0022-3565/03/3052-675–679$7.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 305:675–679, 2003

Vol. 305, No. 2
46797/1052807
Printed in U.S.A.

A Tertiary Alcohol Analog of γ-Hydroxybutyric Acid as a Specific γ-Hydroxybutyric Acid Receptor Ligand

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Received November 11, 2002; accepted January 7, 2003

ABSTRACT

γ-Hydroxybutyric acid (GHB) shows great promise as a treatment for sleeping disorders but is also increasingly abused. The exact mechanism of action of GHB is yet to be delineated, but it is known to interact with specific GHB binding sites or receptors, to act as a weak agonist at $GABA_B$ receptors, and that GHB undergoes metabolism to GABA. In drug discrimination studies, $GABA_B$ agonists, and to a lesser extent $GABA_A$-positive modulators, substitute for GHB. To delineate the relative contributions of each receptor system to the profile of GHB, tertiary alcohol analogs of GHB and its homolog, 5-hydroxypentanoic acid (UMB58), were prepared (UMB68 and UMB75, respectively), which cannot be metabolized to GABA-active compounds. Binding studies against [^{3}H]NCS-382 [(2*E*)-(5-hydroxy-5,7,8,9-tetrahydro-6*H*-benzo[*a*][7]annulen-6-ylidene) ethanoic acid] showed that the tertiary alcohol analog of GHB (UMB68) has similar affinity to GHB, with the longer chain analogs possessing lower affinity. Against [^{3}H]GABA, UMB68 showed no affinity (IC_{50} >100 μM) at $GABA_A$ or $GABA_B$ receptors. In vivo studies showed that, at behaviorally active doses, rats trained to discriminate GHB did not recognize the novel ligands as GHB. Thus, UMB68 is a selective GHB receptor ligand in binding assays, will not undergo metabolism to GABA-active compounds, and does not show the same effects as GHB in vivo. These data suggest that, although UMB68 binds to the GHB receptor, it does not have the observed GABA receptor-mediated effects of GHB in vivo and could provide a novel tool for studying the pharmacology of the GHB receptor in the absence of complicating GABAergic effects.

γ-Hydroxybutyric acid (GHB) (Fig. 1) is an endogenous compound that was initially thought to simply be an inactive metabolite of GABA. However, findings that GHB appears to normalize sleep patterns in narcoleptic patients (Nishino and Mignot, 1997) and its increasing popularity as a recreational drug (Bernasconi et al., 1999) have led to a recent growth of interest in GHB (Nicholson and Balster, 2001). GHB is concentrated in specific regions of the mammalian brain where specific binding sites or receptors are located and, to a lesser extent, in some peripheral tissues (Nelson et al., 1981; Vayer and Maitre, 1988; Snead, 1996). GHB rapidly crosses into the central nervous system and is also rapidly metabolized (Bernasconi et al., 1999), the latter being a major factor contributing to its very short duration of action. GHB has also been proposed to be an effective treatment for alcohol (Addolorato et al., 2000; Gessa et al., 2000) and opioid dependence (Gallimberti et al., 1994).

Although GHB binding sites have been described (Benavides et al., 1982; Snead and Liu, 1984; Hechler et al., 1987; Snead and Nichols, 1987; Castelli et al., 2000, Mehta et al., 2001), the exact mechanism of action of GHB remains elusive due, in part, to apparent GABA-mediated effects (Carai et al., 2001, 2002). GHB is known to interact with $GABA_B$ receptors with low affinity (Bernasconi et al., 1992; Xie and Smart, 1992; Mathivet et al., 1997; Lingenhoehl et al., 1999), and it has been demonstrated that GHB is metabolized to GABA (Hechler at al., 1997; Bernasconi et al., 1999). Thus, at the high doses of GHB that are often required to obtain behavioral effects, it is uncertain whether these effects are mediated through the GHB or GABA systems or indeed due to a combined effect on the two systems. Recent studies have shown that the in vivo effects of GHB in rats are reversed by $GABA_B$ antagonists, but not by the GHB antagonist NCS-382 [(2*E*)-(5-hydroxy-5,7,8,9-tetrahydro-6*H*-benzo[*a*][7]annulen-6-ylidene) ethanoic acid] (Carai et al., 2002). In addition, we recently demonstrated that in rats trained to discriminate

We gratefully acknowledge the National Institute on Drug Abuse (NIDA) for support of this work (DA14986). C.P.F. is supported by a Research Scientist Development Award (DA00211).
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
DOI: 10.1124/jpet.102.046797.

ABBREVIATIONS: GHB, γ-hydroxybutyric acid; NCS-382, (2*E*)-(5-hydroxy-5,7,8,9-tetrahydro-6*H*-benzo[*a*][7]annulen-6-ylidene) ethanoic acid; UMB58, 5-hydroxypentanoic acid; UMB68, 4-hydroxy-4-methylpentanoic acid; UMB75, 5-hydroxy-5-methylhexanoic acid; FR, fixed ratio.

GHB, GABA-positive modulators and $GABA_B$ agonists occasion GHB-appropriate responding (Carter et al., 2003). But, it has been reported that the GHB antagonist NCS-382 (Maitre et al., 1990) blocks the discriminative stimulus effects of GHB (Colombo et al., 1995). It is therefore important to develop potent and selective GHB agonists, which are not converted to GABA active compounds through metabolism, to allow the separation of actions mediated through GHB receptors from those mediated through the GABA system.

A vast amount of work has been performed to determine the structural requirements for recognition at GABA receptors (Allan and Johnston, 1983), but relatively little has been performed on GHB (Bourguignon et al., 1988). A recent review collated all the data currently available for recognition at GHB sites, and even the most promising compounds show micromolar affinity against [^{3}H]GHB (Bourguignon et al., 2000). NCS-356 (Fig. 1), a 4-benzyl-substituted analog of GHB with greater affinity than GHB, is increasingly used to study GHB pharmacology (Gobaille et al., 2002). Metabolism to the 4-amino analog gives a compound with low affinity for GABA sites (Allan and Johnston, 1983), because 4-substituted analogs of GABA generally display low activity toward the GABA system. However, NCS-356 would still be prone to rapid metabolism and would be expected to possess a relatively short half-life. The current hypothesis is that if a large benzyl group can be tolerated at the 4-position of GHB, then two small methyl groups would be expected to be tolerated by the GHB binding site. Such a compound would be a tertiary alcohol and, thus, not a substrate for oxidative enzymes. In addition to removing GABA-active metabolites, limiting metabolism would also be expected to lead to an extended duration of action. This study describes the synthesis and pharmacological evaluation of 4-hydroxy-4-methylpentanonic acid (UMB68) and its pharmacological correlation to GHB, as well as the homolog (UMB75) based on 5-hydroxypentanoic acid (UMB58) because UMB58 was previously shown to possess significant affinity at GHB binding sites (Bourguignon et al., 1988) (all structures are shown in Fig. 1).

Materials and Methods

Radiochemical

[^{3}H]NCS-382 was synthesized as described earlier (Mehta et al., 2001). [^{3}H]GABA was purchased from PerkinElmer Life Sciences (Boston, MA).

Drugs

NCS-382 (Fig. 1) was synthesized as described earlier (Maitre et al., 1990). GABA was purchased from Sigma-Aldrich (St. Louis, MO). (±)Baclofen was purchased from Sigma/RBI (Natick, MA), and GHB was purchased from Sigma-Aldrich as the sodium salt.

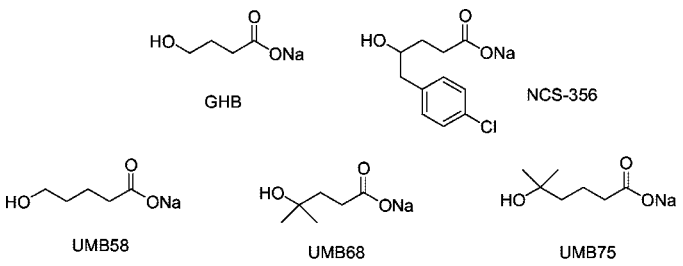

Fig. 1. Structures of GHB, NCS-356, UMB58, UMB68, and UMB75.

Synthesis

All chemicals used in the synthesis of the test compounds were purchased from Sigma-Aldrich. All compounds showed ^{1}H NMR (300 MHz, D_2O) and mass spectral (*m/z* LCQ, negative ion mode; Thermo Finnigan MAT, San Jose, CA) spectra consistent with their assigned structures. Elemental analyses were performed by Atlantic Microlabs Inc. (Norcross, GA) and were within ±0.4% of theory. 5-Hydroxypentanoic acid, sodium salt (UMB58) was prepared by the addition of sodium hydroxide to a MeOH solution of δ-valerolactone, and stirring for 2 h. After removal of the solvent, UMB58 was recrystallized from MeOH (m.p. = 146–147°C). 4-Hydroxy-4-methylpentanoic acid, sodium salt (UMB68) was prepared by a similar hydrolysis of the corresponding lactone prepared by the method of Linstead and Rydon (1933) (m.p. = 159–161°C). 5-Hydroxy-5-methylhexanoic acid, sodium salt (UMB75) was prepared by a similar hydrolysis of the corresponding lactone prepared by the method of Linstead and Rydon (1933) (m.p. = 229–230°C).

Binding Assays

Animals. Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250 to 300 g were used. The animals were maintained at a constant room temperature (22°C) on a 12:12 h light/dark cycle. Food and water were available ad libitum. All experiments were conducted in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Membrane Preparation. Membranes were prepared as described previously (Mehta and Ticku, 2001). Briefly, the rats were decapitated, and the cerebral cortex or cerebellum was dissected. Tissue was stored at −80°C until it was thawed and homogenized in ice-cold 0.32 M sucrose, pH 7.4 (20 ml/g of tissue), and centrifuged at 1,000*g* for 10 min at 4°C. The supernatant was then centrifuged at 140,000*g* for 30 min at 4°C to obtain the mitochondrial plus microsomal (P2 + P3) fraction. This fraction was dispersed in ice-cold double-distilled deionized water, and homogenized by a Brinkmann Polytron (Brinkmann Instruments, Westbury, NY) at a setting of 6 for two 10-s bursts, 10 s apart. The suspension was centrifuged at 140,000*g* for 30 min at 4°C. The pellet was then resuspended in ice-cold Tris buffer (50 mM, pH 7.4) and centrifuged at 140,000*g* for 30 min at 4°C. This step was repeated two more times. After the final centrifugation step, the pellet was suspended in a small volume of ice-cold Tris buffer (50 mM, pH 7.4), and stored frozen at −80°C overnight. On the day of assay, the tissue was thawed and washed two more times with buffer as before (140,000*g*, 30 min, 4°C) and then resuspended in the buffer for use in assay.

[^{3}H]NCS-382 and [^{3}H]GABA Binding Assays. [^{3}H]NCS-382 binding was measured using a centrifugation assay as described by us previously (Mehta et al., 2001). Briefly, aliquots (0.3–0.4 mg of protein) of membrane preparation in Tris buffer (50 mM, pH 7.4) were incubated with [^{3}H]NCS-382 (16 nM) in triplicate at 4°C for 20 min in a 1-ml total volume. Nonspecific binding was determined using NCS-382 (500 μM). The binding reaction was stopped by centrifugation (50,000*g*, 10 min, 4°C). The supernatant liquid was decanted away, and the vials were rapidly rinsed twice with 4 ml of ice-cold Tris buffer (50 mM, pH 7.4) without disturbing the pelleted tissue. Pellets were solubilized with 0.3 ml of Soluene-350 (PerkinElmer Life Sciences) for 4 to 6 h. Scintillation liquid (3 ml) was added to the solubilized material in the bio-vials. Radioactivity was quantified by liquid scintillation spectrometry. For determination of IC_{50} values, [^{3}H]NCS-382 (16 nM) binding was carried out in the absence and presence of a displacer. [^{3}H]GABA (10 nM) binding to the $GABA_A$ receptors in cerebral cortex was also performed in a similar way using 10-min incubation period at 4°C, and GABA (100 μM) was used to define nonspecific binding. For [^{3}H]GABA (10 nM) binding to the $GABA_B$ receptor in rat cerebellum, all the assay tubes contained 40 μM isoguvacine HCl (ICN Pharmaceuticals, Costa Mesa, CA) so as to displace binding to the $GABA_A$ receptors. These

assay tubes also contained calcium chloride (2.5 mM), and the incubation was carried out at 25°C for 10 min. GABA (100 μM) was used to define nonspecific binding. All other assay conditions were similar to those for [^{3}H]NCS-382 binding assays.

Data Analysis. The data are expressed as mean ± S.E.M. IC_{50} data were analyzed using DeltaGraph (DeltaPoint, Monterey, CA). The data were analyzed for each individual experiment, and the mean ± S.E.M. were then calculated.

Behavioral Assays

Animals. Adult male Sprague-Dawley rats (Harlan) were housed individually in plastic cages in a colony room on a 12:12 h light/dark diurnal cycle. Rats were fed 8 to 16 g of chow (rat sterilizable diet; Harlan Teklad, Madison, WI) daily after experimental sessions to maintain body weights at 80% of age-appropriate free-feeding weights. Water was continuously available in the home cage. All animals were maintained in accordance with the Institutional Animal Care and Use Committee, University of Texas Health Science Center at San Antonio, and with the 1996 Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources on Life Sciences, National Research Council, National Academy of Sciences).

Apparatus. Experimental sessions were conducted in sound-attenuating, well ventilated enclosures (model numbers ENV-022M and ENV-008CT; MED Associates Inc., St. Albans, VT) that contained an operant chamber, of which three sides were Plexiglas and the fourth side was a stainless steel response panel equipped with two metal levers 11.5 cm apart. The floor of the operant chamber was a grid comprising 19 rods that were 4.8 mm in diameter, spaced 1.6 cm apart, and oriented parallel to the response panel. A 2.5-cm diameter translucent circle that could be illuminated was located on the response panel above each lever and a 5 cm × 5 cm opening located equidistant between the two levers was available for food pellet delivery. Food pellets (45 mg, PJAI-0045, Noyes Precision Pellets; Research Diets Inc., New Brunswick, NJ) were delivered from a food hopper external to the operant chamber but within the enclosure. Data were collected using MED-PC IV software (MED Associates, Inc.) and a PC interface.

Experimental Sessions. Rats were trained to discriminate 200 mg/kg GHB sodium salt from saline under a fixed ratio (FR) schedule of food presentation. Discrimination training and experimental sessions were carried out as previously described (Carter et al., 2003). Training sessions began with a 15-min pretreatment period, during which the chamber was dark and responses had no programmed consequence, followed by a 15-min response period in which the lights above both levers were illuminated and 10 responses (FR10) on the correct lever resulted in the delivery of a food pellet. A response on the incorrect lever reset the FR requirement on the correct lever. The injection that was administered immediately before the 15-min pretreatment determined which lever was correct and subsequently reinforced. Lever designations were counterbalanced across subjects so that after receiving GHB the left lever was correct for four animals and the right lever was correct for the other four animals. Experimental sessions were conducted 7 days a week and the order of training sessions was generally double alternation (e.g., saline, saline, drug, drug).

All rats had satisfied the following testing criteria prior to commencement of these studies (Carter et al., 2003): at least 90% of the total responses had to be on the correct lever and fewer than 10 responses on the incorrect lever before delivery of the first food pellet. Animals were required to satisfy criteria for at least one saline and one drug training day in 2 of the 3 days before a test (including the day immediately before the test). Test sessions were identical to training sessions with the exception that completion of the FR on either of the two levers resulted in the delivery of a reinforcer. Test compounds (UMB58, UMB68, and UMB75) were evaluated up to a dose of 1778 mg/kg with the order of compounds and the doses tested randomized among subjects.

Drugs. GHB (sodium salt) was dissolved or diluted in sterile water. UMB58, UMB68, and UMB75 were dissolved in sterile water. All injections were given i.p. and were between 0.1 and 1.0 ml in volume and pH 6 to 8.

Data Analysis. Data are reported as the average of at least seven animals ± S.E.M. unless otherwise noted. The percentage of responses on the drug-appropriate lever during the response period of the session is plotted as %DR. Drugs were studied up to doses that markedly decreased rate of responding. Rate is plotted in responses per second. Discrimination data were not plotted when rate of responding was <20% of the control response rate for an individual subject.

Results

Displacement of [^{3}H]NCS-382. Figure 2 clearly shows that the current compounds displace [^{3}H]NCS-382 in a concentration-dependent manner, further indicating that [^{3}H]NCS-382 is an excellent radioligand for measuring the affinity of ligands to GHB receptors. Table 1 shows that GHB displaces [^{3}H]NCS-382 with an IC_{50} of 25 μM. The dimethylated analog of GHB, UMB68, has a similar IC_{50} of 38 μM. The other two compounds possess lower affinity, with UMB58 an IC_{50} of 60 μM and UMB75 a far lower affinity with an IC_{50} of 2000 μM. All three compounds were shown to only weakly displace [^{3}H]GABA from $GABA_A$ receptors at 100 and 1000 μM (Table 2) in a non-dose-related manner. This displacement to about 30% is similar to that seen for GHB and NCS-382. $GABA_B$ receptor affinity was measured in cerebellum where GHB binding to $GABA_B$ receptors has been shown previously (Mathivet et al., 1997). Table 2 shows that GHB does indeed displace [^{3}H]GABA at 100 and 1000 μM, but that UMB58 and UMB68 do not significantly displace the radiolabel.

Drug Discrimination. GHB occasioned dose-related increases in GHB lever responding with >80% of responses on the drug lever after the administration of 200 or 320 mg/kg GHB (Fig. 3, top). GHB also decreased the rate of lever pressing in a dose-related manner, with rates less than 0.2 responses/s after administration of 320 mg/kg GHB. UMB58, UMB68, and UMB75 occasioned predominantly vehicle-lever

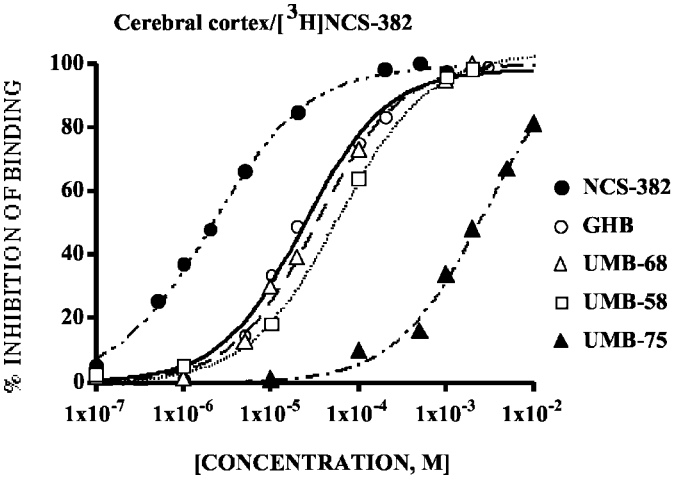

Fig. 2. Representative concentration-dependent inhibition of [^{3}H]NCS-382 (16 nM) binding to the rat cerebrocortical membranes by NCS-382, GHB, and related compounds. Each experiment was performed in triplicate, and these experiments were repeated two to seven times. Average IC_{50} values are summarized in Table 1.

TABLE 1

IC_{50} values of NCS-382, GHB, and related compounds using [^{3}H]NCS-382 (16 nM) as a radioligand in the rat cerebrocortical membranes

Each value is mean ± S.E.M. of number of individual experiments indicated (*n*), and each experiment was performed in triplicate.

Compound	*n*	IC_{50} ± S.E.M.
		μM
NCS-382	7	1.8 ± 0.1[a]
GHB	5	25 ± 1.8[a]
UMB58	2	63 μM, 58 μM[b]
UMB68	3	38 ± 4
UMB75	3	2007 ± 492

[a] Mehta et al. 2001.
[b] These are individual values from two independent experiments.

TABLE 2

Effect of NCS-382, GHB, UMB58, UMB68, and UMB75 on [^{3}H]GABA (10 nM) binding to $GABA_A$ receptors in rat cerebrocortical membranes and $GABA_B$ receptors in rat cerebellum membranes

Each value is mean ± S.E.M. of number of experiments indicated (*n*), and each experiment was performed in triplicate.

Compound	Percentage of Change in Binding (*n*)	
	$GABA_A$ Receptor	$GABA_B$ Receptor
NCS-382, 100 μM	−20 ± 7 (3)	3 ± 3 (3)
NCS-382, 1 mM	−32 ± 10 (3)	1 ± 3 (3)
GHB, 100 μM	−30 ± 5 (3)	−23 ± 4 (6)
GHB, 1 mM	−36 ± 4 (3)	−41 ± 3 (6)
UMB58, 100 μM	−25 ± 11 (3)	−1 ± 5 (3)
UMB58, 1 mM	−25 ± 5 (3)	−8 ± 23 (3)
UMB68, 100 μM	−29 ± 6 (3)	−1 ± 9 (3)
UMB68, 1 mM	−26 ± 10 (3)	2 ± 5 (3)
UMB75, 100 μM	−43 ± 17 (3)	−16 ± 3 (3)
UMB75, 1 mM	−40 ± 15 (3)	−15 ± 2 (3)

responding up to a dose (1778 mg/kg) that decreased markedly or eliminated lever pressing (Fig. 3).

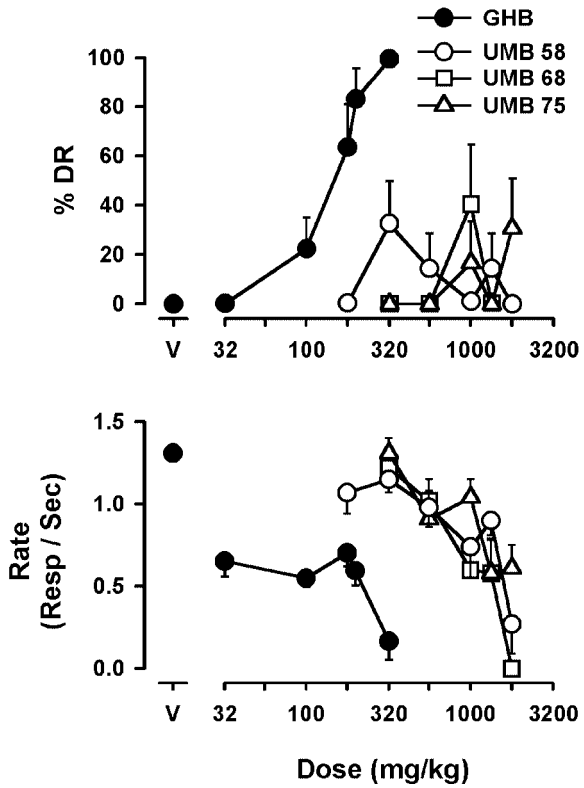

Fig. 3. Discriminative stimulus and rate-altering effects of GHB, UMB58, UMB68, and UMB75 in rats (top panel, %DR = average percentage responding on the GHB-associated lever; bottom panel, average response rate is expressed in responses per second; *V* = vehicle[saline]).

Discussion

Although the pharmacological effects of GHB are reported to be reversible with GHB antagonists (Colombo et al., 1995), it remains unclear as to whether some of the observed in vivo effects are mediated through GABA receptors (Carai et al., 2001, 2002). Such modulation may occur by a direct interaction with $GABA_B$ sites, coupled with the metabolism of GHB to GABA and the interaction of GABA with the GABA system. The current test compounds (UMB58, UMB68, UMB75) were designed to possess no GABA-active metabolites. Binding studies showed that all three displaced [^{3}H]NCS-382, a radioligand for GHB receptors, with IC_{50} values in the low micromolar range. Although all possessed lower affinity than GHB itself, the affinity of UMB68 was less than 2-fold lower than GHB (38 μM versus 25 μM). UMB58, the pentanoic acid analog of GHB, was slightly lower (60 μM), consistent with previous studies with this compound (Bourguignon et al., 1988), and the dimethyl analog, UMB75, possessed much lower affinity at GHB receptors. Although the compounds were designed not to have metabolites with affinity for GABA receptors, an important finding of the current studies is that UMB58 and UMB68 possess no significant affinity at $GABA_B$ sites. Because GHB does possess significant affinity for $GABA_B$ receptors (Bernasconi et al., 1992; Xie and Smart, 1992; Mathivet et al., 1997; Lingenhoehl et al., 1999), this indicates two of the initially complicating, although potentially important, factors from the pharmacology of GHB are avoided with UMB58 and UMB68. Thus, the activity of these compounds in vivo would be expected not to involve GABA systems, allowing the study of the pharmacology of GHB receptors alone. In contrast to the GHB-like discriminative stimulus effects that are obtained under these conditions with the GHB precursor 1,4-butanediol and the $GABA_B$ agonist baclofen (Carter et al., 2003), the three new compounds failed to occasion, on average, greater than 45% GHB-lever responding; for UMB58 and UMB68 this was true up to doses that markedly decreased rates of lever pressing. UMB75 possesses a low affinity for GHB binding sites, so no interpretation of its activity in vivo in terms of GHB can be made. UMB58 and UMB68, however, possess a similar affinity to GHB, yet show no GHB-like activity in behavioral assays, adding more evidence to the hypothesis that the activity of GHB in some behavioral assays is due to activation of the GABA system. Although UMB58 would not be metabolized to a potent GABA metabolite, the primary alcohol of UMB58 would still leave it prone to rapid oxidation and, thus, a short half-life. The fact that UMB68 contains a tertiary alcohol that cannot undergo metabolic oxidation, coupled with its relatively high affinity at GHB receptors, suggests that UMB68 may be an important pharmacological tool to study the GHB system.

Although the literature on GHB is extensive and growing rapidly, interpretation of available data is complicated by the fact that GHB has multiple mechanisms of action in vivo, including direct and indirect actions on the GABA systems. There is a significant need for GHB receptor specific assays that can be used to dissect these multiple mechanism of action. For example, Carai et al. (2002) have reported that

central effects such as the sedative/hypnotic effects of GHB are mediated through the $GABA_B$ receptor and not through the GHB receptor since these effects were blocked by $GABA_B$ antagonists, whereas the GHB antagonist NCS-382 failed to block these effects. Indeed, even the purported GHB antagonist (NCS-382) appears to possess GABA-like activity in vivo (Carter et al., 2003). UMB68 and UMB58 are the first GHB analogs with similar affinity to GHB, which do not possess the behavioral effects seen for GHB. As such, it could be argued that these compounds are GHB antagonists, but without a robust functional assay for measuring the effects of activating the GHB receptors alone, no concrete conclusions can be formulated. However, the fact that a decrease in the rate of responding was observed strongly suggests a central effect. Studies are currently underway to use UMB68 as a tool to determine a functional assay for GHB receptors, without the complication of GABAergic actions.

In conclusion, two compounds, UMB58 and UMB68, interact with GHB receptors with a similar affinity to GHB, have no significant affinity at $GABA_B$ receptors, cannot be metabolized to GABA-active compounds, and do not substitute for GHB in rats. Because the discriminative stimulus and other effects of GHB include significant GABA components of action, the selectivity of the compounds described in this study could prove especially useful for dissecting effects that are mediated specifically through GHB receptors.

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