response curve had 40 animals.

^aDiffers significantly ($P < .01$) from the value of one expected for additivity.

at different concentrations while 3 α 5 β P was varied, while in the remainder, the reverse procedure was followed. The fixed concentrations, EC₅₀s determined, and α values of these experiments, as well as all same site mixture experiments in this study, are all reported in Table 2, and the isobologram analysis is given in Figure 2c. None differed significantly from the expected additive value of one.

For simplicity, we expressed the concentration of each drug as a fraction of its own EC₅₀ and plotted the data according to Equation (1) in Figure 3c. In this method, the α values are simply the sum of the coordinates of each point. Of the six points tested, all are in close proximity to the line of additivity, with two below and four above it. The α values ranged from 0.93 to 1.31, as shown in Table 2.

The Site 1 anaesthetics, etomidate and azi-etomidate, were tested in the same manner as above, and the isobologram is displayed in Figure 3a. Of the six points tested, all are in close proximity to the line of additivity, with the α values ranging from 0.90 to 1.15.

The Site 2 anaesthetics, R-mTFD-MPAB and R-mCF₃-MPAB, were tested in the same manner and the isobologram is displayed in Figure 3b. Of the six points tested, all are in close proximity to the line of additivity, with the α values ranging from 0.88 to 1.26. In addition, ANOVA was conducted between these three sets of same-site mixtures, showing no significant differences.

3.3 | Two site-selective anaesthetics acting on different sites interact synergistically

Etomidate and alphaxalone, Sites 1 and 3 agents respectively, were tested in combination with one another in the same manner as described above. The fixed concentrations, EC₅₀s, and α values of these experiments, as well as all experiments of combinations of anaesthetics acting on different sites, are displayed in Table 3, and the isobologram analysis is shown in Figure 4a. All α values for sets of two site-selective

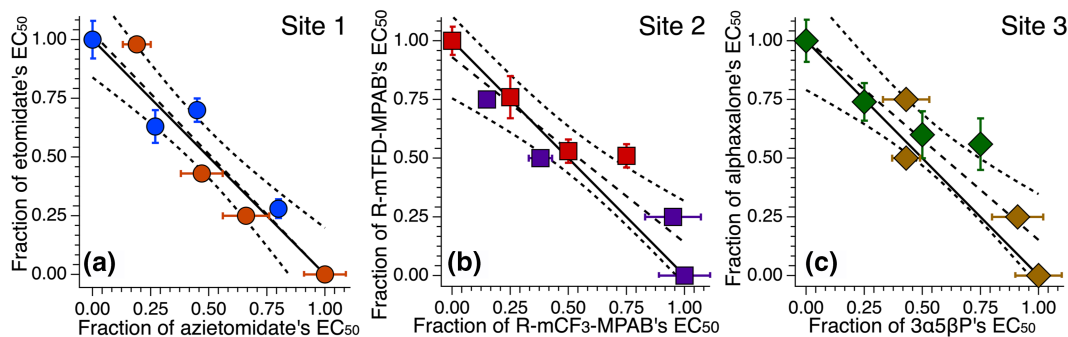


FIGURE 3 Isobolograms of mixtures of two anaesthetics that both act on the same site show additivity. The fractional concentrations of the drug mixtures required to produce 50% loss of righting reflexes (LoRR) are plotted with the theoretical line for additivity. Each point represents the concentrations of the two drugs, one of which was held constant and the other varied to determine the concentration-response curve and EC_{50} . L. R. is the linear least squares fit of the data and C.I. is its confidence interval. Shown are combinations of (a): etomidate (Eto) and azietomidate (Azi), both Site 1 anaesthetics; (b): R-mTFD-MPAB (MPAB) and R-mCF₃-MPAB (CF₃), both Site 2 anaesthetics, and (c): alphaxalone (Alphax) and 3 α 5 β P, both Site 3 anaesthetics.

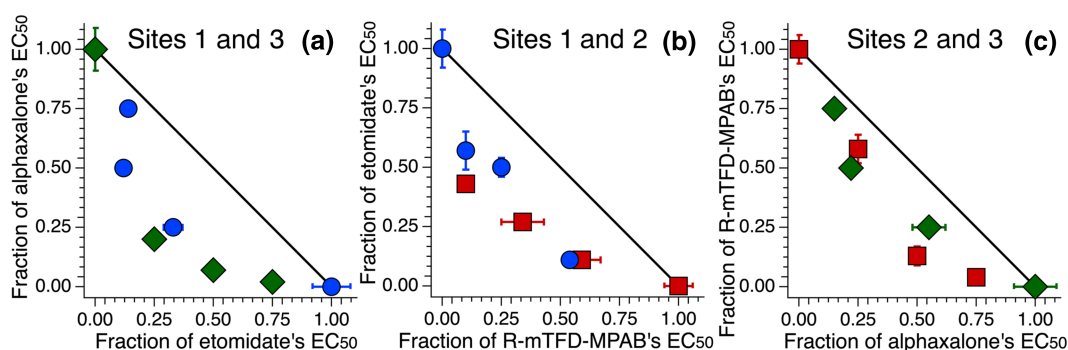


FIGURE 4 Isobolograms of mixtures of two anaesthetics acting on different sites show synergy. The fractional concentrations of the drugs required to produce 50% LoRR are plotted as in Figure 3. Shown are combinations of (a): etomidate (Eto) and alphaxalone (Alphax), Site 1 and Site 3 anaesthetics, respectively; (b): etomidate and R-mTFD-MPAB (MPAB), Site 1 and Site 2 anaesthetics, respectively, and (c): R-mTFD-MPAB and alphaxalone, Site 2 and Site 3 anaesthetics, respectively.

anaesthetics were significantly lower than the value of one expected for additivity. Of the six points tested, all lay well below the line of additivity, with the α values ranging from 0.45 to 0.89.

Similarly, etomidate was then tested with the Site 2 agent R-mTFD-MPAB, and the data are shown in Figure 4b. Of the six points tested, all lay well below the line of additivity, with the α values ranging from 0.53 to 0.75. Lastly, R-mTFD-MPAB was tested with alphaxalone, and the data are shown in Figure 4c. Of the six points tested, all lay well below the line of additivity, with the α values ranging from 0.63 to 0.90. In addition, ANOVA was conducted between these three sets of different-site mixtures, showing no significant difference.

3.4 | Three site-selective anaesthetics acting on the same site interact nearly additively

Three Site 1 anaesthetics, etomidate, azietomidate, and carboetomidate, were tested in three-way combination with one another by fixing the concentration of two drugs in solution and varying the third to determine the EC_{50} shift. The data from the nine mixtures tested, as well as all the other three-way same-site combinations,

are shown in Table 2. Like the isobolograms of two-way combinations, an isobologram was constructed with the fractional concentration of each drug on each axis, and the data are displayed as blue discs in the 3D isobologram in Figure 5a. Of the nine points tested, all lay below the surface of additivity with α values ranging from 0.77 to 0.98 but did not differ significantly from the expected value of one.

The three Site 2 anaesthetics, R-mTFD-MPAB, R-mCF₃-MPAB, and R-miPr-MPAB, were tested in the same manner as above, and the data are displayed in the same figure, but as red cubes. Of the nine points tested, all lay below the surface of additivity with the α values ranging from 0.56 to 0.95 and differed significantly from the expected value of one.

The three Site 3 anaesthetics, alphaxalone, 3 α 5 β P, and THDOC, were tested, and the data are also displayed in the same figure, but as green spheres. Of the nine points tested, all lay below the surface of additivity with the α values ranging from 0.71 to 0.94, which differed significantly from the expected value of one.

Taken together, these data suggest in each of the three sets above that there is a small degree of synergy that reached the threshold of significance in two of the three sets. ANOVA showed that the α values

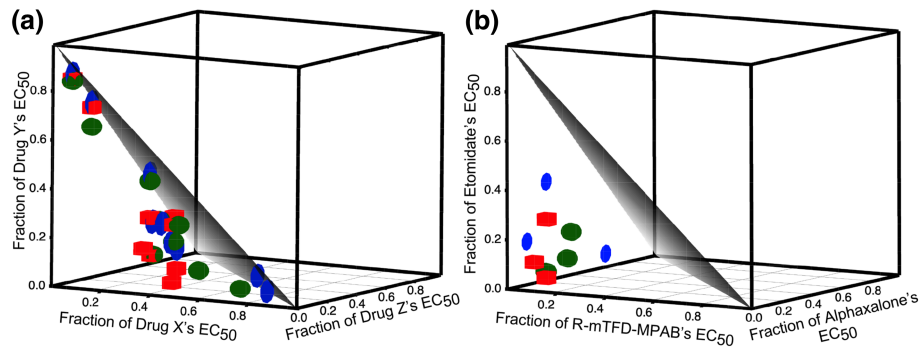


FIGURE 5 Three dimensional isobolograms of three-way mixtures of site-selective anaesthetics that either (a) act on a single site or (b) act on different sites show additivity and synergy, respectively. The triangular grey surface is the result expected for additivity (Surface of add.). (a) The drugs corresponding to the x, y, and z axes are: for the Site 1 agents, azietomidate, etomidate, and carboetomidate; for the Site 2 agents, R-mTFD-MPAB, R-miPr-MPAB, and R-mCF₃-MPAB and for the Site 3 agents, THDOC, 3 α 5 β P, and alphaxalone. (b) Data shown are from experiments where etomidate was varied and the other two (alphaxalone and R-mTFD-MPAB) were held constant, where R-mTFD-MPAB was varied while the other two anaesthetics were held constant and where alphaxalone was varied and R-mTFD-MPAB and etomidate were held constant.

of these three sets of agents did not differ from each other. The average α of the three sets of three-way same-site anaesthetics was 0.84 ± 0.15 ($n = 27$), which was significantly different from the value of one expected for additivity. However, this small degree of synergy was significantly less than that experienced when two agents acted on different sites ($\alpha = 0.69 \pm 0.13$; $n = 18$).

3.5 | Three site-selective anaesthetics acting at three different sites interact super synergistically

The anaesthetics etomidate, R-mTFD-MPAB, and alphaxalone, each site-specific anaesthetics acting on Sites 1, 2, and 3 respectively, were tested in three-way combinations in the same manner as described

above, and the results are shown in Table 2 and Figure 5b. Of the nine points tested, all are well below the surface of additivity, with the α values ranging from 0.23 to 0.63. The mean and SD of α for this data set ($n = 9$) was significantly less than that for the three sets of three-way same-site anaesthetics above ($n = 27$).

3.6 | Propofol is synergistic with anaesthetics acting on Site 1, 2, or 3

Although propofol and alphaxalone interact synergistically (Cao et al., 2018), propofol has only weak or no selectivity between Sites 1 and 2. (Chiara et al., 2013). We first confirmed that alphaxalone and propofol interact synergistically (Table 4 and Figure 6b).

TABLE 4 Fixed concentrations, EC₅₀s, and α s of propofol with Site 1, 2, and 3 anaesthetics

Drug A	[Drug A] μ M	[Propofol] μ M	α	Average α
Etomidate	0.29 ± 0.03	0.158	0.38 ± 0.01	0.58 ± 0.17^a
	0.25 ± 0.03	0.315	0.61 ± 0.01	
	0.07 ± 0.02	0.473	0.78 ± 0.01	
	0.555	0.09 ± 0.01	0.39 ± 0.02	
	1.11	0.03 ± 0.01	0.54 ± 0.01	
	1.655	0.01 ± 0.01	0.75 ± 0.01	
R-mTFD-MPAB	0.24 ± 0.04	0.158	0.35 ± 0.02	0.56 ± 0.17^a
	0.14 ± 0.02	0.315	0.56 ± 0.01	
	0.03 ± 0.01	0.473	0.76 ± 0.01	
	0.585	0.09 ± 0.01	0.39 ± 0.02	
	1.17	0.03 ± 0.01	0.54 ± 0.01	
	1.755	0.01 ± 0.01	0.75 ± 0.01	
Alphaxalone	0.34 ± 0.04	0.158	0.59 ± 0.03	0.64 ± 0.20^a
	0.29 ± 0.05	0.315	0.79 ± 0.04	
	0.09 ± 0.01	0.473	0.84 ± 0.01	
	0.355	0.08 ± 0.02	0.33 ± 0.03	
	1.710	0.03 ± 0.01	0.53 ± 0.01	
	1.065	0.03 ± 0.01	0.78 ± 0.01	

Note. α values are reported as \pm SD. SD of average α s are Calculated according to Equation (2). Each concentration-response curve had 40 animals except for the first two rows that had 50 and 55 animals respectively.

^aDiffers significantly ($P < .01$) from the value of one expected for additivity.

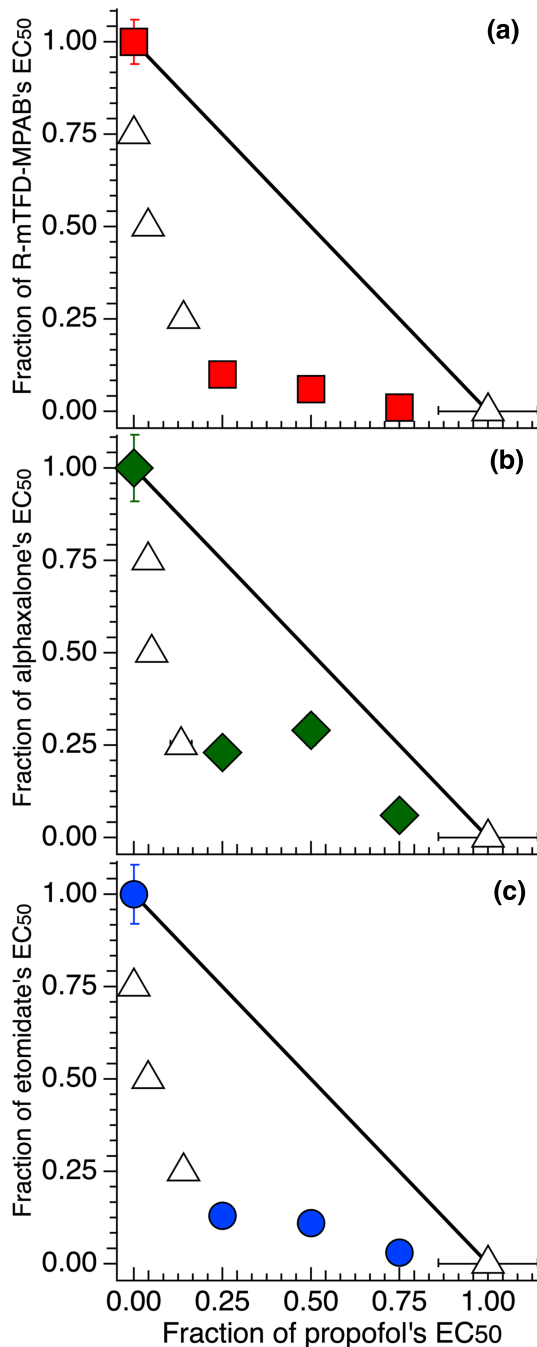


FIGURE 6 Isobolograms of propofol with either R-mTFD-MPAB, alphaxalone or etomidate all show synergy. Isobolograms are plotted as in Figure 3. Shown are combinations of propofol (both a Site 2 and 3 anaesthetic) with: (a) R-mTFD-MPAB (Site 2), (b) alphaxalone (Site 3) or (c) etomidate (Site 1).

Combinations of R-mTFD-MPAB and propofol interacted synergistically with α values ranging from 0.35 to 0.75 (Table 4 Figure 6a). Furthermore, combinations of etomidate and propofol also interacted synergistically with α values ranging from 0.38 to 0.78 (Table 4 and Figure 6c). In addition, ANOVA showed that the α values of these mixtures with propofol did not differ significantly from one another.

4 | DISCUSSION

Our work establishes, for the first time, some principles for predicting whether combinations of general anaesthetics that act on GABA_A receptors will interact additively or synergistically. Early studies of anaesthetic mixtures were carried out with the relatively low affinity volatile and gaseous anaesthetics and all demonstrated additivity (Clarke et al., 1978; Eger et al., 2008; Hendrickx, Eger, Sonner, & Shafer, 2008). Richards and White (1981) were the first to recognize that higher potency intravenous agents with stricter structure activity relationships would be more likely to reveal interactions between binding sites. Monitoring sleep time in rats, they showed that mixtures of alphaxalone with either etomidate or methohexitone were synergistic whereas mixtures with alphadalone, a steroid, were additive. They postulated that allosteric interactions between binding sites were responsible. These studies on the interaction of steroid anaesthetics with intravenous agents were later extended to alphaxalone + propofol and to 3 α 5 β P + etomidate and parallels with actions on heterologously expressed GABA_A receptors were established (Cao et al., 2018; Li et al., 2014; Shin, Germann, Covey, Steinbach, & Akk, 2019; Shin, Germann, Steinbach, & Akk, 2017). In this study, we have extended the pharmacology to include synergy between the three distinct TMD sites shown in Figure 1.

To avoid pharmacokinetic uncertainties associated with studies in mammals, we used the traditional model of tadpoles because they equilibrate with the bath concentration of the agents investigated. We set out to test the hypothesis that the general anaesthetic potencies of mixtures of site-selective agents binding to the same or to different sites would combine additively or synergistically respectively. The results proved to be remarkably consistent with this hypothesis, a success that may be attributed to the highly site-selective agents employed and to the low occupancy of these sites at general anaesthetic concentrations (Rusch, Zhong, & Forman, 2004). First, we found that for each of the three sites, the potencies of two or three agents each acting on a single site were simply additive and consistent with Equation (1) with $\alpha = 1$. Second, we found that pairs of selective agents acting on any two of the three possible pairwise combinations of sites always acted synergistically and that within the broad errors associated with in vivo studies, no pair interacted more strongly than any other based on their values of α , although it remains possible that studies in model systems such as those at the receptor level may reveal subtle differences (Cao et al., 2018). Third, combinations of three agents that acted selectively on different sites were even more synergistic.

Although our work firmly establishes the principles governing additivity and synergy of anaesthetic mixtures of site-selective agents that act on GABA_A receptors, clinically used non-steroidal intravenous general anaesthetics often have low selectivity between the etomidate and R-mTFD-MPAB sites and do not bind the steroid sites at all. For example, the selectivity for the R-mTFD-MPAB sites over the etomidate sites is just 13-fold for **phenobarbital**, eightfold for **pentobarbital**, 1.6-fold for **thiopental**, and 0.6-fold to twofold for propofol (Chiara et al., 2013; Shin et al., 2018). These selectivities

are much lower than those for the canonical agents etomidate and R-mTFD-MPAB, which have selectivity for their preferred sites of ~1000- and 60-fold respectively (Chiara et al., 2013). We chose propofol to test the hypothesis that agents with weak selectivities would act synergistically with both etomidate- and R-mTFD-MPAB-selective agents because it is thoroughly characterized, it is a widely used intravenous anaesthetic in the clinical setting, and its interaction with the steroid site has been reported to be synergistic in mice and in vitro (Cao et al., 2018), a finding we have confirmed here in tadpoles (Table 2). Consistent with the hypothesis, we found that when propofol was mixed with either etomidate or R-mTFD-MPAB, the resultant combination produced LoRR synergistically (Table 3). That is, whichever site is occupied by a site-selective anaesthetic, propofol can bind to the other site to produce a synergistic interaction. The corollary of this observation is that propofol must act synergistically with itself, which might account for the high potency of such a simple molecule.

Pharmacokinetics may compromise the use of mixtures of intravenous anaesthetics in mammals including humans (Hendrickx et al., 2008; McKay, 1991), but in the few cases where plasma levels have been measured, no major issues have emerged to date (Borowicz, Luszczki, & Czuczwar, 2004). Furthermore, the little data using site-selective agents in mammals are consistent with our findings. For example, mixtures of alphaxalone and etomidate are synergistic in rats (Richards & White, 1981) and mixtures of propofol and thiopentone, which interact with little selectivity on both Site 1 and 2, are additive in humans (Jones et al., 1999; Vinik, Bradley, & Kissin, 1999).

A goal of using combinations of general anaesthetics in the clinical situation would be to reduce off-target side effects. Compared to its use alone, the concentration of an agent used in a site-selective triple mixture is reduced 6–7 fold. Whether this is sufficient to reduce a side effect that only one agent in the mixture exerts depends on the target. For example, in the case of etomidate's high affinity interaction with **steroid 11- β hydroxylase (CYP 11B1)**, this would be far from sufficient (Zolle et al., 2008). However, for reducing side effects that are only encountered at high anaesthetic concentrations, for example, respiratory and cardiac depression, the strategy might have some success. Furthermore, for other members of the pentameric ligand-gated ion channel superfamily such as **nicotinic ACh receptors** in the autonomic nervous system, where the pharmacology does not parallel that of general anaesthesia, the strategy might be advantageous (Forman, 1998; Forman & Miller, 2011).

Side effects that are mediated by GABA_A receptors are a special case. Although much remains to be learnt about GABA_A receptor isoforms, most are expected to have two β^+/α^- interfaces because GABA binds in these interfaces in the ECD. Thus, mixtures of Site 1 and 3 agents might be expected to act equally on all GABA_A receptor isoforms. But Site 2 agents and future agents that might target the undrugged α^+/γ^- interface might have their action confined to a subset of largely synaptic receptors. However, further characterization of Site 2 agents in non-synaptic GABA_A receptors is needed because they can also target the β^+/β^- interface in $\alpha\beta$ and even some $\alpha\beta\delta$ receptors (Chiara et al., 2016).

The principles discussed above may well be generalizable to other behaviours mediated by heteromeric pentameric ligand-gated ion channel receptors. However, a prerequisite will be the development of agents that selectively target specific subunit interfaces, which in turn requires a knowledge of the stoichiometry and arrangement of the five subunits. In non-synaptic GABA_A receptors, the γ subunit is, with the exception of $\alpha 5$ subunit-containing receptors, replaced by either an additional β subunit or a δ subunit so that more than two homologous sites are likely to exist in all GABA_A receptors. An additional complexity is that some receptors may have no well-defined stoichiometry. This is thought to be the case in δ subunit containing GABA_A receptors and in $\alpha 4\beta 2$ nAChRs (Nelson, Kuryatov, Choi, Zhou, & Lindstrom, 2003; Wongsamitkul, Baur, & Sigel, 2016).

In conclusion, the $\beta 3$ N265M knock-in mouse established that the general anaesthetic actions of etomidate were mediated by GABA_A receptors (Jurd et al., 2003). The fact the steroids and the mephobarbital agents studied here allosterically enhance the action of etomidate strengthens the hypothesis that their general anaesthetic actions are also mediated by GABA_A receptors, without providing definitive proof.

The concept that general anaesthetics interact allosterically with function in the pentameric ligand-gated ion channel superfamily has long been recognized (Olsen, 2015; Changeux, 2013). Our work and that of Akk and colleagues adds to this concept by considering allosteric interactions between anaesthetic sites (Cao et al., 2018). We have shown that combinations of anaesthetics that act on just one of the three known sites interact additively, whereas those that act on one or two different sites interact synergistically leading to significant reductions in drug load. The principles demonstrated here may have wider implications for the development of drugs that act allosterically and for drug screening.

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AUTHOR CONTRIBUTIONS

K.W.M. conceived the project. K.W.M. and D.E.K. designed the experiments, which were performed and analysed by D.E.K., K.S.B. and P.Y. S. performed the synthesis of the barbiturates and azietomidate.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for [Design & Analysis](#), and [Animal Experimentation](#), and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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