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γ-Hydroxybutyric Acid-Ethanol Drug-Drug Interaction: Reversal of toxicity with MCT1 inhibitors

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No author has an actual or perceived conflict of interest with the contents of this article.

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Running title: Reversal of toxicity of GHB-Ethanol Drug-Drug Interaction

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Abbreviations:

ABEC: area below the effected curve; A_e: total amount of GHB excreted unchanged in the urine; ARC: AR-C155858; AUC: area under the curve; AZD: AZD-3965, C_{max}: maximum concentration; CL: total clearance; CL_{NR}: non-renal clearance; CL_R: renal clearance; DDI: drug-drug interaction; E_{max}: maximum effect; EtOH: ethanol; GHB: gamma-hydroxybutyric acid; IACUC: Institutional Animal Care and Use Committee; i.g.: intragastric; i.v.: intravenously; LRR: loss-of-righting reflex; MCT: monocarboxylate transporters; p.o.: orally; RRR: return-to-righting reflex; RT: room temperature; SMCT: sodium -coupled monocarboxylate transporter; TD: toxicodynamics; TK: toxicokinetics

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Abstract:

The drug of abuse, γ-hydroxybutyric acid (GHB), is commonly co-ingested with ethanol, resulting in a high incidence of toxicity and death. Our laboratory has previously reported that GHB is a substrate for the monocarboxylate transporters (MCT), necessary for its absorption, renal clearance, and tissue distribution, including across the blood-brain barrier. Our goal was to investigate the drug-drug interaction (DDI) between GHB-ethanol and evaluate MCT1 inhibition as a strategy to reverse toxicity. The toxicokinetics of this DDI were investigated, including brain to plasma concentration ratios, in the presence and absence of ethanol. The toxicodynamic parameters examined were respiratory depression (breathing frequency, tidal volume) and sedation (time of return-of-righting reflex). Ethanol was administered (2 g/kg i.v.) 5 min before the i.v. or oral administration of GHB, and MCT1 inhibitors, AZD-3965 and AR-C155858 (5mg/kg i.v.) were administered 60 min after GHB administration. Ethanol administration did not alter the toxicokinetics or respiratory depression caused by GHB after i.v. or oral administration; however, it significantly increased the sedation effect, measured by return-to-righting time. AZD-3965 or AR-C155858 significantly decreased the effects of the co-administration of GHB and ethanol on respiratory depression and sedation of this DDI, and decreased brain concentrations and the brain/plasma concentration ratio of GHB. The results indicate that ethanol co-administered with GHB increases toxicity and MCT1 inhibition is effective in reversing toxicity by inhibiting GHB brain uptake when given post-GHB/ethanol administration.

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Significance statement:

The aim of this project was to investigate the enhanced toxicity observed clinically when GHB is co-ingested the alcohol, and evaluate strategies to reverse this toxicity. The effects of the novel MCT1 inhibitors AR-C155858 and AZD-3965 on this drug-drug interaction have not been studied before, and our preclinical studies indicate that MCT1 inhibitors can decrease brain concentrations of GHB by inhibiting brain uptake, even when administered at times after GHB/ethanol. AZD-3965 represents a potential treatment strategy for GHB/ethanol overdoses.

Introduction:

Gamma-hydroxybutyric acid (GHB) is a Schedule I/III class drug that is currently used clinically for the treatment of narcolepsy (sodium oxybate, Xyrem®), for alcohol and opioid withdrawal (Alcover®), and as an anesthetic (Somsanit®)(Carter et al., 2009). However, its clinical use is limited due to high abuse potential, since its ingestion produces euphoria, sociability, sexual arousal, relaxation and altered states of consciousness (Bosch et al., 2017; Raposo Pereira et al., 2019). In recent years GHB use has been associated with ‘chemsex’, which is sex under the influence of psychoactive drugs. The abuse of GHB in this scenario is due to its effects on sexual arousal and longevity (Frankis et al., 2018).

GHB overdose can lead to seizures, dizziness, nausea, vomiting, as well as respiratory depression that can lead to coma or death (Morse et al., 2012; Roiko et al., 2012; Vijay et al., 2015). The use of physostigmine and naloxone has been studied as treatments for GHB overdose with minimal success, and current treatment is limited to

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supportive care (Morris and Felmlee, 2008). European Drug Emergencies Network reported GHB as the fourth most commonly used drug, after heroin, cocaine, and cannabis (2013-2014) (Hockenhull et al., 2017). Reports related to GHB toxicity showed that in 33-41% of the cases, GHB was co-ingested with alcohol (Zvosec et al., 2011; Liakoni et al., 2016a). Our laboratory has previously reported toxicodynamic interactions between GHB and ethanol (Morse and Morris, 2013b). Our studies reported no difference in GHB toxicokinetics but significant decreases in tidal volume, which is a compensatory mechanism when respiration decreases, and also a significant increase in sleep time and lethality when GHB was co-administered with ethanol. Other investigators have examined other pharmacodynamic endpoints in investigating this interaction (Cook et al., 2006; Thai et al., 2006a; Johnson and Griffiths, 2013). Studies performed in mice by Cook et al. reported that the co-administration of GHB and ethanol decreased locomotor activity when administered together (Cook et al., 2006). Thai et al. observed in their clinical studies that the combination of GHB and ethanol resulted in significantly decreased oxygen saturation, and diastolic and systolic blood pressure (Cook et al., 2006; Thai et al., 2006a).

The pKa of GHB is ~4.7, making the drug ionized at physiological pH. Due to the ionization state, permeation through lipid membranes is limited; therefore, membrane transporters are crucial for its tissue distribution. It has been shown previously that GHB is a substrate for the monocarboxylate transporter (MCT) family (SLC16A) (Wang et al., 2006; Wang et al., 2007), and for the sodium-coupled monocarboxylate transporter (SMCT) family (SLC5A) (Cui and Morris, 2009). The MCT family consists of 14 members but only MCT 1-4 are proton-linked monocarboxylate transporters that

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transport GHB (Wang et al., 2006; Wang et al., 2007; Halestrap, 2013). On the other hand, SMCT only consists of 2 members SMCT1 (SLC5A8) and SMCT2 (SLC5A12), which share similar substrates with MCT1-4 (Vijay et al., 2015). MCT1 is present ubiquitously in the body, while the expression of SMCTs is more restricted and present mainly in the kidney and intestine (Morris and Felmlee, 2008; Cui and Morris, 2009). Only MCT1 is expressed at the blood brain barrier (BBB), where it plays an important role in transport of its substrates into and out of the brain (Vijay and Morris, 2014).

MCT1 inhibition has been shown extensively in our laboratory to improve GHB toxicokinetics and toxicodynamics. The MCT1 substrate and inhibitor L-lactate can reverse toxicity following GHB overdoses. L-lactate can increase the renal and total clearances of GHB, resulting in decreased toxicity. Additionally, at high doses, L-lactate can inhibit the MCT1-mediated uptake of GHB into the brain (Roiko et al., 2012; Morse and Morris, 2013b; Roiko et al., 2013). Other more specific and potent MCT1 inhibitors have been developed by AstraZeneca (figure 1). AR-C155858 has been shown to improve GHB respiratory depression (Vijay et al., 2015), with a K_i of 2.3 nM for the inhibition of MCT1-mediated lactate transport. In rat kidney KNRK cells, AR-C155858 inhibited uptake of GHB with a Ki of 6.5 nM (Vijay et al., 2015). AZD-3965, an analog of AR-C155858 has similar Ki values, and is currently in a Phase I clinical trial in patients with solid tumors or lymphoma (NCT01791595) (Curtis et al., 2017; Halford et al., 2017; Noble et al., 2017). This inhibitor was shown to improve the respiratory depression observed after the oral administration of GHB in preclinical studies (Follman and Morris, 2019).

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The objectives of this investigation were to study 1) the drug-drug interaction between GHB and ethanol *in vivo* when administered together, and 2) the effect of MCT1 inhibitors, AR-C155858 and AZD-3965, on this drug-drug interaction, when administered 60 minutes after GHB/ethanol administration.

Material and Methods:

Chemicals and reagents

The National Institute on Drug Abuse provided sodium GHB. AZD-3965 was obtained from MedKoo Biosciences (Chapel Hill, NC), and AR-C155858 from Chemscene (Monmouth Junction, NJ). Ethyl alcohol USP (200proof) was purchased from Decon Labs (King of Prussia, PA). Deuterated GHB (GHB-d6) was purchased from Cerilliant Corporation (Round Rock, TX). High-performance liquid chromatography grade acetonitrile was purchased from Honeywell Burdick& Jackson (Muskegon, MI).

Animals and Animal Surgery

Male Sprague-Dawley rats (Envigo, Somerset, NJ) weighing 225–305g were used for experiments. Animals were housed under controlled temperature and humidity with artificial 12-hours light/dark cycles and water/food availability ad libitum. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University at Buffalo. The animals were allowed to acclimate to their environment for one week before any procedure. Surgical implantation of the jugular cannula was performed under anesthesia with ketamine/xylazine solution. After surgery, cannulas were flushed daily with 40 IU/mL heparinized saline to maintain patency. Animals were

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allowed to recover a minimum of 3 days after surgery before any experiment was conducted.

Toxicokinetic/Toxicodynamic Interaction studies

Respiratory Depression Studies. The effect of ethanol on GHB induced respiratory depression was measured using whole-body plethysmography, as previously performed in our laboratory (Morse et al., 2012; Morse and Morris, 2013b; Morse and Morris, 2013a; Vijay et al., 2015). Briefly, rats were placed in plethysmography chambers (model PLY4213; Buxco Research Systems, Wilmington, NC) for one hour before the study and allowed to acclimate for 45 minutes before baseline readings were recorded over 15 minutes. Ethanol was administered after these readings were recorded and 5 minutes before GHB was administered. GHB administration was considered time 0, and respiration measures were recorded at 2.5, 5, 7.5, 10, 15, 20, 25, 30, and every 15 minutes after that for a duration of 8 (i.v. administration) or 15 (p.o. administration) hours. The respiratory parameters measured were breathing frequency (rate), tidal volume, and minute volume (rate*tidal volume). Ethanol was administered at a dose of 2 g/kg i.v. as a 50%(v/v) solution in sterile water. GHB was administered 600 mg/kg i.v. or 1500 mg/kg p.o. as a 300 mg/mL solution in sterile water. Intravenous solutions were administered via the jugular vein cannula, and oral administration was performed by oral gavage. To access the effects of MCT1 inhibitors (AR-C155858 and AZD-3965), studies were carried out as described before, with each inhibitor administered 60 minutes after administration of GHB at a dose of 5 mg/kg. Both AR-C155858 and AZD-3965 were administered as a 5 mg/ml i.v. solution in 20 % cyclodextrin/ normal saline via the jugular vein cannula. To study the toxicokinetic

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parameters, blood and urine samples were collected after GHB administration during the study. After oral administration blood samples were collected at times up to 921 min and urine at intervals of 0-2, 2-6, 4-6, 6-12, and 12-15 hours. After i.v. drug administration blood samples were collected at times up to 481 min, and urine at intervals of 0-1, 1-2, 2-4, 4-6, and 6-8 hours.

Sedation Studies. The sedative effect of ethanol and GHB was determined using the return-to-righting reflex (RRR) as the endpoint, as previously performed in our laboratory (Wang et al., 2008; Felmlee et al., 2010a; Morse and Morris, 2013b). Briefly, rats were administered 2.0 g/kg ethanol i.v., 1500 mg/kg GHB p.o. or co-administration of GHB and ethanol. The co-administration consisted of 2 g/kg ethanol i.v. 5 minutes before GHB (1500 mg/kg p.o.). To assess the effects of the MCT1 inhibitor AZD-3965, the drug was administered as a 5 mg/kg i.v. dose via the jugular vein cannula 60 minutes after administering GHB. In all treatment groups, the time of loss-of-righting reflex (LRR) and time of RRR were recorded, and sleep time was determined as RRR-LRR. LRR was determined as the time when the animal could not right itself after being placed on its back and RRR was determined as the time when the animal could right itself. All animals were euthanized at the time of RRR and blood, and brain samples were collected. Brain samples were snap-frozen in liquid nitrogen and stored at -80°C until analysis. Blood samples were centrifuged, and plasma was stored at -80°C until analysis.

Plasma/Brain concentration over time study. The brain to plasma partitioning of GHB was analyzed after administration of oral GHB, as previously performed in our laboratory (Morse and Morris, 2013b; Follman and Morris, 2019). Three different

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groups, with four animals per group, were evaluated: GHB alone, GHB-ethanol, GHB-ethanol with AZD-3965. Animals were administered 1500 mg/kg GHB p.o. or co-administered GHB and ethanol. The co-administration consisted of 2 g/kg ethanol i.v. 5 minutes before GHB (1500 mg/kg p.o.). To assess the effects of AZD-3965, the MCT1 inhibitor was administered 60 minutes after administration of GHB, as a 5 mg/kg i.v. dose via the jugular vein cannula. Groups were sacrificed at 90 minutes after GHB administration, corresponding to 30 min after AZD-3965 administration. Terminal plasma and whole brain samples were collected at the time of sacrifice. Brain samples were snap-frozen in liquid nitrogen and stored at -80°C until analysis. Blood samples were centrifuged, and plasma was stored at -80°C until analysis.

Sample analysis

The plasma, urine, and brain concentrations of GHB were determined using previously validated liquid chromatography coupled to mass spectrometry assays (Morse et al., 2012). Briefly, plasma samples collected before the 241 min period were prepared by diluting 5µL of the sample with 45µL of blank plasma; for plasma samples, after this time point, 50µL of the sample was utilized. The standard curve concentration ranged from 1 to 500µg/mL. GHB standards were prepared by adding 5µL of stock solution to 50µL of blank plasma. GHB-d6, internal standard, (5µL) was added to all samples. The addition of 800µL acetonitrile achieved protein precipitation; samples were centrifugated for 20 minutes at 10,000 rpm at 4^{o}C. Supernatant was dried under a stream of nitrogen and reconstituted in 250µL of the mobile phase. Urine samples were diluted 100-fold with blank urine and 5µL of the internal standard. The standard curve was prepared by adding 5µL of stock solutions to 25µL of blank urine and 5µL of the

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internal standard. Methanol (1mL) was added, and double-distilled water (470µL for samples, 465µL for samples). Samples were centrifugated for 20 minutes at 10,000 rpm at 4^{o}C. Supernatant was transferred to a clean vial for analysis.

Data and statistical analysis

The pharmacokinetic parameters were determined using Excel add-ins PK solver (Zhang et al., 2010). The area under the plasma concentration-time curve (AUC) was determined using the trapezoidal method. The total clearance (CL) was determined as dose/AUC, the renal clearance (CL_R) was determined as A_e/AUC, where A_e is the total of GHB excreted unchanged in the urine, the non-renal clearance (CL_{NR}) was determined as CL-CL_R, and terminal half-life ($t_{1/2}$) was determined as ln(2)/λ, and V_{ss} was determined as mean residence time (MRT)*CL. Maximal concentration (C_{max}) and time of maximal concentration (T_{max}) after oral administration were also determined. The pharmacodynamics parameters analyzed were the area below the effect curve (ABEC) and maximum effect (E_{max}). These parameters were obtained using Graph Pad Prism 7 (GraphPad Software, La Jolla, CA). One-way analysis of variance followed by Tukey's test was performed on the parameters obtained for all studies to determine statistical significance using Graph Pad Prism 7. Differences resulting in $p < 0.05$ were considered significant.

Control toxicokinetic and toxicodynamic data was obtained from previous work in our laboratory (Morse and Morris, 2013b; Morse and Morris, 2013a; Vijay et al., 2015). The historical data was used to reduce the number of animals utilized in these studies. All experiments were conducted utilizing the same equipment and protocols.

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Results:

Effect of ethanol and MCT1 inhibitors on GHB toxicokinetics after intravenous administration. The plasma concentrations of GHB after it was administered alone or co-administered with ethanol are shown in figure 2 and table 1. The administration of ethanol did not alter GHB plasma concentrations, clearance or the apart volume of distribution. Notably, there was an increase in the terminal half-life. When AR-C155858 or AZD-3965 was administered 60 minutes after GHB and ethanol administration, there was a non-significant increase in renal CL and small decrease in non-renal CL. However, the area under the curve (AUC) and total clearance values were not significantly different from GHB/ethanol treated controls. The apparent volume of distribution values of GHB after GHB/ethanol/AR-C155858 or AZD-3965 administration were lower than that after GHB alone, although the value was just significant after AR-C155858 administration. The half-life of GHB was significantly greater after AR-C155858 administration compared with GHB administered alone.

Effect of ethanol and MCT1 inhibitors on GHB-induced respiratory depression after administration of intravenous GHB. GHB-induced respiratory depression after the administration of GHB alone, GHB/ethanol and with treatment with the MCT1 inhibitors AZD-3965 and AR-C155858 is shown in figure 3. The administration of ethanol did not have an effect on GHB-induced respiratory depression (figure 3A). MCT1 inhibition reversed GHB-induced respiratory depression (figure 3B), resulting in decreases in frequency ABEC, with both MCT1 inhibitors, AZD-3965 and AR-C155858. The administration of AZD-3965 also significantly decreased the tidal volume ABEC. No

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effect on E_{max} was expected since the maximal effect of GHB on respiration occurred before the MCT1 inhibitors were administered. The findings are summarized in table 2.

Effect of ethanol and MCT1 inhibitor, AZD-3965, on GHB toxicokinetics after oral administration. The plasma concentrations of GHB after its oral administration alone or following co-administration with ethanol are shown in figure 4 and table 3. The administration of ethanol did not result in statistically significant alterations in the toxicokinetics of GHB. When AZD-3965 was administered 60 min after GHB administration, there were significant decreases in AUC, C_{max}. and T_{max}, and significant increases in CL/F and CL_{NR}/F, V_{ss}/F and CL_R compared to GHB alone.

Effect of ethanol and MCT1 inhibitors AZD-3965 on GHB-induced respiratory depression after administration of oral GHB. The effects of GHB on respiration and tidal volume are shown in figure 5. The administration of ethanol did not have an effect on GHB-induced respiratory depression (figure 5A). The administration of the AZD-3965 (figure 5B) 60 min after GHB dose (dashed line) resulted in a statistically significant reduction in the frequency ABEC (Table 4). There is a significant increase in time to reach maximum respiratory frequency effect (E_{max}) after the administration of AZD-3965. We also observed a significant decrease in tidal volume ABEC, due to the reversal of the respiratory depression.

Effect of ethanol and MCT1 inhibitor AZD-3965 on GHB sedation after administration of oral GHB. As shown in figure 6, although ethanol administered alone has no sedation effect, the co-administration of ethanol with GHB significantly increased sleep time, compared to GHB alone. Treatment with AZD-3965, 60 minutes after GHB-ethanol, significantly decreased the sleep time compared to GHB-ethanol alone. In table

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5, the plasma and brain concentrations of GHB at RRR are presented. Note that these are concentrations of GHB in plasma and brain determined at 114 min after IV GHB administration alone, 283 minutes after the concomitant administration of IV GHB and ethanol, and 66.5 minutes after the administration of IV GHB/ethanol when AZD-3965 was administered at 60 minutes. Both plasma and brain concentrations at time of RRR are lower following GHB-ethanol administration, compared with those after GHB administration alone. Following AZD-3965 administration to animals receiving GHB/ethanol, there was a significant increase in plasma concentrations since RRR occurred at an earlier time, but brain concentrations were similar to those animals treated with GHB/ethanol, and lower than animals receiving GHB alone. The ratio of brain concentrations to plasma concentrations after AZD-3965 treatment was decreased, compared with GHB ratios after GHB alone or GHB/ethanol.

Effect of ethanol and MCT1 inhibitor AZD-3965 on GHB brain to plasma table 6, the plasma and brain concentrations of GHB at 90 minutes after the GHB dose are presented. Coadministration of 2.0 g/kg ethanol significantly increased the plasma concentration observed with 1500 mg/kg GHB alone at 90 minutes after administration. Both these plasma concentrations are similar to the average plasma concentrations at 90 minutes from the pharmacokinetic study presented in figure 4. Treatment with AZD-3965, 60 minutes after GHB-ethanol, decreased the GHB plasma concentration compared with GHB-ethanol alone at the same time point, i.e., 30 minutes after AZD-3965. As shown in figure 7 and administration. As shown in table 6, significantly lower

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GHB brain concentrations and brain/plasma ratios were observed with the administration of AZD-3965 compared with GHB-ethanol alone.

Discussion:

GHB is most often abused in combination with ethanol, and the combination results in a greatly increased risk for toxicity and death (Kim et al., 2007; Liakoni et al., 2016b; Hockenhull et al., 2017). Therefore, understanding the impact of ethanol on GHB TK and TD is crucial to understanding the potentially enhanced toxicity of GHB when co-ingested with ethanol and for identifying a successful treatment for GHB/ethanol overdose (Liakoni et al., 2016b). In our laboratory, the transport of GHB by MCT1 has been extensively studied (Morris and Felmlee, 2008; Cui and Morris, 2009; Felmlee et al., 2010b; Morse et al., 2012; Morse and Morris, 2013b; Vijay et al., 2015), and studies have reported efficacy of the MCT1 inhibitors, AR-C155858, and AZD-3965, as potential treatment strategies for GHB when GHB is administered alone (Vijay et al., 2015; Follman and Morris, 2019). However, GHB is often ingested with alcohol and therefore it is important to understand if MCT1 inhibitors are also effective in the presence of ethanol. In the current study we have investigated the effect of ethanol on GHB TK, including brain concentrations, and TD, using two measures of toxicity, namely respiratory depression and sedation. We also evaluated the potential of using a specific MCT1 inhibitor to treat overdoses when GHB and ethanol are administered together, by administering the inhibitor 60 minutes post-dose of GHB/ethanol.

Ethanol administration after IV administration of GHB resulted in no change in CL, CL_R and in the apparent volume of distribution, although there was a significant

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increase in half-life. Following oral administration of GHB, there was a trend towards decreased AUC and increased CL/F and CL_{NR}/F. Since changes in CL were not seen after IV GHB administration, the likely mechanism involves changes in bioavailability, which may involve changes in first pass extraction, resulting in a higher value for oral CL (CL/F). These findings are consistent with previous publications demonstrating no or small effects of ethanol on GHB AUC or clearance.

After administration of GHB intravenously and orally, we did not observe any change in GHB respiratory depression when ethanol was co-administered, similar to previous animal and human studies where this DDI has been studied (Thai et al., 2006b; Morse and Morris, 2013b). While ethanol had little to no effect on respiration at the doses used in this study, it is known that ethanol can decrease response to elevated CO_2 concentrations and decrease oxygenation (Thai et al., 2006b). On the other hand, sedation after oral GHB administration is enhanced; there was a significant increase in sleep time (RRR-LRR). Although ethanol itself did not cause sedation, sleep time was doubled, and plasma and brain concentrations at RRR were both lower than those following GHB alone, determined at RRR. Previous studies in our laboratory have demonstrated a brain concentration-sedation relationship in which animals have the same brain concentration at RRR, regardless of the dose of GHB and RRR time after dosing (Felmlee et al., 2010). However, synergistic effects of ethanol on GHB-mediated sedation have been reported in other investigations, including by Cook *et al.* after intragastric administration in mice and Morse and Morris after i.v. administration in rats (Cook et al., 2006; Morse and Morris, 2013b). While ethanol itself does not produce sedation at the dose used in this study, ethanol enhances the sedation observed with

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GHB. While we did not see significant changes in the brain-to-plasma ratios, it is clear that ethanol-GHB interactions in the brain result in changes in sedation, mediated potentially by GABA or other receptors. Our previous studies have examined reversal of the effects of GHB/ethanol on sedation using both $GABA_A$ and $GABA_B$ receptor antagonists (Morse and Morris, 2013b). While a $GABA_A$ antagonist (bicuculline) had no effect on sedation, $GABA_B$ antagonists (SCH50911 and SGS742) were effective, although they did not completely reverse sedation. Therefore, ethanol affects the brain GHB concentration- sedation relationship, in that RRR occurs at lower brain concentrations of GHB in animals receiving GHB and ethanol than with GHB alone. Previous studies have also reported increased lethality when GHB and ethanol are administered together in rats, at doses of GHB and ethanol that were not associated with lethality for either drug (Morse and Morris, 2013b). This is consistent with clinical reports of enhanced toxicity and death of GHB when co-ingested with ethanol (Liakoni et al., 2016b; Hockenhull et al., 2017).

When evaluating changes in the plasma and brain concentrations of GHB, determined 90 minutes after GHB oral administration, we see a significant increase in plasma concentrations when ethanol was co-administered. The plasma concentration values obtained for GHB at this 90-minute time point are consistent with the plasma concentrations observed in our pharmacokinetic study; the estimated mean GHB plasma concentration in the PK study was 838 µg/ml for GHB/ethanol administration, 389 µg/ml for GHB administration. Since GHB is not protein-bound (Morris et al., 2005), this plasma concentration represents the free plasma concentration of GHB. The brain

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concentration was also increased even though it was not significant, and the brain to plasma ratio was not changed, compared to GHB administration alone.

In the current investigation, the effect of the potent and specific MCT1 inhibitors, AR-C155858 and AZD-3965, are studied for the first time following GHB and ethanol co-administration to determine effects on GHB TK and TD. Treatments were administered 60 min after intravenous or oral GHB administration to recreate a realistic situation where treatment after an overdose will be delayed. AZD-3965 and AR-C155858 administration, when administered 60 minutes after the administration of intravenous GHB, resulted in no change in AUC and total clearance. Interestingly, non-renal clearance was significantly decreased after the administration of AR-C155858; this could be to the inhibition of uptake in the liver by MCTs or inhibition of hepatic metabolism, since GHB is extensively metabolized by mitochondrial and cytosolic enzymes in the liver, and metabolism is the major clearance mechanism. The major route of metabolism is oxidation by GHB dehydrogenase to succinic semialdehyde which is converted to succinic acid, followed by further metabolism via the Krebs cycle to the end products carbon dioxide and water (Busardò and Jones, 2015). The differences between the two MCT1 inhibitor analogues on GHB kinetics likely reflect differences in their physicochemical properties and disposition. AZD-3965 is more lipophilic than AR-C155858, and differences in the renal elimination and protein binding of the two compounds may result in the small differences observed in effects on the renal or non-renal CL of GHB (Påhlman et al., 2010; Guan and Morris, 2019). After oral administration of GHB with ethanol, AZD-3965 increased the renal clearance through

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inhibition of the renal reabsorption of GHB, which is dependent on MCT1, as well as decreasing C_{max}, potentially through changes in MCT1-mediated absorption.

Treatment with MCT1 inhibitors reduced the respiratory depression produced by GHB, after i.v. or oral administration alone, or in the presence of ethanol, when administered 60 minutes after GHB/ethanol administration. Published and preliminary studies have demonstrated that AR-C155858 and AZD-3965 have no effect on respiration, and that changes in L-lactate concentrations that may occur with MCT1 inhibitor concentrations do not affect respiration (Vijay et al., 2015; Morse et al. 2014). AZD-3965 administration also significantly decreased sleep time after the oral-administration of GHB/ethanol. Evaluation of plasma and brain concentrations, 30 minutes after AZD-3965 administration, found that brain concentrations were decreased, as was the brain-to-plasma concentration ratio, suggesting that the main mechanism of the effect of AZD-3965 on GHB toxicodynamics was inhibition of MCT1-mediated brain uptake of GHB. MCT1 is the only MCT isoform present at the BBB and responsible for the uptake of GHB and other monocarboxylic acids including L-lactate (Vijay and Morris, 2014; Morris et al., 2017).

This represents the first evaluation of AR-C155858 and AZD-3965 on respiratory depression after the administration of ethanol with GHB, suggesting that MCT1 inhibition may be a strategy to treat overdoses of GHB/ethanol. GHB is often co-administered with ethanol, which results in enhanced toxicity; based on our current and previous pre-clinical studies in rats, there is increased sedation and lethality. An MCT1 transporter inhibitor is effective in reversing toxicity following the co-administration of

oral GHB and ethanol, resulting in decreased brain uptake of GHB, and supporting its use for the treatment of GHB overdoses.

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Authorship Contributions

Participated in research design: Morris, Rodriguez-Cruz

Conducted experiments: Rodriguez-Cruz

Contributed new reagents or analytic tools: Morris, Rodriguez-Cruz

Performed data analysis: Morris, Rodriguez-Cruz

Wrote or contributed to the writing of the manuscript: Morris, Rodriguez-Cruz

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Footnotes:

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Figure legends:

Figure 1. Molecular structures of AR-C155858 (A), and AZD-3965 (B).

Figure 2. GHB plasma concentrations (A) and amount of GHB excreted unchanged (B) after administration of 600 mg/kg GHB i.v., alone, with ethanol and with MCT1 specific inhibitors (administered 60 min after GHB administration). Dashed line represents the time of administration of the treatments. GHB alone data was obtained from previous publications by our laboratory. Data presented as mean (n = 4-5).

Figure 3. Effect of ethanol and treatment with specific MCT1 inhibitors on GHB-induced respiratory depression after administration of GHB i.v. Ethanol was administered as 2 g/kg i.v. bolus. The administration of 600 mg/kg GHB i.v. was 5 min after the ethanol bolus administration (A). AZD-3965 (AZD) and AR-155858 (ARC) (B) were administered 60 minutes after GHB administration at a dose of 5 mg/kg i.v. The dashed line (B)

represents the time of administration of each treatment at 60 min after GHB administration. Data presented as mean ± S.D. (n = 4-6).

Figure 4. Effect of ethanol and treatment with specific MCT1 inhibitors on GHB toxicokinetics after administration oral administration. (A) GHB plasma concentrations over time. (B) The amount of GHB excreted unchanged in the urine. Ethanol was administered as 2 g/kg i.v. bolus. The administration of 1500 mg/kg GHB p.o. was 5 min after the ethanol administration. Administration of AZD-3965 was 60 minutes after GHB administration at a dose of 5 mg/kg i.v. The dashed line represents the time of administration of treatment. Data presented as mean ± S.D. (n = 4-5). Data from 1500 mg/kg GHB alone were used from a previous study (Morse and Morris, 2013b).

Figure 5. Effect of ethanol and treatment with specific MCT1 inhibitors on GHB-induced respiratory frequency and tidal volume after administration of GHB p.o. Ethanol was administered as 2 g/kg i.v. bolus. The administration of 1500 mg/kg GHB p.o. was 5 min after the ethanol administration (A, C). Administration of AZD-3965, 60 minutes after GHB administration at a dose of 5 mg/kg i.v. (B, D). The dashed line represents the time of administration of AZD-3965 at 60 min after GHB administration. Data presented as mean ± S.D. (n = 4). Data from 1500 mg/kg GHB alone were used from a previous study (Morse and Morris, 2013b).

Figure 6. Effect of ethanol co-administration and treatment with AZD-3965 on the toxicodynamic sedative effect of GHB. EtOH (2 g/kg i.v.) was administered 5 min before

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GHB administration. GHB was administered at a dose of 1500 mg/kg by oral gavage and AZD 3965 treatment was administered 60 min after GHB administration at a 5mg/kg i.v. dose. Ethanol infusion was given to see the sedative effects of ethanol alone. Animals were euthanized at RRR. One-way ANOVA followed by Dunnett's post hoc test was used to determine statistically significant differences between groups. Data presented as mean ± S.D. (n = 3–7). *Significantly different from GHB + ethanol. # Significantly different from GHB alone.

Figure 7. Effect of ethanol and AZD-3965 treatment on GHB plasma (A), brain (B), and brain to plasma ratio (C) concentrations at 90 minutes post oral GHB dose. Ethanol (2.0 g/kg i.v.) was administered 5 minutes before GHB. GHB (1500 mg/kg p.o.) was administered at time zero, and AZD-3965 (5 mg/kg) was given intravenously 60 minutes after GHB. Animals were euthanized at 90 minutes post GHB dose. One-way analysis of variance followed by Dunnett's post hoc test was used to determine statistically significant differences compared with GHB plus ethanol. Data are presented as the mean ± S.D. (n = 4-6). *Significantly different from GHB + ethanol.

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Tables:

Table 1. Effect of ethanol and treatment with specific MCT-1 inhibitors on GHB toxicokinetics after intravenous administration.

Parameter	GHB (n=5)	GHB + EtOH (n=4)	GHB + EtOH + AZD (n=4)	GHB + EtOH + ARC (n=4)
AUC (mg*min/mL)	109 ± 4	108 ± 18	115 ± 14	121 ± 15
CL (mL/min/kg)	5.51 ± 0.23	5.72 ± 0.99	5.27 ± 0.58	5.00 ± 0.59
CL_R (mL/min/kg)	1.42 ± 0.56	1.72 ± 0.34	2.22 ± 0.24	2.45 ± 0.60
CL_{NR} (mL/min/kg)	4.09 ± 0.36	4.00 ± 0.71	3.05 ± 0.47	2.55 ± 0.38*#
V_{ss} (ml)	97.5 ± 4.3	95.0 ± 10.6	66.6 ± 36.0	41.5 ± 9.8*
Half-life (min)	15.2 ± 2.7	50.7 ± 14.2*	62.1 ± 52.5	83.8 ± 79.9*

AZD-3965 (AZD) and AR-C155858 (ARC) were administered at a dose of 5 mg/kg 60 minutes after GHB (600 mg/kg iv). Ethanol was administered at a dose of 2 g/kg i.v. 5 minutes before GHB. Data presented as mean ± S.D.

* Significantly different from GHB alone ($P < 0.05$).

Significantly different from GHB + ethanol ($P < 0.05$).

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Table 2. Effect of ethanol and treatment with specific MCT1 inhibitors on GHB-induced respiratory depression after i.v. administration.

Toxicodynamic Parameter	**GHB (n=5)**	**GHB + EtOH (n=4)**	**GHB + EtOH + AZD (n=4)**	**GHB + EtOH + ARC (n=4)**
Frequency ABEC (breaths)	4130 ± 1192	5819 ± 1191	1820 ± 743$^{\#}$	1215 ± 544$^{\#\#}$
Frequency E_{max} (breaths/min)	39.3 ± 2.5	40.5 ± 6.2	44.2 ± 12.2	38.9 ± 5.0
Tidal Volume ABEC (mL/breath/min)	94.5 ± 32.5	88.2 ± 31.1	29.7 ± 4.7*$^{\#}$	42.4 ± 20.9
Tidal volume E_{max} (mL)	3.37 ± 0.61	3.10 ± 0.97	2.58 ± 0.48	3.12 ± 0.95

AZD-3965 (AZD) and AR-C155858 (ARC) were administered at a dose of 5 mg/kg 60 minutes after GHB (600 mg/kg i.v.). Ethanol was administered at a dose of 2 g/kg i.v. 5 minutes before GHB. Data presented as mean ± S.D.

*Significantly different from GHB alone ($P < 0.05$)

$^{\#}$Significantly different from GHB + ethanol ($P < 0.05$), $^{\#\#}P<0.01$

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Table 3. Effect of ethanol and treatment with specific MCT1 inhibitor (AZD-3965) on toxicokinetics after GHB oral administration.

Parameter	GHB (n=4)	GHB + Ethanol (n=4)	GHB + Ethanol+ AZD (n=4)
AUC (mg*min/mL)	230 ± 30	167 ± 7	139 ± 18*
CL/F (mL/min/kg)	6.60 ± 0.86	9.01 ± 0.41	10.9 ± 1.60*
CL_R (mL/min/kg)	1.67 ± 0.55	1.68 ± 0.39	2.72 ± 0.43#
CL_{NR}/F (mL/min/kg)	4.92 ± 1.10	7.33 ± 0.74	8.24 ± 1.68*
V_{ss}/F (mL)	584 ± 161	394 ± 60	814 ± 227#
Urinary recovery (%)	25.8 ± 9.87	18.8 ± 4.93	25.2 ± 5.4
C_{max}	906 ± 111	906 ± 112	489 ± 167#
T_{max}	330 ± 60	121 ± 0	211 ± 114*
$t_{1/2}$ (min)	40.4 ± 22.4	63.2 ± 14.3	74.1 ± 13.0*

AZD-3965 (AZD) was administered at a dose of 5 mg/kg 60 minutes after GHB (1500 mg/kg p.o.). Ethanol was administered at a dose of 2 g/kg i.v. 5 minutes before GHB. Data presented as mean ± S.D.

* Significantly different from GHB alone ($P < 0.05$).

Significantly different from GHB + ethanol ($P < 0.05$).

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Table 4. Effect of ethanol and treatment with specific MCT1 inhibitor (AZD-3965) on GHB-induced respiratory depression after oral administration.

Parameters	**GHB (n=4)**	**GHB + EtOH (n=4)**	**GHB + EtOH + AZD (n=4)**
ABEC (breaths)	8797 ± 39	8978 ± 17	1569 ± 6*
E_{max} (breaths/min)	32.7 ± 9.0	28.1 ± 11.3	61.1 ± 5.2**
Tidal Volume ABEC (mL/breath/min)	239 ± 161	188 ± 89	6.78 ± 3.54[#]
Tidal volume E_{max} (mL)	2.36 ± 0.45	2.51 ± 0.63	1.79 ± 0.28

AZD-3965 (AZD) was administered at a dose of 5 mg/kg 60 minutes after GHB (1500 mg/kg p.o.). Ethanol was administered at a dose of 2 g/kg i.v. 5 minutes before GHB. Data presented as mean ± S.D.

*Significantly different from GHB + EtOH and GHB ($P < 0.05$), **($P < 0.005$)

[#] Significantly different from GHB alone ($P < 0.05$).

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Table 5. Effects of Ethanol and AZD-3965 treatment on GHB plasma and brain concentrations at RRR.

Treatment	Time of RRR (min)	C_{plasma} (µg/mL)	C_{brain} (µg/mL)	Brain/Plasma ratio
GHB (n=3)	114 ± 126*	434 ± 133	171 ± 9	0.426 ± 0.159
GHB + EtOH (n=4)	283 ± 36[#]	308 ± 53	112 ± 21	0.369 ± 0.055
GHB + EtOH + AZD (n=4)	66.5 ± 3.7*	557 ± 50*	84.9 ± 11.5[#]	0.156 ± 0.026*

AZD-3965 (AZD) was administered at a dose of 5 mg/kg 60 minutes after GHB (1500 mg/kg p.o.). Ethanol was administered at a dose of 2 g/kg i.v. 5 minutes before GHB. Data presented as mean ± S.D.

*Significantly different from GHB + EtOH ($P < 0.05$)

[#]Significantly different from GHB alone ($P < 0.05$)

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Table 6. Effects of MCT1 inhibitor and ethanol on the brain to plasma partitioning of GHB 90 minutes post-dose

Treatment	**C_{plasma} (µg/mL)**	**C_{brain} (µg/mL)**	**Brain/Plasma ratio**
GHB (n=4)	353 ± 77*	93.6 ± 24.6	0.268 ± 0.048
GHB + EtOH^ (n=6)	778 ± 206	235 ± 71	0.301 ± 0.039
GHB + EtOH + AZD (n=4)	360 ± 87*	49.2 ± 20.4**	0.145 ± 0.031*

AZD-3965 (AZD) was administered at a dose of 5 mg/kg 60 minutes after GHB (1500 mg/kg p.o.). Ethanol was administered at a dose of 2 g/kg i.v. 5 minutes before GHB. Data presented as mean ± S.D.

*Significantly different from GHB + EtOH ($P < 0.05$), **($P < 0.01$)

^ Outlier (with values more than 2SD from mean of other animals) was removed.

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Figure 1

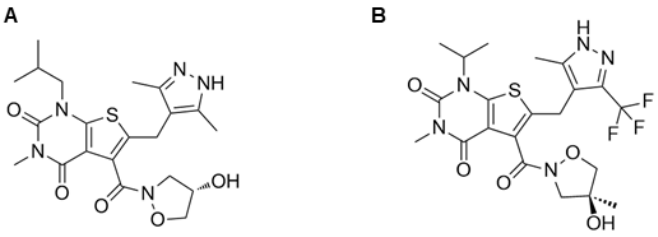

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Figure 2

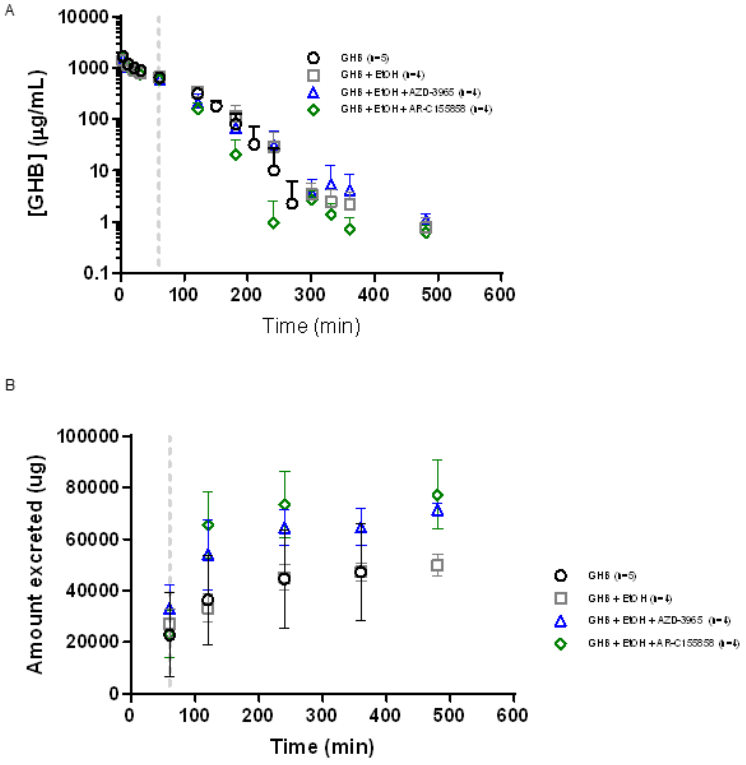

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Figure 3

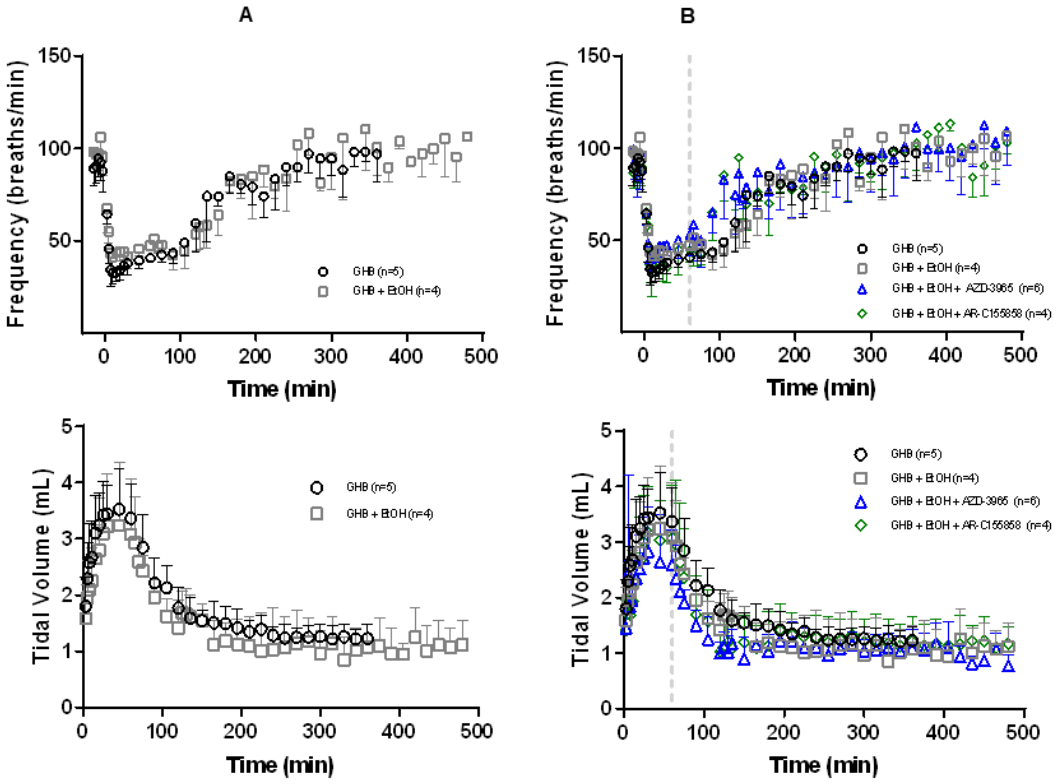

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Figure 4

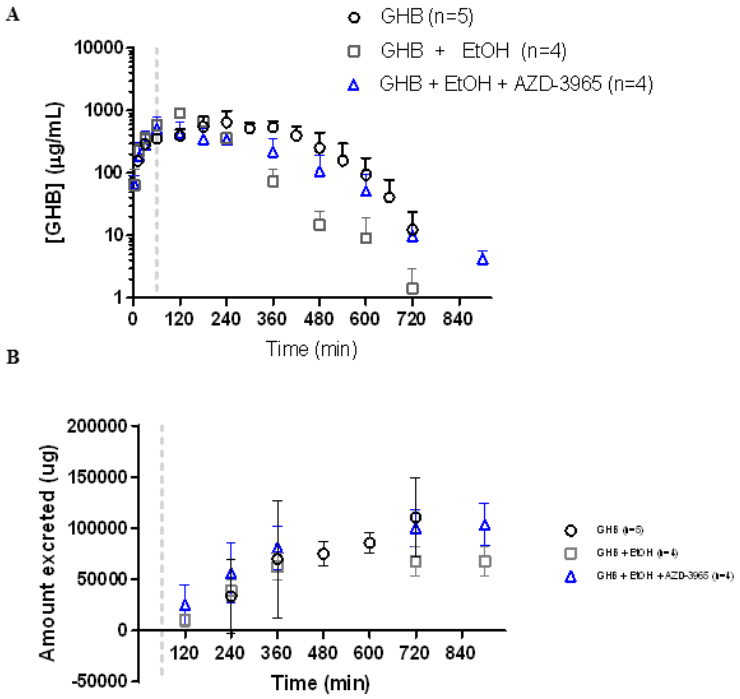

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Figure 5

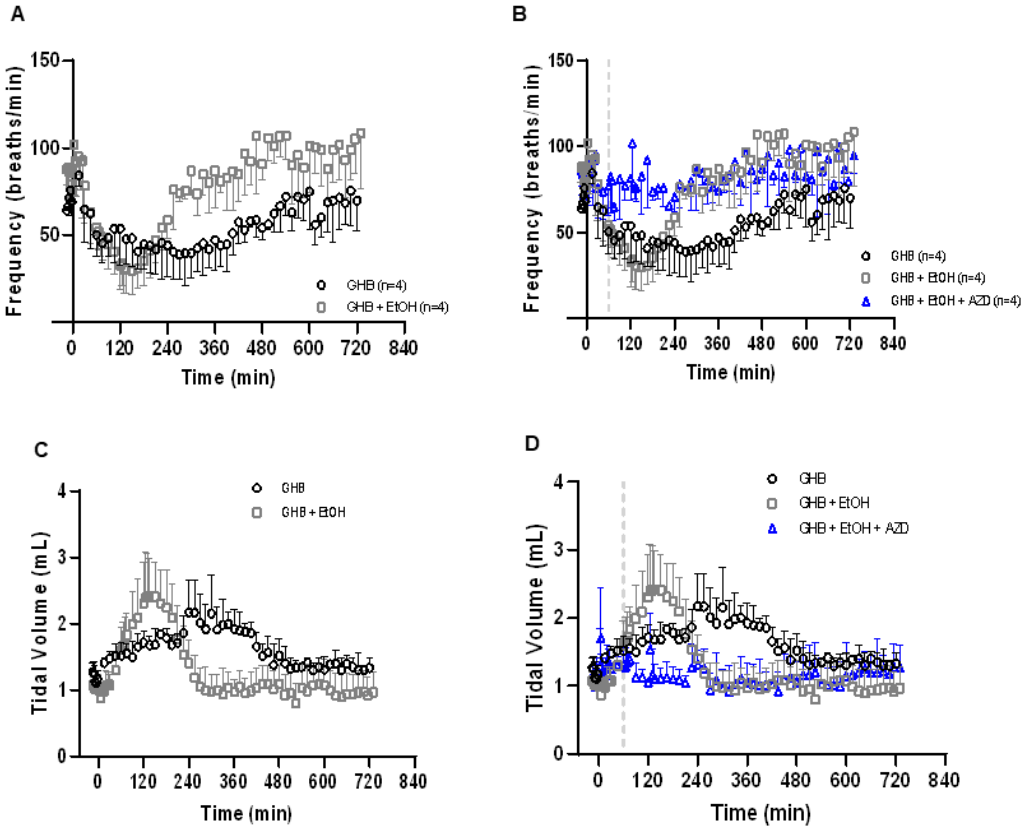

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Figure 6

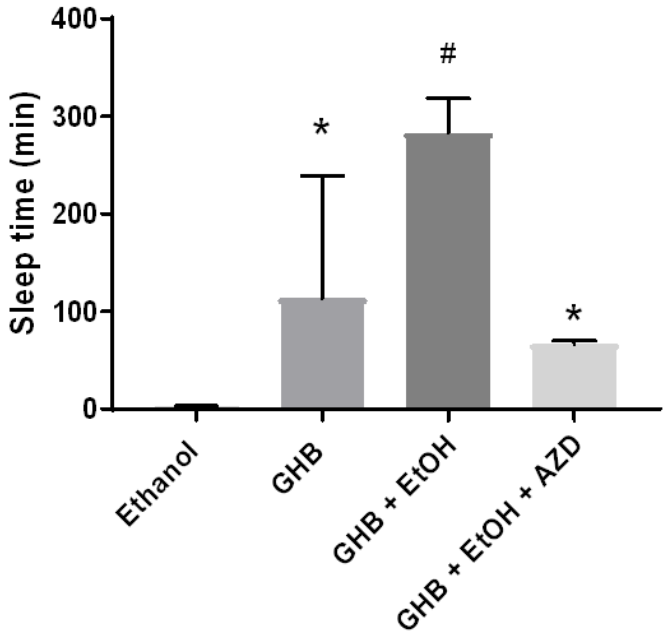

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Figure 7