JPET Fast Forward. Published on March 30, 2016 as DOI: 10.1124/jpet.115.228890
This article has not been copyedited and formatted. The final version may differ from this version.

JPET #228890

Title: Differential potency of 2,6 dimethylcyclohexanol isomers for positive modulation of $GABA_A$ receptor currents

Luvana Chowdhury, Celine J. Croft, Shikha Goel, Naina Zaman, Angela C-S Tai, Erin M. Walch, Kelly Smith, Alexandra Page, Kevin M. Shea, C. Dennis Hall, D. Jishkariani, Girinath G. Pillai and Adam C. Hall

Neuroscience Program, Department of Biological Sciences, Smith College, Northampton, MA 01063, USA (L.C., C.J.C., S.G., N.Z., A.C.-S.T., E.M.W., A.C.H.); Department of Chemistry, Smith College, Northampton, MA 01063, USA (K.S., A.P., K.M.S.); Department of Chemistry, University of Florida, Gainesville, FL 32611, USA (C.D.H., D.J., G.G.P.); and Department of Chemistry, University of Tartu, Ravila 14a, 50411, Estonia (G.G.P.)

JPET #228890

Running title: Modulation of $GABA_A$ currents by 2,6 dimethylcyclohexanols

Author for correspondence and for proofs:

Dr. Adam C. Hall, Neuroscience Program,

Department of Biological Sciences,

Ford Hall 202a, Smith College,

Northampton, MA 01063, USA

Tel: 1-413-585-3467; Fax: 1-413-585-3786;

e-mail: ahall@science.smith.edu

Number of text pages: 20

number of tables: 3

number of figures: 8

number of references: 33

number of words in the Abstract: 250

number of words in the Introduction: 452

number of words in the Discussion: 1710

GABA (γ-aminobutyric acid)

JPET Fast Forward. Published on March 30, 2016 as DOI: 10.1124/jpet.115.228890
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

$GABA_A$ receptors meet all the pharmacological requirements necessary to be considered important targets for the action of general anesthetic agents in the mammalian brain. In the following patch-clamp study, the relative modulatory effects of 2,6-dimethylcyclohexanol diastereomers were investigated on human γ-aminobutyric acid type A ($GABA_A$, $\alpha_1\beta_3\gamma_{2s}$) receptor currents stably expressed in human embryonic kidney cells. *Cis,cis*, *trans,trans*; and *cis,trans* isomers were isolated from commercially available 2,6-dimethylcyclohexanol and were tested for positive modulation of sub-maximal GABA responses. For example, the addition of 30 μM *cis,cis* isomer resulted in ~2-3 fold enhancement of the ~EC_{20} GABA current. Co-applications of 30 μM 2,6-dimethylcyclohexanol isomers produced a range of positive enhancements of control GABA responses with a rank order for positive modulation: *cis,cis* > *trans,trans* ≥ mixture of isomers >> *cis,trans* isomer. In molecular modeling studies, the three cyclohexanol isomers bound with the highest binding energies to a pocket within transmembrane helices M1 and M2 of the β_3 subunit through hydrogen-bonding interactions with a glutamine at the 224 position and a tyrosine at the 220 position. The energies for binding to and hydrogen bond lengths within this pocket corresponded with the relative potencies of the agents for positive modulation of $GABA_A$ receptor currents (*cis,cis* > *trans,trans* > *cis,trans* 2,6 dimethylcyclohexanol). In conclusion, the stereochemical configuration within the dimethylcyclohexanols is an important molecular feature in conferring positive modulation of $GABA_A$ receptor activity and for binding to the receptor, a consideration that needs to be taken into account when designing novel anesthetics with enhanced therapeutic indices.

JPET Fast Forward. Published on March 30, 2016 as DOI: 10.1124/jpet.115.228890
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Intravenous sedatives and general anesthetics are some of the most commonly used therapeutic agents during surgery. Several of these agents (*e.g.* propofol and etomidate) are postulated to sedate patients and render them unconscious through positive modulation of $GABA_A$ receptor currents in the central nervous system (Franks and Lieb, 1994; Krasowski and Harrison, 1999; Olsen and Li, 2011). $GABA_A$ receptors are membrane-spanning chloride-selective ion channel complexes activated through the binding of GABA (Barnard et al., 1998) and are the predominant ionotropic receptor type for fast inhibitory neurotransmission in the mammalian central nervous system (CNS). Their pentameric structure is composed of different subunits ($\alpha_{1\text{-}6}$, $\beta_{1\text{-}4}$, $\gamma_{1\text{-}3}$, δ, ε, π, and θ) with the predominant $GABA_A$ receptor combination of $\alpha_1\beta_2\gamma_2$ in mammalian neurons (McKernan and Whiting, 1996). Investigations of the action of anesthetics at $GABA_A$ receptors have revealed that for select agents, the potentiation of GABA currents correlates with anesthetic potency *in vivo* (Krasowski et al., 2001; Watt et al., 2008; Hall et al., 2011).

Given the interest in developing less toxic sedatives and anesthetics, several studies have explored the structure-activity relationship for agents that enhance GABA-evoked currents and provide anesthesia (for example, Krasowski et al., 2001; Pejo et al., 2014). Previously we demonstrated the potential for cyclohexanols to act as positive modulators of $GABA_A$ receptor currents and as general anesthetics (Hall et al., 2004; Watt et al, 2008). The structure-activity relationship for a range of cyclohexanol analogues was further explored and among those tested 2,6-dimethylcyclohexanol was determined to be the most potent for both receptor modulation and as a general anesthetic (Hall et al., 2011).

Stereoselectivity for positive modulation of $GABA_A$ receptor currents is not unprecedented, particularly in regard to enantiomers of general anesthetics (*e.g.* Hall et al., 1994; Tomlin et al., 1998). Likewise, cyclohexanol-based compounds (*e.g.* menthol) have also been shown to exhibit stereoselectivity of action for these receptors (Corvalan *et al.*, 2009). In the following study, we used WSS-1 cells to investigate modulation of wild-type $GABA_A$ receptors ($\alpha_1\beta_3\gamma_{2s}$) by *cis,cis, trans,trans* and *cis,trans* diastereomers of 2,6-dimethylcyclohexanol (Figure 1). In the most stable of the two possible chair conformations, *cis,cis*-dimethylcyclohexanol has both methyl groups equatorial and the hydroxyl group axial. The *cis,trans* isomer has the hydroxyl group and one methyl substituent equatorial while the other methyl is axial. In the *trans,trans* isomer, the hydroxyl group and both methyl groups are all equatorial making it the most stable configuration and closest to planarity. Molecular modelling studies were carried out for the *cis,cis, trans,trans* and *cis,trans* diastereomers of 2,6-dimethylcyclohexanol against the β_3-subunit of the human $GABA_A$ receptor. The results indicate that stereochemical configuration within the dimethylcyclohexanols is an important molecular feature in conferring positive modulation of $GABA_A$ receptor activity and for binding to the receptor.

2. Methods

2.1 Cell Culture

WSS-1 cells (ATCC, CRL-2029) were used for all electrophysiology experiments. WSS-1s are human embryonic kidney cells (HEK) that have been stably transfected with cDNAs encoding for the rat α_1 and γ_{2s} subunits of the $GABA_A$ receptor along with expression of an endogenous human β_3 subunit (Wong *et al.*, 1992; Davies *et al.*, 2000) and thus are a convenient cell line for generating GABA-evoked currents consistently. WSS-1 cells were grown in standard media (90% Dulbecco's modified Eagle's medium, 10% fetal bovine serum, with 100 units/ml penicillin, 100 μg/ml streptomycin) including 500 μg/ml geneticin (G-418) in order to select for cells expressing $GABA_A$ receptors (Wong *et al.*, 1992). Cells were maintained in culture flasks in a humidified incubator with 5% CO_2 / 95% air at 37 °C and passaged on a weekly basis. During passaging, cells were either plated on poly-L-lysine (Trevigen)-coated glass coverslips for electrophysiological recording or used to reseed another flask. Cells were used for up to 30 passages after purchase from the ATCC. All culturing reagents were purchased from Life Technologies unless stated otherwise.

2.2 Electrophysiology

Electrophysiological recordings were performed using standard whole-cell patch clamp technique at room temperature. Coverslips were transferred to a recording chamber that was continuously superfused at 3 ml/min with extracellular recording medium containing (mM): 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 11 Glucose, 5 HEPES (pH 7.4 with NaOH). The electrode solution contained (mM): 140 KCl, 2 $MgCl_2$, 11 EGTA, 0.1 Mg^{2+}-ATP and 10 HEPES (pH 7.4 with KOH). Pipettes, fabricated using a Flaming/Brown micropipette puller (Sutter

Instruments), typically had resistances in the range of 2-4 MΩ. Pipettes were maneuvered onto cells to form 'gigaohm' seals using a micromanipulator (MP-225, Sutter Instruments). Junction potentials were zeroed in the chamber prior to all recordings, the liquid junction potential was negligible (~2 mV) and cells were routinely voltage-clamped at -50 mV.

Drugs were superfused onto cells using a motor-driven exchange device (BioLogic Rapid Solution Changer, RSC-100) controlled *via* Clampex 10 acquisition software (Molecular Devices, Axon Instruments). Flow of extracellular solutions onto cells was driven by a multichannel infusion pump (KD Scientific). Currents evoked by GABA with and without the modulators in extracellular solution were amplified *via* an Axopatch 200A (Molecular Devices, Axon Instruments), filtered at 1 kHz *via* a low-pass Bessel filter (Frequency Devices) and digitized using a Digidata 1440 (Molecular Devices, Axon Instruments). All currents were measured using Clampex 10 (Molecular Devices, Axon Instruments) and further analyzed using Origin software (OriginLab Corp., Northampton, MA). Data are expressed as the mean ± standard error of the mean (S.E.M.) calculated from at least n = 5 individual cells for each data point reported (unless stated otherwise). Positive modulation of GABA-induced currents was defined as the percentage increase of the control GABA response (average of pre- and post-drug). Concentration-response data were fitted with the Hill equation (below) using Origin software (OriginLab Corp., Northampton, MA):

$$I = I_{max} \cdot [agonist]^{nH} / ([agonist]^{nH} + (EC_{50})^{nH})$$

where I is the agonist-evoked current at a given concentration, I_{max} is the peak current at saturating [agonist], EC_{50} is the concentration of agonist that elicits a half-maximal response, and nH is the Hill coefficient.

2.3 Drugs and reagents

All reagents were purchased from Sigma Aldrich unless stated otherwise. During experiments GABA and the cyclohexanol isomers were co-applied to assess the level of current modulation. Stock solutions (10-100 mM) of the 2,6 dimethylcyclohexanol isomers in DMSO were diluted daily to the required concentrations in extracellular medium with DMSO concentrations in final solutions never exceeding 0.1% (a concentration that had no effect on GABA-activated currents).

2.4 Isolation of 2,6 dimethylcyclohexanol isomers

2,6-Dimethylcyclohexanol from Acros was a mixture of 45% *cis,cis*, 32% *trans,trans*, and 23% *cis,trans* isomers (Figure 1). TLC: eluant, 10% diethyl ether in petroleum ether : Rf = 0.1, a mixture of trans,trans and cis,trans diastereomers; Rf = 0.2, cis,cis diastereomer; both spots visualized with vanillin. 2,6-Dimethylcyclohexanone was obtained from Sigma Aldrich as a mixture of 80% *cis* and 20% *trans* isomers.

TLC: eluant, 5% diethyl ether in petroleum ether : R_f= 0.23 (*trans*), 0.42 (*cis*), visualized with $KMnO_4$.

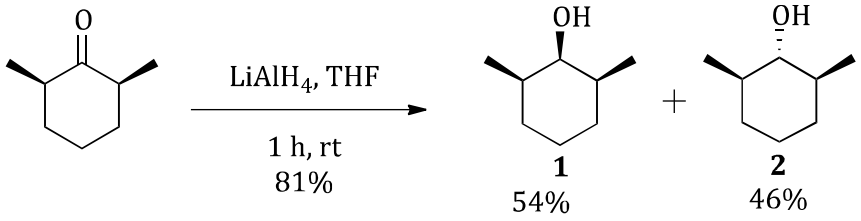
2,6-Dimethylcyclohexanone (1.50 g, 11.9 mmol) was applied to a column of silica gel (150 g) and eluted with 5% ethyl ether in petroleum ether to yield *cis*-2,6-dimethylcyclohexanone (1.01 g, 8.00 mmol), a mixture of both isomers consisting of 20% *cis*-

2,6-dimethylcyclohexanone and 80% *trans*-2,6-dimethylcyclohexanone (115 mg, 0.910 mmol), and *trans*-2,6-dimethylcyclohexanone (72.1 mg, 0.570 mmol).

Cis-2,6-dimethylcyclohexanone: TLC: eluant, 5% diethyl ether in petroleum ether: R_f= 0.42, , visualized with $KMnO_4$. ^{1}H NMR (300 MHz, $CDCl_3$): 1.0 (d, 6 H), 1.2-1.4 (m, 2 H), 1.7-1.9 (m, 2 H), 2.0-2.2 (m, 2 H), 2.3-2.5 (m, 2 H)

Trans-2,6-dimethylcyclohexanone: TLC: eluant, 5% diethyl ether in petroleum ether: R_f= 0.23, , visualized with $KMnO_4$. ^{1}H NMR (300 MHz, $CDCl_3$): 1.1 (d, 6 H), 1.5-1.6 (m, 2 H), 1.7-1.8 (s, 2 H), 1.9-2.0 (m, 2 H), 2.5-2.6 (m, 2 H).

Reduction of *cis*-2,6-dimethylcyclohexanone with lithium aluminum hydride



An oven-dried 100-mL 3-neck flask, equipped with magnetic stirrer, rubber septum and gas inlet was filled with N_2. Tetrahydrofuran (anhydrous, 20 ml) was added followed by a 1 M solution of lithium aluminum hydride in tetrahydrofuran (13.1 ml, 13.1 mmol, 1.10 equiv). *Cis*-2,6-dimethylcyclohexanone (1.46 g, 11.5 mmol) was then added dropwise, *via* syringe and the reaction was stirred at room temperature for 1 h.

The reaction was quenched with 2 ml of water added dropwise, then diluted with 5 ml of 15% NaOH solution, followed by another 2 ml of water. The mixture was filtered through silica gel under vacuum and rinsed with diethyl ether (~50 ml). The filtrate was transferred to a 250-

ml separatory funnel. The aqueous layer was extracted with ethyl ether (3 x 50 ml), and the combined ether layers were dried over anhydrous $MgSO_4$. An ^1H NMR on the crude product (1.65 g, 100%) showed a mixture of *cis,cis*-2,6-dimethylcyclohexanol (54%) and *trans,trans*-2,6-dimethylcyclohexanol (46%). Flash column chromatography of the crude product using 240 g of silica gel, and 10:1 petroleum ether to ethyl ether as eluant gave the *cis,cis* isomer (646 mg, 42%), a combination of both isomers (41 mg, 3%), and the *trans,trans* isomer (551 mg, 36%). The total yield was 1.24 g (9.65 mmol, 81%).

Cis,cis-2,6-dimethylcyclohexanol (Isomer 1)

TLC: R_f= 0.2, eluant 10% diethyl ether in petroleum ether, visualized with vanillin

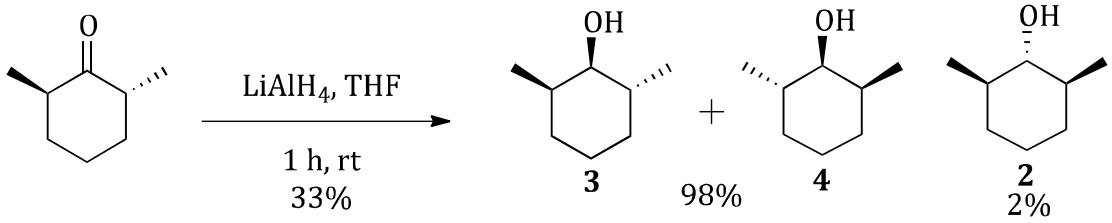
^1H NMR (300 MHz, $CDCl_3$): 0.98 (d, 6 H), 1.16 (d, 1 H), 1.28-1.38 (m, 5 H), 1.45-1.59 (m, 2 H), 1.65-1.74 (m, 1 H), 3.51-3.56 (m, 1 H)

Trans,trans-2,6-dimethylcyclohexanol (Isomer 2)

TLC: R_f= 0.1, eluant 10% diethyl ether in petroleum ether, visualized with vanillin

^1H NMR (300 MHz, $CDCl_3$): 1.07 (d, 6 H), 1.20-1.29 (m, 2 H), 1.30-1.42 (m, 2 H), 1.47 (d, 1 H), 1.56-1.65 (m, 2 H), 1.66-1.76 (m, 2 H), 2.72 (t of d, 1 H)

Reduction of *trans*-2,6-dimethylcyclohexanone with lithium aluminum hydride



NOTE : 3 and 4 are enantiomers and may (or may not) have equal activity dependent on whether the site is chiral (not the same activity) or achiral (same activity)

An oven-dried 25-ml 2-neck flask, equipped with magnetic stirrer, rubber septum, and gas inlet was filled with dry N_2. Tetrahydrofuran (anhydrous, 1.5 ml) was added together with a 1 M anhydrous solution of lithium aluminum hydride in tetrahydrofuran (1.10 ml, 1.10 mmol, 1.10 equiv). A solution of *trans*-2,6-dimethyl cyclohexanone (128.5mg, 1.01mmol) in anhydrous THF (1ml) was transferred by cannula from a pear-shaped flask and the mixture was stirred at room temperature for 1 hr.

The reaction was quenched with a few drops of water then diluted with 9% NaOH solution added dropwise. The mixture was filtered through silica gel under vacuum, rinsed with diethyl ether (~15 ml) and the filtrate was transferred to a 50-ml separatory funnel. The aqueous layer was extracted with ethyl ether (3x10 ml). and the ether layers were combined and dried over $MgSO_4$. An ^{1}H NMR on the crude product (42.9 mg, 33%) revealed a mixture of *cis,trans*-2,6-dimethylcyclohexanol (98%) and *trans,trans*-2,6-dimethylcyclohexanol (2%).

Cis,trans- and _trans,cis_-2,6-dimethylcyclohexanol (Enantiomers 3 and 4)

TLC: R_f= 0.1, eluant 10% diethyl ether in petroleum ether, visualized with vanillin

^{1}H NMR (300 MHz, $CDCl_3$): 0.97(t, 6 H), 1.36-1.55 (m, 6 H), 1.63-1.80 (m, 2 H), 1.90-2.04 (m, 1 H), 3.28-3.35 (m, 1 H)

A sample of the *cis,cis* isomer was also isolated directly from the commercially available mixture *via* column chromatography of the mixture (5g) on silica gel (300g) using hexane/ethyl acetate (20:1) to yield 1.1g of pure *cis,cis* isomer (by ^{1}H NMR) as a colorless liquid. Samples of

the *cis,cis* isomer from both isolation procedures produced similar modulation of GABA currents.

2.5 Molecular Modeling

Molecular docking studies were carried out to define the mode of interaction between the $GABA_A$ receptor, propofol and each diastereomer of 2,6-dimethylcyclohexanol. Given previous literature highlighting the role of β-subunits in the binding of propofol to $GABA_A$ receptors (Yip et al., 2013) and the availability of a crystal structure (PDB ID: 4COF, Miller and Aricescu, 2014), the β_3 subunit of human γ-aminobutyric acid type A receptor was the considered target. This target was prepared for the docking process by protonating, minimizing and examining the missing side chain residues in the protein using Chimera Software (Pettersen et al, 2004, Goddard et al, 2005). The prepared target was uploaded to ProBis server - http://probis.nih.gov/ (Carl et al., 2010) for the detection of binding sites using protein binding site structure similarities. The ProBiS program aligns and superimposes protein binding sites, and enables pairwise alignments and fast database searches for similar binding sites.

We focused on propofol binding sites highlighted in previous studies (Nury et al., 2011; Chiara et al., 2013; Yip et al., 2013) and sites based on the structural similarities of the following proteins (PDB IDs): 2M6B –(structure of transmembrane domains of human glycine receptor α_1 subunit, Mowrey et al., 2013), 4X5T (α_1 glycine receptor transmembrane structure fused to the extracellular domain of GLIC, Moraga-Cid et al., 2015), and 3P50 (structure of propofol bound to GLIC, Nury et al., 2011). Based on these previous studies, we explored intra-subunit binding sites within chain A of a single subunit of 4COF (Miller and Aricescu, 2014). The best binding site with a Z-Score of 4.21 included the key amino acid residues TYR220, PHE221, GLN224,

HIS267 and THR271. A second binding site with key amino acid residues: TYR143, THR225, PRO228, ILE264, and LEU268 gave a Z-score of 4.01. The high scoring binding sites were then merged as there was considerable spatial overlap between the key amino acids. Although it is recognized that inter-subunit sites have been proposed for propofol binding to $GABA_A$ receptors (*e.g.* Bali and Akabas, 2004), sites between subunits (*e.g.* Chain A and B) were not considered because steric clashes were encountered when modeling multiple subunits at these sites.

The protein was loaded to MGL-Autodock Tools to define the custom binding site grid box for the docking process. In brief, polar hydrogen atoms and Kollman charges were assigned to the protein by converting to PDBQT format of Autodock (Morris et al., 2009). The program AutoGrid was used to generate grid maps for the custom binding site on the protein and the grid was generated based on selected residues from binding site analysis. In order to generate grid maps for different types of ligands (with possible hydrogen bonding), the grid parameter file was modified to include O-H bond types from ligands. To generate the custom grid maps, we defined grid points as x=40, y=40, z=50 and grid center as x = 10.70, y = -13.28, z = 157.35 with a spacing of 0.38 in the protein 3D structure. The binding site for 2,6-dimethylcyclohexanols included TYR143, TYR220, PHE221, GLN224, THR225, PRO228, ILE264, HIS267, LEU268, and THR271 amino acids from the M1 and M2 domains of Chain A(Fig. 2).

Propofol, *cis,cis, trans,trans* and *cis,trans* diastereomers of 2,6-dimethylcyclohexanol were all considered as ligands. The geometries were drawn in Marvin Sketch and converted to 3D using MM2 forcefield. The diastereomeric conformers were frozen for further quantum chemical optimization using the DFT/B3LYP level of theory and 6-31G basis set in HyperChem software; (see Table 1 for quantum chemical properties). The lowest energy conformer was uploaded to

MGL Tools to assign Gasteiger partial charges and for the detection of torsions to rotate the bonds during the docking procedure. For all ligands, random initial positions, fixed conformers and torsions were parameterized. The number of active torsions and the number of torsional degrees of freedom were set to default values indicated in AutoDock. A Lamarckian Genetic Algorithm was used for minimization using optimum parameters (from initial docking evaluations) to generate all possible energies in order to rank the conformers (Morris et al., 1998). For energy evaluations, we used the following docking parameters: 250000 evaluations, 250 Genetic Algorithm iterations, and a population size of 150 to generate 250 docked conformers. For reliable docking results, the root mean square deviation (rmsd) of the lowest energy conformer and the rmsd to one another were analyzed in order to group families of similar conformations using clustering.

3. Results

Cis,cis and trans,trans 2,6-dimethylcyclohexanols isomers are positive modulators of $GABA_A$ receptor currents

We investigated the modulation of sub-maximal GABA currents by the three isolated 2,6-dimethylcyclohexanol diastereomers *cis,cis, cis,trans; trans,trans* along with the mixture of the isomers. WSS-1 cells (HEKs stably expressing $\alpha_1\beta_3\gamma_{2s}$ $GABA_A$ receptors) were routinely exposed to applications of 10 μM GABA that evoked ~EC_{20} currents (effective concentration that evoked 20% of maximal current). Co-applications of 30 μM 2,6-dimethylcyclohexanols produced potentiations of the GABA responses (Fig. 3). For example, the addition of 30 μM *cis,cis* isomer resulted in ~2-3 fold enhancement of the EC_{20} GABA current (Fig. 3B). Pre-exposure to cyclohexanols did not affect the extent of current modulation upon subsequent co-application and no direct activation of $GABA_A$ receptor currents was observed by the cyclohexanols even at the highest concentrations (300 μM) of the isomers tested (data not shown).

Co-applications of 30 μM 2,6-dimethylcyclohexanol isomers produced a range of positive enhancements of control GABA responses with the rank order for positive modulation of GABA EC_{20} currents: *cis,cis* > *trans,trans* ≥ mixture of isomers >> *cis,trans* isomer (Fig. 3). We confirmed the relative extent of the potentiations of the GABA receptor activity in the presence of increasing concentrations of the isomers (1-300 μM, Fig. 4). For instance, on average with addition of 30 μM *cis,cis* isomer the positive modulation of the current (above control) was 165 ± 27 % (n=6) while the equivalent for the *trans,trans* isomer was 92 ± 24 % (n=5). Current modulation by the *cis,trans* isomer was negligible even at the highest

concentration tested (at 300 μM, 5 ± 5 %, n=6). All the cyclohexanol effects were fully reversible upon washout (as previously reported, Hall *et al.*, 2011).

The relative potencies for positive modulation of GABA currents were further supported by recording and plotting the leftward shifts in the GABA concentration-response curves in the presence of 30 μM 2,6-dimethylcyclohexanol isomers (Fig. 5). For instance, the EC_{50} for the control GABA currents (~21 μM) was shifted to ~10 μM by the *trans,trans* isomer and to ~7 μM in the presence of the *cis,cis* isomer. The *cis,trans* isomer produced a negligible shift in the concentration-response relationship (Fig. 5).

Modeling the binding of the isomers to the β3 subunit of the $GABA_A$ receptor

Molecular modeling focused on regions of the β3 subunit of the $GABA_A$ receptor (4COF, Miller and Aricescu, 2014) that, in previous studies, were implicated in propofol binding to $GABA_A$ receptor β-subunits or GLIC channels (Yip et al., 2013; Nury et al., 2011; Chiara et al., 2013). The binding site prediction server ProBis http://probis.nih.gov/ (Carl et al., 2010) gave the best binding site score with key amino acid residues, TYR143, TYR220, PHE221, GLN224, THR225, PRO228, ILE264, HIS267,THR271 and LEU268.

This binding site is located between the transmembrane M1 and M2 helices of the β_3 subunit (Fig.2) and includes a phenylalanine at the position 221 (M1) and a histidine at the position 267 (M2) that were previously photolabeled with an ortho-propofol diazirine derivative (Yip *et al.*, 2013). In the same study, replacement of the phenylalanine at 221 by a tryptophan residue was shown to attenuate propofol's potentiation of GABA-evoked currents. Interestingly, the three cyclohexanol isomers were found to bind within this pocket through hydrogen-bonding interactions with a glutamine at the 224 position and a tyrosine at the 220 position plus

hydrophobic interactions with the leucine at position 268, phenylalanine at position 221 and tyrosine at position 220 (Tables 1, 2, & 3, Figure 6). By comparison, propofol's hydrogen bonding was modeled only through the glutamine residue and hydrophobic interactions with the leucine at 268, tyrosine at 220 and threonine at 225 positions (Figure 7). The ligand efficiency, van der Waals energy, electrostatic and H-bond energy were all considered in the calculation of binding energies and intermolecular energies for this intra-subunit site. Within the site the binding energies for the interactions (Table 2) had a rank of order: propofol > *cis, cis* > *trans,trans* > *cis,trans* 2,6 dimethylcyclohexanol corresponding with the rank order of potencies for positive modulation of $GABA_A$ receptor currents recorded electrophysiologically (compare with Figures 3 & 7 from Davies et al, 2000; Hall et al., 2011, respectively).

Cis, trans 2,6 dimethylcyclohexanol as an inhibitor of propofol's modulatory action at $GABA_A$ receptors

Finally, given the lack of modulation observed by *cis,trans* 2,6 dimethylcyclohexanol and the modeling of its binding to the receptor at a site similar to that of propofol's, we explored the possibility that this isomer might competitively inhibit propofol's modulatory action at the receptor. As expected, sub-maximal GABA (3 μM) responses were potently enhanced by propofol (10 μM, Figure 8). However, this positive modulation was only moderately attenuated by the co-application 100 μM *cis,trans* 2,6 dimethylcyclohexanol (Figure 8). In summary, positive modulation by propofol was attenuated by 14.1 +/- 1.3 % (n=4) and by 13.4 +/- 2.5 % (n=5) by 100 and 300 μM *cis,trans* 2,6 dimethylcyclohexanol, respectively.

Discussion

In the search for novel anesthetic and sedative compounds, the current study investigated the potential of 2,6-dimethylcyclohexanol stereoisomers as positive modulators of $GABA_A$ receptor mediated currents. In this study , the GABA EC_{50} in HEK cells expressing $\alpha_1\beta_3\gamma_{2s}$ receptors was determined to be ~20 μM with a Hill coefficient of 1.6, reasonably consistent with other reports for similar receptor combinations using the same expression system (*e.g.* Ueno *et al*., 1996). The cyclohexanols in this study all have their aliphatic groups in the 2,6-position relative to the hydroxyl group equivalent to the commonly used intravenous anesthetic, propofol (2,6-di-isopropylphenol). Following on from studies that demonstrated the efficacy of 2,6-dimethylcyclohexanol for positive modulation of $GABA_A$ receptor currents and for inducing anesthesia (Hall *et al.*, 2011), the major electrophysiological findings of the current study are i) the mixture of 2,6-dimethylcyclohexanol isomers (3-300 μM) enhanced GABA currents evoked in HEK cells expressing $\alpha_1\beta_3\gamma_{2s}$ receptors; ii) the *cis,cis* and *trans,trans* isomers of 2,6-dimethylcyclohexanol positively modulated GABA currents with the former being marginally more potent and iii) *cis,trans* 2,6-dimethylcyclohexanol had minimal effects on GABA currents.

The modulatory effects of cyclohexanols on GABA receptors have been previously investigated (Hall *et al*., 2011), but in this instance they were studied in oocytes expressing $\alpha_1\beta_2\gamma_{2s}$ receptors (the most prevalent combination found in the mammalian brain). By comparison, the potentiation of GABA currents by 30 μM 2,6-dimethylcyclohexanol was greater in the oocyte studies (~4-fold enhancement, Hall *et al.*, 2011) than in HEK cells expressing $\alpha_1\beta_3\gamma_{2s}$ receptor composition (~1.5 fold enhancement, current study). Subunit composition,

JPET Fast Forward. Published on March 30, 2016 as DOI: 10.1124/jpet.115.228890
This article has not been copyedited and formatted. The final version may differ from this version.

differences in expression systems and speed of agonist/modulator application probably contribute to the discrepancies in the extent of the modulations reported.

While the 2,6-dimethylcyclohexanol mixture of stereoisomers enhanced GABA currents at concentrations of 3-300 μM, the mixture is composed of isomers that may have different modulatory effects on the receptors. Stereoselective action has been previously observed for GABA receptor modulation by anesthetic agents. For instance, the S(+)-isoflurane isomer was observed to be more effective in potentiating GABA-induced currents than the R(-)-isoflurane (Hall *et al*. ,1994) with steroselectivity also observed for (+)- and (-)-pentobarbital (Tomlin *et al*., 1998). It seemed plausible therefore that individual isomers of 2,6-di-methylcyclohexanol would exhibit a range of potencies due to differing chemical configurations. 2,6-Di-isopropylphenol (propofol), a potent positive modulator of GABA receptor currents, consists of a hydroxyl group and two ortho iso-propyl groups in the plane of the benzene ring. By contrast, all three 2,6-di-methylcyclohexanols are chair shaped and not planar molecules. The *trans, trans* configuration of 2,6-di-methylcyclohexanol is the closest to planarity with the hydroxyl and two methyl groups in equatorial positions in the most stable conformer. Thus, we expected the *trans,trans* isomer would be the most potent modulator since it might fit most effectively into an equivalent propofol binding pocket within the $GABA_A$ receptor. Indeed previous studies reported that cyclohexanols and propofol may share similar sites of action on $GABA_A$ receptors (Watt et al., 2008). The *cis,cis* configuration of 2,6-dimethylcyclohexanol was postulated to be the next most potent since the most stable conformation would have the hydroxyl group axial and the methyl groups equatorial. Finally, the *cis,trans* configuration of 2,6-dimethycyclohexanol was expected to be the least potent since one methyl must necessarily be axial thereby reducing planarity. Contrary to expectations, although the *trans,trans* isomer was still an effective modulator , the

cis,cis isomer was the most potent diastereomer for positive modulation of GABA responses. As anticipated, the *cis,trans* isomer had minimal impact on the modulation of GABA receptor currents suggesting that the 23% of this isomer present in the commercially available mixture acts as pharmacological ballast with regard to $GABA_A$ receptor modulation.

In previous studies a range of 2,6-substituted cyclohexanols with varying sizes of the aliphatic chains were assessed for anesthetic potency and for loss of righting reflex in a tadpole assay (Hall *et al.*, 2011). 2,6-Dimethylcyclohexanol was one of the most potent in this regard with an EC_{50} ~ 13 μM with 2,6-di-isopropylcyclohexanol demonstrating equivalent potency (EC_{50} ~ 14μM). To date, yields of isolated isomers from mixtures of both 2,6-dimethylcyclohexanol and 2,6-di-isopropylcyclohexanol have been insufficient to enable similar analyses of the relative anesthetic potencies of the *cis,cis vs trans,trans* isomers in *in vivo* tadpole assays.

Although our electrophysiological studies were conducted on a $GABA_A$ receptor consisting of α_1, β_3 and γ_{2s} subunits, the closest approximating crystal structure for the molecular modeling studies was for a homopentamer of human β3 subunits (4COF, Miller and Aricescu, 2014). The binding pocket for the cyclohexanol isomers, revealed through our modeling studies of a $GABA_A$ receptor β3-subunit, was comprised primarily of residues from M1 and M2 transmembrane helices. This intra-subunit binding site was predicted by reference to previous propofol binding studies, by similarity in the templates used for the homology modeling and by identifying key amino acid residues within two sites that produced the highest Z-scores. The site included a phenylalanine at the 221 position and a histidine at 267 (Figure 7) that have already been implicated in propofol's positive modulation of receptor currents and in propofol's binding (Yip et al 2013). Both active isomers, *cis,cis* and *trans,trans* were also observed to bind

to the receptor subunit *via* the histidine 267 and through hydrophobic interactions with the phenylalanine 221 (Figure 6a & b). It should be noted that Yip et al (2013) observed interactions of an equivalent binding site with the main chain of the neighboring subunit. In our modeling studies inter-subunit sites of action were not explored because of steric clashes between subunits (e.g. Chain A and B) at these sites that were severe even after extensive protein preparation (*i.e.* minimization to refine protein structure). As this questions the availability of the proposed site in an assembled receptor, future studies will require a thorough protein minimization to refine the structure and MD simulations to address other potential intra and inter-subunit binding sites.

Our modeling studies revealed several important points regarding the binding of the 2,6-dimethylcyclohexanols at the chosen site. First the binding energies (BE, Table 2) vary from -4.42 through -4.38 to -4.21 kcalmol^{-1} for the *cis,cis*; *trans, trans* and *cis,trans* isomers, respectively. Such small differences, although corresponding to the rank order of potency for receptor modulation, are unlikely to explain changes in current modulation from ~300% (300 μM *cis,cis*) to ~ 0% (300 μM *cis,trans*). Binding energy comprises a combination of hydrophobic interactions, van der Waal's forces and hydrogen bonding. Figure 6 shows that the H-bond length to glutamine 224 (GLN224) increases from 1.898Å (*cis,cis*) through 2.014Å (*trans,trans*) to 2.152Å (*cis,trans*). Interestingly, these H-bond lengths are inversely related to the extent of current enhancements derived electrophysiologically, suggesting that H-bonding may be an important factor in determining the extent of receptor positive modulation (Table 3).

It is instructive to compare the modeling data for the cyclohexanols with those for propofol which shows a binding energy of -4.96 kcalmol^{-1}. If binding energy was the determining factor, this only corresponds to an estimated modulation enhancement over *cis,cis*-2,6-dimethylcyclohexanol of ~3-fold while the observed enhancements using propofol are

considerably greater (*e.g.* compare Figure 3B with Figure 8). According to our modeling, propofol also forms a H-bond with GLN224 with a bond length of 1.874Å only slightly shorter than that with *cis,cis* 2,6-dimethylcyclohexanol. There is, however, a fundamental difference. Possibly due to the acidity of propofol (pK_a ca 9), the H-bond is described between the *hydrogen* of the OH group and the basic amide *oxygen* of GLN224. By contrast, the H-bond between the cyclohexanols and GLN224 is between the *NH_2* group (in $CONH_2$) of the glutamine and the *oxygen* atom of the OH group. Therefore, one may conclude that it is the *nature* and *lengths* of the hydrogen bonds, that determine the degree of receptor positive modulation although additional contributions from the hydrophobic and van der Waal's interactions must also contribute to the binding energy..

Although we focused on a site selected from pre-docking studies with the highest Z-scores, many other sites had the potential to accommodate the isomers albeit with lesser scores. Indeed, many other sites have been proposed for propofol's interactions and modulation of $GABA_A$ receptor activity including other sites in M1 (Chang et al., 2003), M2 (Siegwart et al., 2002) and also M3 and M4 helices (Siegwart et al., 2002; Krasowski et al., 1998; and Richardson et al., 2007). For example, in previous studies a tyrosine in the 444 position of β_2 subunits proved important for modulation of the GABA currents by propofol (Richardson et al., 2007) and by menthol, a monoterpenoid with a neutral cyclohexanol chair structure (Watt et al, 2008).

The drugs investigated in this study were all positive modulators and therefore likely stabilize the 'open' state of the ligand-gated ion channel through an allosteric mechanism. By constrast, the 4COF structure used for our modeling studies is described by Miller & Aricescu (2014) as representative of a 'desensitized' state. This state is evidently distinct from that

JPET Fast Forward. Published on March 30, 2016 as DOI: 10.1124/jpet.115.228890
This article has not been copyedited and formatted. The final version may differ from this version.

predicted to be stabilized by positive modulators. Moreover, the 4COF structure represents a modified β3 subunit of the $GABA_A$ receptor with extensive intracellular loop domains removed. Therefore, although 4COF provides an approximation of the relevant structure for binding studies, interpretation of the molecular modeling data must be approached with caution. Furthermore, the structure of 4COF is that of a homopentameric receptor while our electrophysiological recordings were derived from receptors with two additional subunits (α_1 and γ_{2s}), which could contribute either directly to drug binding or indirectly to binding site conformations. Given these caveats, other candidate binding sites may be equally or more relevant to producing the positive modulations of the receptor activity observed electrophysiologically.

Finally, we performed competition experiments to determine if the *cis,trans* 2,6-dimethylcyclohexanol could act as a competitive inhibitor of propofol's action at the receptor (Figure 8). In these recordings we observed only modest inhibition of the positive modulation induced by propofol. This result suggests that, while *cis,trans* cyclohexanol produced no modulation of GABA responses, its ability to compete for a propofol binding site is limited, presenting a further caveat for over-interpretation of the the modeling data.

In conclusion, the enhanced activity of *cis,cis*-2,6-dimethylcyclohexanol presents an interesting lead in the development of novel anesthetics given that cyclohexanols in general are typically well tolerated (Thorup *et al*., 1983). Further refinement of such agents through isolation of individual isomers may lead to novel anesthetics with improved therapeutic indices.

Acknowledgements

We thank Harrison Hunter and Salma Bargach for helping to set up the patch-clamp electrophysiology. We also thank Dr. Andrew Jenkins (Dept. Anesthesiology, Emory University, Atlanta, GA) for his reading and comments on the manuscript. G.G. Pillai is grateful to the graduate school “Functional materials and technologies,” University of Tartu.

Authorship Contributions

Participated in research design: Chowdhury, Croft, Goel, Zaman, Tai, Walch, Smith, Page, Shea, C.D. Hall, Jishkariani, Pillai and A.C. Hall

Conducted experiments: Chowdhury, Croft, Goel, Zaman, Tai, Walch, Smith, Page, Shea, C.D. Hall, Jishkariani, Pillai and A.C. Hall

Synthesised new reagents or analytic tools: Smith, Page, Shea, C.D. Hall, Jishkariani

Performed data analysis: Chowdhury, Croft, Goel, Zaman, Tai, Walch, Smith, Page, Shea, C.D. Hall, Jishkariani, Pillai and A.C. Hall

Wrote or contributed to the writing of the manuscript: Chowdhury, Croft, Goel, Zaman, Tai, Walch, Smith, Page, Shea, C.D. Hall, Jishkariani, Pillai and A.C. Hall

References

Bali M, Akabas MH. (2004) Defining the propofol binding site location on the GABAA receptor. *Mol Pharmacol*. **65**: 68–76.

Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G, Braestrup C, Bateson AN, Langer SZ (1998) Subtypes of γ-aminobutyric $acid_A$ receptors: classification on the basis of subunit structure and receptor function. *Pharmacol. Rev.* **50**: 291-313.

Carl N, Konc J, Vehar B, and Janezic D (2010) Protein-protein binding site prediction by local structural alignment. *J Chem Inf Model* **50**:1906–1913.

Chang C-S, Olcese R, Olsen RW (2003) A single M1 residue in the β_2 subunit alters gating of $GABA_A$ receptor in anesthetic modulation and direct activation. *J. Biol. Chem*. **278**: 42821-42828.

Chiara DC, Gill JF, Chen Q, Tillman T, Dailey WP, Eckenhoff RG, Xu Y, Tang P, Cohen JB (2013) Photoaffinity labeling the propofol binding site in GLIC. *Biochem*., **53**: 135-142.

Corvalan NA, Zygadlo JA, Garcia DA (2009) Stereo-selective activity of menthol on $GABA_A$ receptor. *Chirality* **21**: 525-530.

Davies PA, Hoffmann EB, Carlisle HJ, Tyndale RF, Hales TG (2000) The influence of an endogenous b_3 subunit on recombinant $GABA_A$ receptor assembly and pharmacology in WSS-1 cells and transiently transfected HEK293 cells. *Neuropharmacol*. **39**: 611-620.

Franks NP, Lieb WR (1994) Molecular and cellular mechanisms of general anesthesia. *Nature* **367**: 607-614.

Goddard TD, Huang CC, Ferrin TE (2005) Software extensions to UCSF chimera for interactive visualization of large molecular assemblies. *Structure*, **13**: 473-482.

Hall AC, Lieb WR, Franks NP (1994) Stereoselective and non-stereoselective actions of isoflurane on the $GABA_A$ receptor. *Br. J. Pharmacol*. **112**: 906-910.

Hall AC, Turcotte CM, Betts BA, Yeung W-Y, Agyeman AS, Burk LA (2004) Modulation of human $GABA_A$ and glycine receptor currents by menthol and related monoterpenoids. *Eur. J. Pharmacol*. **506**: 9-16.

Hall AC, Griffith TN, Tsikolia M, Kotey FO, Gill N, Humbert DJ, Watt EE, Yermolina YA, Goel S, El-Ghendy B, Hall CD (2011) Cyclohexanol analogues are positive modulators of $GABA_A$ receptor currents and act as general anesthetics *in vivo*. *Eur J Pharmacol* **667**: 175-181.

Krasowski MD, Koltchine VV, Rick CE, Ye Q, Finn SE, Harrison NL (1998) Propofol and other intravenous anesthetics have sites of action on the γ-aminobutyric acid type A receptor distinct from that for isoflurane. *Mol Pharm*. **53**: 530-538.

Krasowski MD, Harrison NL (1999) General anesthetic actions on ligand-gated ion channels. *Cell. Mol. Life Sci*. **55**: 1278-1303.

Krasowski MD, Jenkins A, Flood P, Kung AY, Hopfinger AJ, Harrison NL (2001) General anesthetic potencies of a series of propofol analogs correlate with potency for potentiation of γ-aminobutyric acid (GABA) current at the $GABA_A$ receptor but not with lipid solubility. *J. Pharmacol. Exp. Ther*. **297**: 1-14.

McKernan RM, Whiting PJ (1996) Which $GABA_A$ receptors really occur in the brain? *Trends Neurosci*. **19**: 139-143.

Miller PS and Aricescu AR (2014) Crystal structure of a human $GABA_A$ receptor. *Nature* **512**: 270-275.

Moraga-Cid G, Sauguet L, Huon C, Malherbe L, Girard-Blanc C, Petres S, Murail S, Taly A, Baaden M, Delarue M, and Corringer P-J (2015) Allosteric and hyperekplexic mutant

phenotypes investigated on an α1 glycine receptor transmembrane structure. *Proc Natl Acad Sci U S A* **112**:2865–70.

Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK, and Olson AJ (1998) Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J Comput Chem* **19**:1639–1662.

Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, and Olson AJ (2009) AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J Comput Chem* **30**: 2785–91.

Mowrey DD, Cui T, Jia Y, Ma D, Makhov AM, Zhang P, Tang P, and Xu Y (2013) Open-channel structures of the human glycine receptor α1 full-length transmembrane domain. *Structure* **21**:1897–904.

Nury H, Renterghem CV, Weng Y, Tran A, Baaden M, Dufresne V, Changeux J-P, Sonner JM, Delarue M, Corringer P-J (2011) X-ray structures of general anesthetics bound to a pentameric ligand-gated ion channel. *Nature* **468**: 428-433.

Olsen RW, Li GD (2011) GABA(A) receptors as molecular targets of general anesthetics: identification of binding sites provides clues to allosteric modulation. *Can J Anaesth*. **58**: 206-15.

Pejo E, Santer P, Jeffrey S, Gallin H, Husain SS, Raines DE (2014) Analogues of etomidate: modifications around etomidate's chiral carbon and the impact on in vitro and in vivo pharmacology. *Anesthesiol*. **121**: 290-301.

Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, and Ferrin TE (2004) UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem* **25**:1605–12.

Richardson JE, Garcia PS, O'Toole KK, Derry JM, Bell SV, and Jenkins A (2007) A conserved tyrosine in the β2 subunit M4 segments is a determinant of γ-aminobutyric acid type-A receptor sensitivity to propofol. *Anesthesiol.***107**: 412-418.

Siegwart R, Jurd R, Rudolph U (2002) Molecular determinants for the action of general anesthetics at recombinant $\alpha_2\beta_3\gamma_2$ γ-aminobutyric $acid_A$ receptor. *J. Neurochem*. **80**: 140-148.

Thorup L, Wurtzen G, Carstensen J, Olsen P (1983) Shortterm toxicity in rats dosed with pulegone and menthol. *Toxicol. Lett*. **19**, 207-210.

Tomlin SL, Jenkins A, Lieb WR, Franks NP (1998) Stereoselective effects of etomidate optical isomers on gamma-aminobutyric acid A receptors and animals. *Anesthesiol*. **88**: 708-717.

Ueno S, Zorunski C, Bracamontes J, Steinbach JH (1996) Endogenous subunits can cause ambiguities in the pharmacology of exogenous γ-aminobutyric $acid_A$ receptors expressed in human embryonic kidney 293 cells. *Mol. Pharmacol*. **50**: 931-938.

Watt EE, Betts BA, Kotey FO, Humbert DJ, Griffith TN, Kelly EW, Veneskey KC, Gill N, Rowan KC, Jenkins A, Hall AC (2008) Menthol shares general anesthetic activity and sites of action on the $GABA_A$ receptor with the intravenous agent, propofol. *Eur J. Pharmacol*. **590**: 120-126.

Wong G, Sei Y, Skolnick P (1992) Stable expression of type I γ-aminobutyric $acid_A$/benzodiazepine receptors in a transfected cell line. *Mol. Pharmacol*. **42**, 996-1003.

Yip GMS, Chen Z-W, Edge CJ, Smith EH, Dickinson R, Hohenester E, Townsend RR, Fuchs K, Sieghart W, Evers AS, Franks NP (2013) A propofol binding site on mammalian $GABA_A$ receptors identified by photolabeling. *Nature Chem. Biol*. **11**: 715-20

Footnotes

This work was supported by grants from the Howard Hughes Medical Institute to Celine Croft and Shikha Goel, from a Tomlinson Award to Luvana Chowdhury, and from Blakeslee Foundation funding to Adam Hall. G.G. Pillai received funding from the European Social Fund under project 1.2.0401.09-0079, University of Tartu.

Legends for Figures

Figure 1. Structures of 2,6-dimethylcyclohexanol diastereomers and enantiomers.

Figure 2. Binding site and hydrophobicity of the target $GABA_A$ β3-subunit protein Chain A PDB ID : 4COF with *cis,cis* 2,6-dimethylcyclohexanol. Binding site is in the adjacent subunit of membrane region (M1 and M2) close to the Cys-loop.

Figure 3. Current recordings illustrating modulation by 2,6-dimethylcyclohexanol isomers of $GABA_A$ receptor activity.

WSS-1 cells were held at -50 mV and 10 μM GABA was applied for the duration (2 s) of the shaded boxes above each trace. Upper traces indicate current evoked in the presence of GABA alone. Superimposed lower traces indicate current evoked in presence of 10 μM GABA + 30 μM of the 2,6-dimethylcyclohexanol isomer(s) specified. A) with co-application of the commercially available mixture of 2,6-dimethylcyclohexanol isomers. B) with co-application of *cis,cis* 2,6-dimethylcyclohexanol. C) with co-application of *trans,trans* 2,6-dimethylcyclohexanol. D) with co-application of *cis,trans* 2,6-dimethylcyclohexanol.

Figure 4. *cis,cis* 2,6-dimethylcyclohexanol is the most potent of the isomers for positive modulation of $GABA_A$ receptor currents.

Relative modulations of GABA (10 μM) responses were compared by co-applying increasing concentrations (1-300 μM) of 2,6-dimethylcyclohexanol isomer(s). With the exception of *cis,trans* 2,6-dimethylcyclohexanol, the isomers were effective as positive modulators of GABA

currents with *cis,cis* 2,6-dimethylcyclohexanol demonstrating the most potent current enhancement. Data points represent mean ± S.E.M. modulations for n ≥ 5 cells.

Figure 5. GABA concentration-response curves are shifted to the left with co-application of *cis,cis* and *trans,trans* 2,6-dimethylcyclohexanol isomers.

Currents were normalized against the maximum current evoked by any concentration of agonist ± co-application of 2,6-dimethylcyclohexanol. Data (n ≥ 5 , mean ± S.E.M.) were fitted with the Hill equation (mentioned in the Materials and Methods) in the absence (filled square) and presence of *cis,trans* 2,6-dimethylcyclohexanol (open circles), *cis,cis* 2,6-dimethylcyclohexanol (open triangles), and *trans,trans* 2,6-dimethylcyclohexanol (closed inverted triangles).

Figure 6. 3D interpretations (6a, 6b, 6c) and 2D depictions (6a', 6b', 6c') of molecular docking interactions between the ligands and $GABA_A$ receptor (β_3-subunit). 6a represents the *cis,cis* 2,6-dimethylcyclohexanol, 6b represents *trans,trans* 2,6-dimethylcyclohexanol, and 6c represents *cis,trans* 2,6-dimethylcyclohexanol with hydrogen bond interactions (red color), bond distance in Å (red color) and hydrophobic interaction regions in the blue colored surfaces. H-Bond distances - 6a/6a' : O - GLN224 = 1.898Å; H - TYR 220 = 1.904Å, 6b/6b' : O - GLN224 = 2.014 Å; H - TYR 220 = 1.891Å, and 6c/6c' : O - GLN224 = 2.152Å; H - TYR 220 = 2.054Å. The amino acid residues represented in the blue discs of 6a', 6b', 6c' show the hydrophobic residues in 2D depiction, the green color disc represents van der Waals residue interactions and the purple discs represent electrostatic interactions. Definitions for the various interacting elements represented in the 2D depiction (6a', 6b', 6c) are given below. 'Circled' residues in the 2D diagrams are indicative of residues within hydrophobic interaction regions and H-bonding interaction regions

JPET Fast Forward. Published on March 30, 2016 as DOI: 10.1124/jpet.115.228890
This article has not been copyedited and formatted. The final version may differ from this version.

while those outside are other relevant binding site residues. Note, in each case only residues within 4Å distance from the ligand are depicted.

Element	**Description**
	Residues involved in hydrogen-bond, charge or polar interactions are represented by purple circles.
	Residues involved in van der Waals interactions are represented by green circles.
	The solvent accessible surface of an interacting residue is represented by a blue halo around the residue. The diameter of the circle is proportional to the solvent accessible surface.
	Hydrogen-bond interactions with amino acid main chains are represented by a green dashed arrow directed towards the electron donor.
	Hydrogen-bond interactions with amino acid side-chains are represented by a blue dashed arrow directed towards the electron donor.

Figure 7. 3D interpretations (7) and 2D depictions (7') of molecular docking interactions between the propofol and $GABA_A$ receptor (β_3-subunit). H-Bond distances: H - GLN224 = 1.874Å. The amino acid residues represented in the blue circle of 7' shows the hydrophobic residues in 2D depiction and green color disc represents van der Waals residue interactions and

purple represents electrostatic interactions. 'Circled' residues in the 2D diagrams are indicative of residues within hydrophobic interaction regions and H-bonding interaction regions while those outside are other relevant binding site residues. Note, in each case only residues within 4Å distance from the ligand are depicted.

.

Figure 8. Current recording illustrating minimal attenuation by *cis,trans* 2,6-dimethylcyclohexanol isomer of the positive modulation of $GABA_A$ receptor activity by propofol. A WSS-1 cell was held at -50 mV and 3 μM GABA was applied for the duration (2 s) of the open boxes above the trace. During the period of the checkered box a current was evoked by the presence of 3 μM GABA + 10 μM propofol. . During the filled box a current was evoked by the presence of 3 μM GABA + 10 μM propofol + 100 μM *cis,trans* 2,6-dimethylcyclohexanol resulting in only moderate inhibition of the propofol-induced positive modulation. Positive modulation by propofol was attenuated by 14.1 +/- 1.3 % (n=4) and by 13.4 +/- 2.5 % (n=5) by 100 and 300 μM *cis,trans* 2,6 dimethylcyclohexanol, respectively

Table 1. Quantum Chemical properties in vacuum.

Properties/ Ligand	E(RHF) Hatree	Dipole Moment Debye	E (Thermal) kcal/mol	Heat Capacity cal/mol-K	Entropy cal/mol-K	Zero-point Energy Correction Hatree
cis,cis	-386.97113	1.815	161.11	35.239	91.083	0.247
trans,trans	-386.97115	2.169	160.99	35.556	91.592	0.247
cis,trans	-386.96832	1.891	161.18	35.374	90.988	0.247

JPET Fast Forward. Published on March 30, 2016 as DOI: 10.1124/jpet.115.228890
This article has not been copyedited and formatted. The final version may differ from this version.

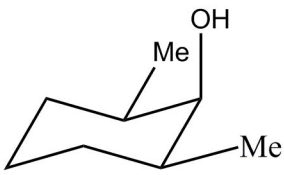
Table 2. Molecular Docking Results: Docked ligand scoring parameters and interacting amino acid residues

Properties/ Ligand	**B.E. (kcal/mol)**	**prKi (µM)**	**L.E**	**I.E. (kcal/mol)**	**vHd.E. (kcal/mol)**	**H-Bond**	**Hydrophobic Interactions**
cis,cis	-4.42	572.09	-0.49	-4.72	-4.51	TYR220, GLN224	TYR220, PHE221, LEU268
trans,trans	-4.38	620.36	-0.49	-4.67	-4.42	TYR220, GLN224	TYR220, PHE221, LEU268
cis,trans	-4.21	813.66	-0.47	-4.51	-4.36	TYR220, GLN224	TYR220, PHE221, LEU268
propofol	-4.96	231.96	-0.38	-5.85	-5.71	GLN224	TYR220, THR225, LEU268, GLN224

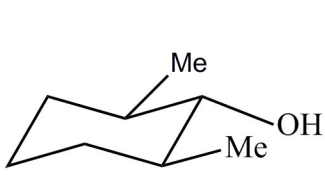
B.E. = Binding Energy, prKi = Predicted Inhibition Constant, L.E. = Ligand Efficiency, I.E. = Intermolecular Energy, vHd.E. (van der Waal's desolvation Energy) = vdW + Hbond + desolv Energy

Table 3. Molecular Docking Results: H-Bond distance (Å)

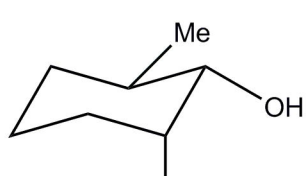
Residues/ Ligand	**TYR 220**	**GLN 224**
cis,cis	1.904	1.898
trans,trans	1.891	2.014
cis,trans	2.054	2.152
propofol	-	1.874



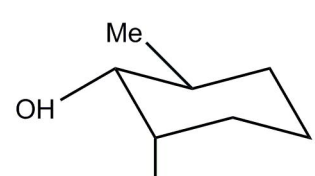
cis,cis



trans,trans



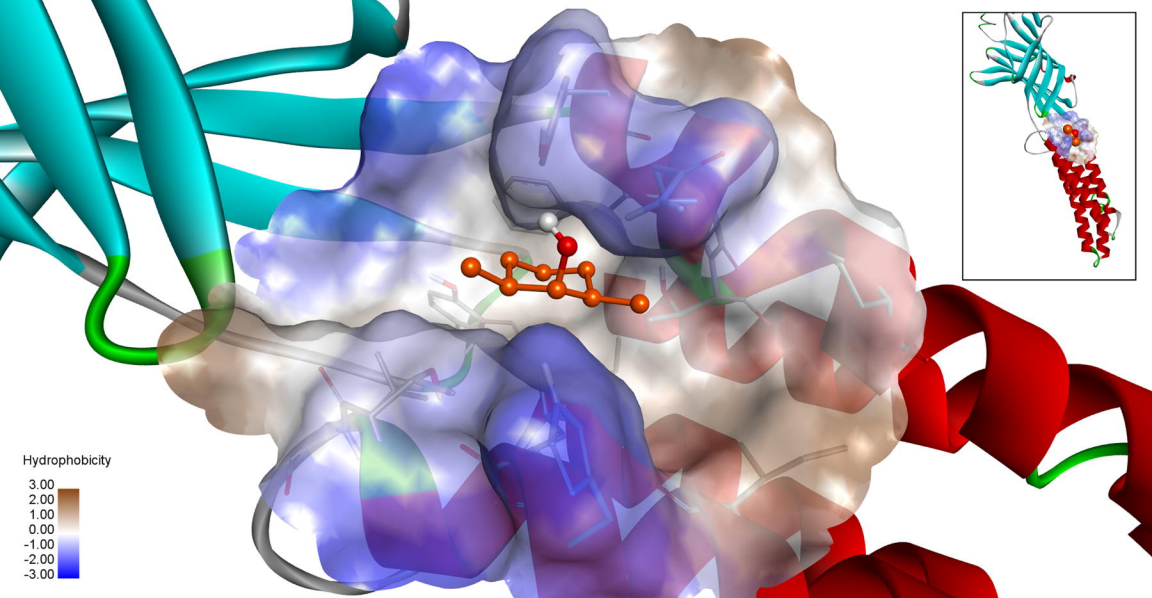
cis,trans



cis,trans

Enantiomers

Figure 1


Hydrophobicity
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00

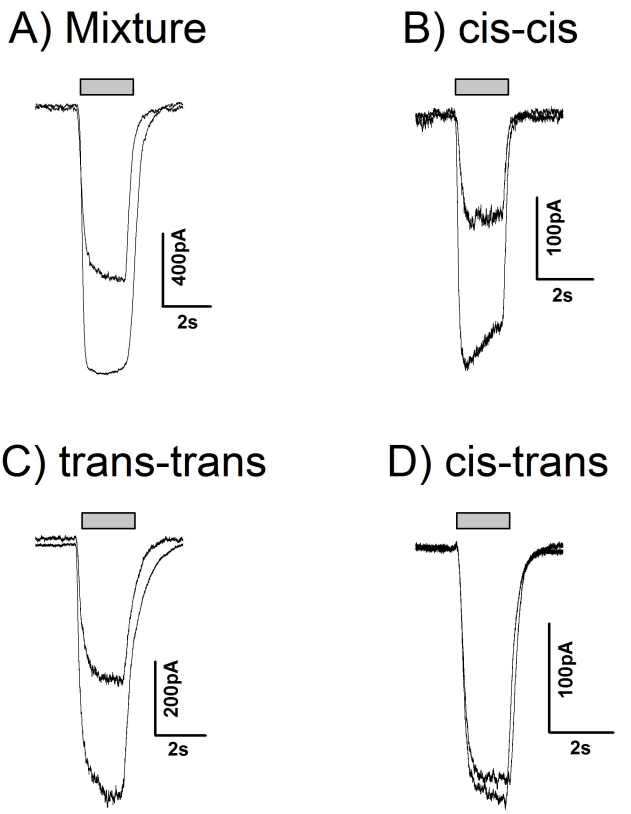

A) Mixture
400pA
2s
B) cis-cis
100pA
2s
C) trans-trans
200pA
2s
D) cis-trans
100pA
2s

Figure 3

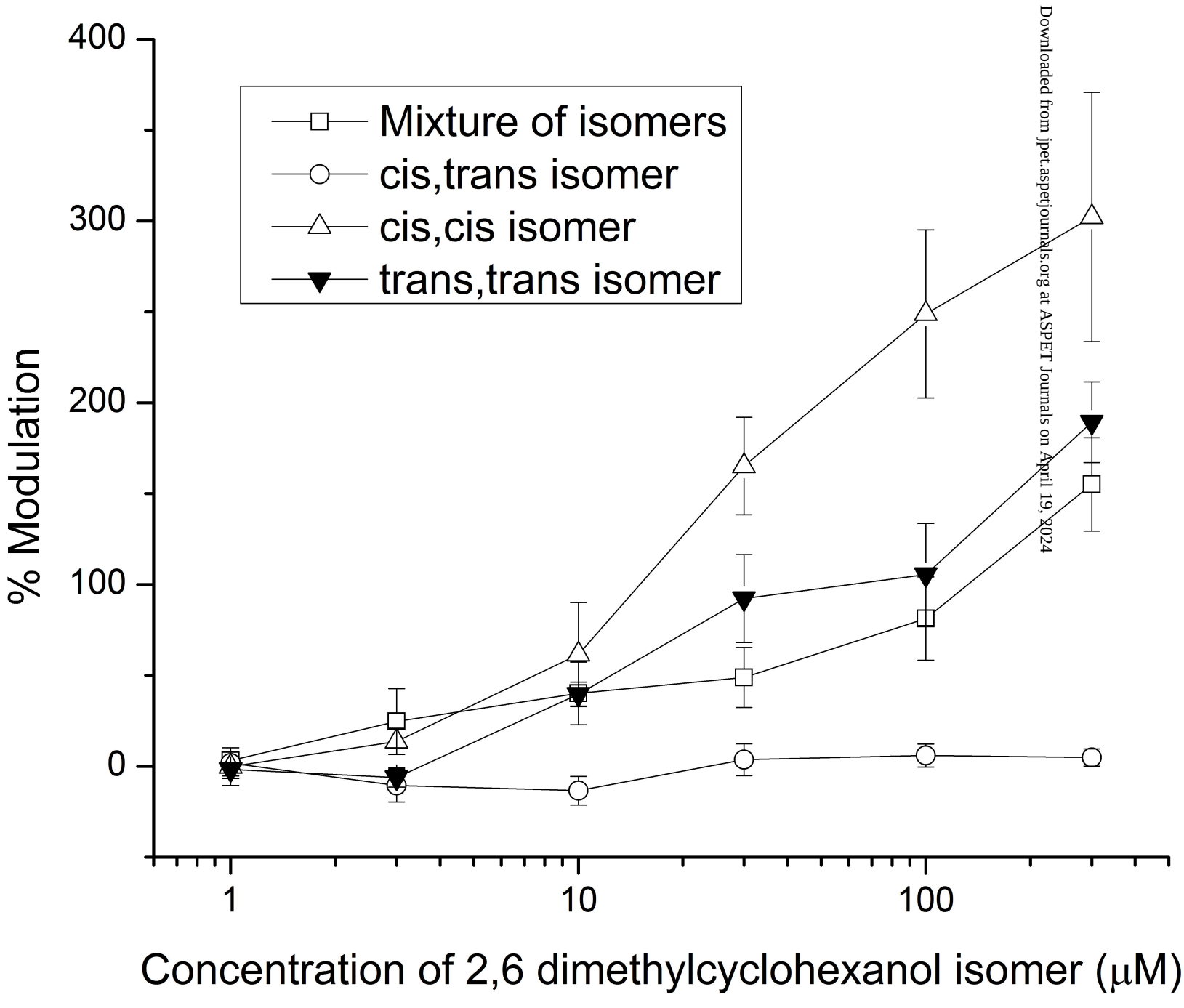


Figure 4

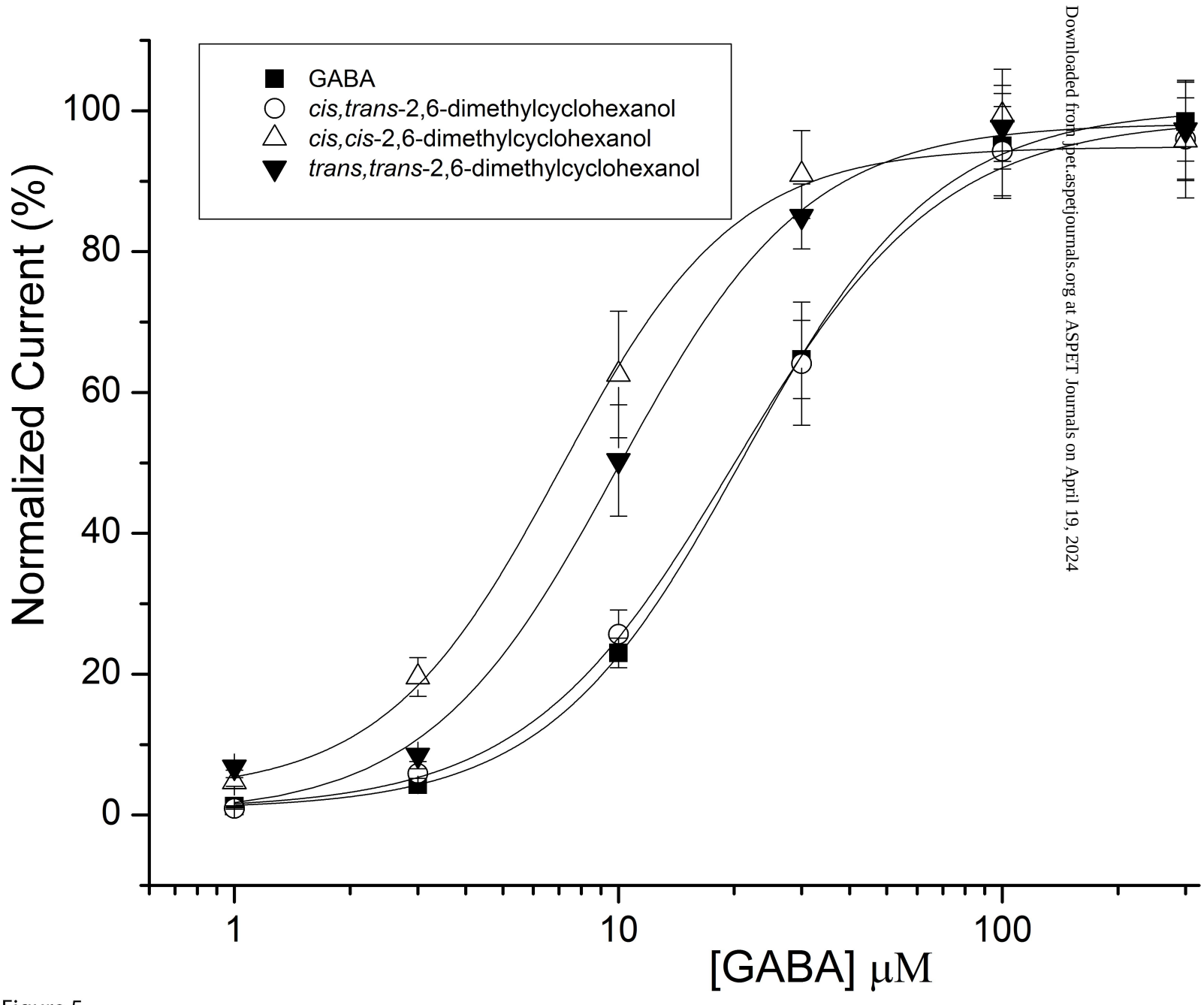
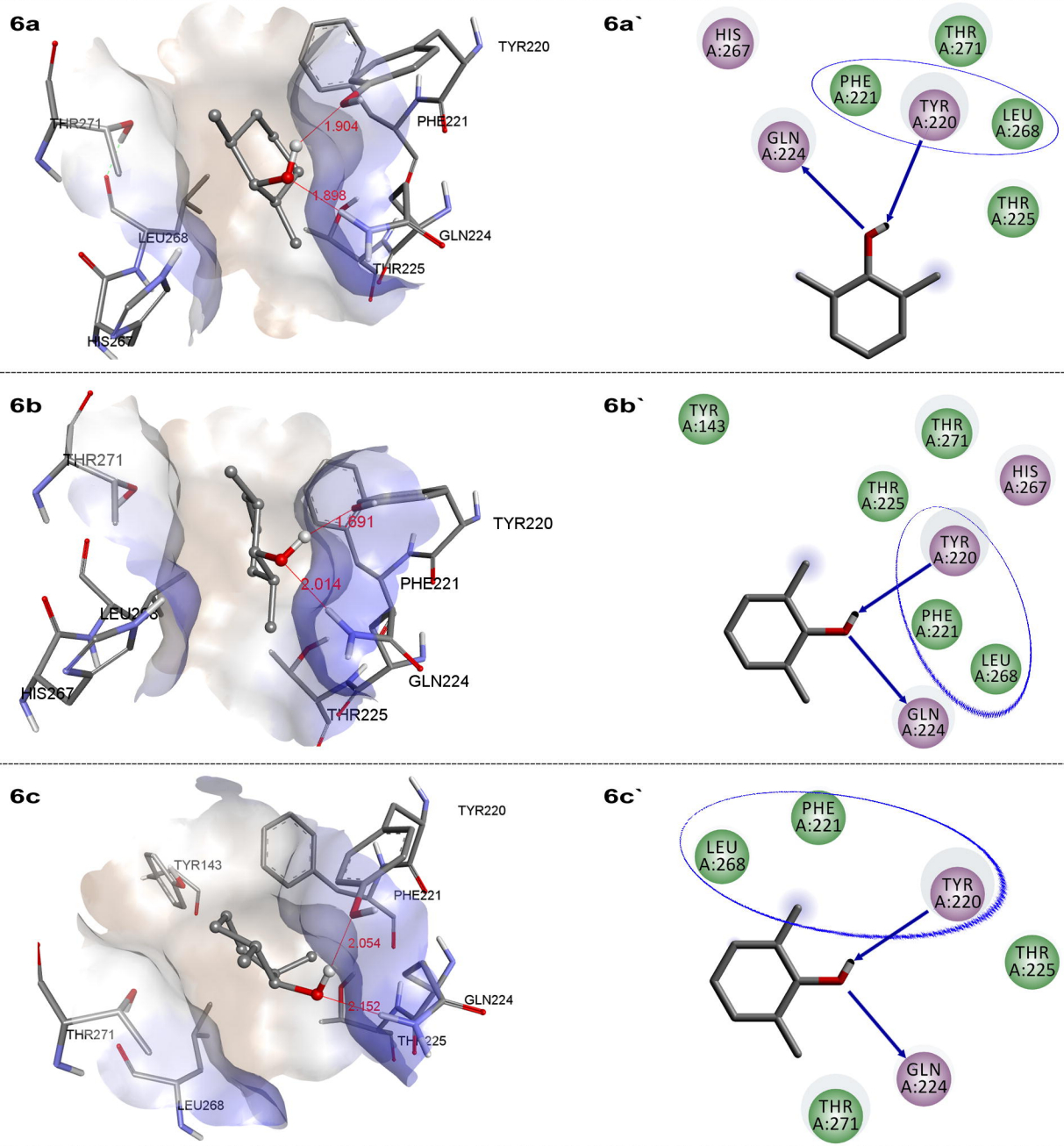
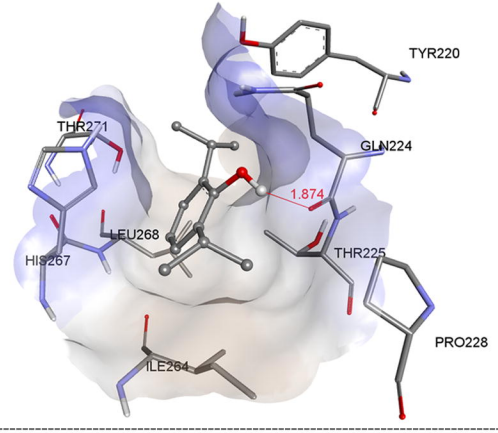


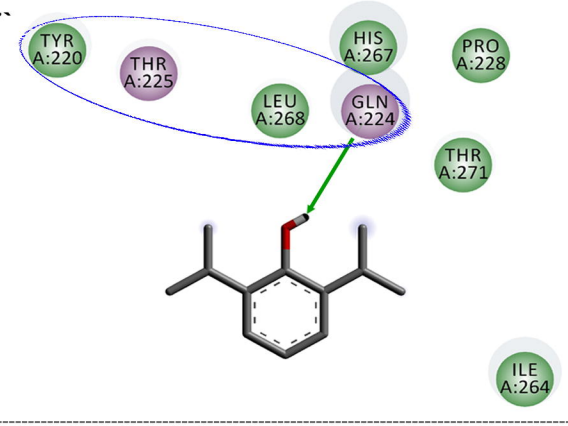
Figure 5


6a
TYR220
THR271
1.904
PHE221
1.898
LEU268
GLN224
THR225
HIS267
6a`
HIS A:267
THR A:271
PHE A:221
TYR A:220
LEU A:268
GLN A:224
THR A:225
6b
THR271
1.891
TYR220
2.014
PHE221
LEU268
HIS267
GLN224
THR225
6b`
TYR A:143
THR A:271
HIS A:267
THR A:225
TYR A:220
PHE A:221
LEU A:268
GLN A:224
6c
TYR220
TYR143
PHE221
2.054
GLN224
2.152
THR271
THR225
LEU268
6c`
PHE A:221
LEU A:268
TYR A:220
THR A:225
GLN A:224
THR A:271

7


TYR220
THR271
GLN224
1.874
LEU268
HIS267
THR225
PRO228
ILE264

7`


TYR A:220
THR A:225
HIS A:267
PRO A:228
LEU A:268
GLN A:224
THR A:271
ILE A:264