



Comprehensive evaluation of the pharmacological and toxicological effects of γ -valerolactone as compared to γ -hydroxybutyric acid: Insights from *in vivo* and *in silico* models

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ABSTRACT

Γ -valerolactone (GVL), marketed online as “Tranquilli-G” and “excellent Valium”, is used as a legal substitute for γ -hydroxybutyric acid (GHB); however, until now, GVL has only been connected to one Drug-Facilitated Sexual Assault (DFSA) case. Moreover, the pharmaco-toxicological effects of GVL are poorly studied. The aim of this study was to investigate the 1) *in vivo* effects of gavage administration of GVL (100–3000 mg/kg) on neurological (myoclonia, convulsions), sensorimotor (visual, acoustic, and overall tactile) responses, righting reflex, thermoregulation, motor activity (bar, drag, and accelerod test) and cardiorespiratory changes (heart rate, breath rate, oxygen saturation, and pulse distension) in CD-1 male mice and the 2) *in silico* ADMET profile of GVL in comparison to GHB and the open active form γ -hydroxyvaleric acid (GHV). The present study demonstrates that GVL inhibits, in a dose-dependent manner, sensorimotor and motor responses and induces cardiorespiratory depression (at a dose of 3000 mg/kg) in mice. The determination of the ED₅₀ in sensorimotor and motor responses revealed that GVL is about 4–5 times less potent than GHB. *In silico* prediction of ADMET profiles revealed toxicokinetic similarities between GHB and GHV, and differences with GVL. These results suggest that GVL could be used as a substitute for GHB and should be added to forensic toxicology screenings.

1. Introduction

Over the last decade, an increasing number of new substances known

as Novel Psychoactive Substances (NPS) have been discovered in the European illicit drug market, with an average of two new compounds detected every week (European Monitoring Centre for Drugs and Drug

Abbreviations: ADMET, Absorption, Distribution, Metabolism, Excretion, Toxicity; ANNE, Artificial Neural Network Ensemble; BBB, Blood Brain Barrier; BR, Breath rate; DFCs, Drug-Facilitated Crimes; DFSA, Drug-Facilitated Sexual Assault; EMCDDA, European Monitoring Centre for Drugs and Drug Addiction; GABA, γ -aminobutyric acid; GBL, γ -Butyrolactone; GHB, γ -Hydroxybutyric acid, 4-Hydroxybutanoic acid, 4-Hydroxybutyric acid; GHBR, γ -hydroxybutyrate receptor; GHV, γ -Hydroxyvaleric; GVL, γ -Valerolactone; HR, Heart rate; NPS, Novel Psychoactive Substances; SpO₂, Oxygen Saturation.

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Addiction: EMCDDA, 26). At the end of 2021, the EMCDDA monitored about 880 NPSs, 52 of which appear to have been reported for the first time in Europe (EMCDDA, 2022). Most of these NPS are old molecules that have either not been approved or never undergone clinical testing; on the other hand, some NPS are being synthesized *ex novo*. These new drugs were originally designed to avoid prohibition laws and produce similar or higher psychotropic effects compared to “older” drugs (El Balkhi et al., 2020). The EMCDDA is monitoring a broad range of NPS that have been reported through the EU Early Warning System. These substances are divided into different categories: synthetic cannabinoids, synthetic cathinones, phenethylamines, opioids, tryptamines, arylalkylamines and a range of other substances including sedative compounds like benzodiazepines, the well-known γ -hydroxybutyric acid (GHB), its analogs such as its precursors γ -butyrolactone (GBL) and 1, 4-butanediol, and its “legal substitute” γ -valerolactone (GVL; EMCDDA, 2021; Andersen-Streichert et al., 2013). The EMCDDA reported a large seizure of GHB or its precursor GBL in 18 countries, amounting to 48 tonnes and just under 500 liters. The use of GHB analogs in the industrial field, imbed a clear interpretation of the data regarding their seizure. In fact, the latest EMCDDA report states Holland still converts GBL to GHB on a large scale (EMCDDA, 2022). Moreover, in parallel with data regarding a large number of seizures of GHB and its analogs, recent clinical reports revealed that GHB was the fifth most common drug reported by Euro-DEN Plus hospitals in 2019 to be present in 10.6% of acute drug toxicity presentations and 27% of critical care admissions (EMCDDA, 2021).

GHB is a short-chain fatty acid, an endogenous neurotransmitter and neuromodulator mainly present in the hypothalamus and basal ganglia of the mammal brain (Bosch et al., 2012; Fishbein and Bessman, 1966). The sedating and narcotic effects of this compound led to its first use in anesthesia, later in psychiatry (Langlois et al., 1960), sleep disorders (narcolepsy with catalepsy; EMCDDA, 20) and drug or alcohol addiction and craving (its sodium salt is approved as a prescription drug; Addolorato et al., 2009; Leone et al., 2010; Le and Richards, 2022). Presumably, exogenous supraphysiological levels of GHB produce qualitatively different neuronal actions than those produced by endogenous GHB (Mortali and Berretta, 2020).

There are concerns about the possible risks associated with certain niche settings, such as the presence of sedative drugs like GHB and its alternatives in ‘chemsex’ (Wise, 2020) and crime scenes. Although accurate data on the incidence of sexual assault are not available (Busardò et al. 2019), the phenomenon of drug-facilitated crimes (DFCs) has dramatically increased in recent years (García et al., 2021). Therefore, these compounds are probably the most difficult to deal with in terms of chemical analysis and forensic interpretations because of their specific characteristics. These compounds are colorless, odorless, and tasteless, they are rapidly dissolved in any drink and therefore could easily be bioavailable in the victim’s system (García et al., 2021). Moreover, these drugs are rapidly eliminated from the body which prevents their detection by the investigators (Abanades et al., 2017; Brailsford et al., 2012; Haller et al., 2006).

The prohibition of GHB led to the research for legal substitutes in the illicit drug market. Among these, GVL is freely marketed as a green solvent and is frequently used in the food industry as an additive (Kumar et al., 2019). GVL has been pushed online as “excellent Valium” and/or as a GHB alternative (Andersen-Streichert et al., 2013; Fishbein and Bessman, 1966). GVL was detected in humans for the first time in 2013 in three individuals through toxicology analysis (Andersen-Streichert et al., 2013). One of the individuals was suspected to be the victim of a drug-facilitated sexual assault (DFSA) and the other two individuals were suspected to regularly use GVL. The reason for the low number of GVL identifications in literature may be due to low levels of use, but the authors of the case report comment that it is more likely because toxicology laboratories do not routinely test for GVL. The structure of GVL is characterized by a lactone ring containing a methyl group in the fifth position of a dihydrofuran-2(3H)-one ring (Wong et al., 2017; Fig. 1).

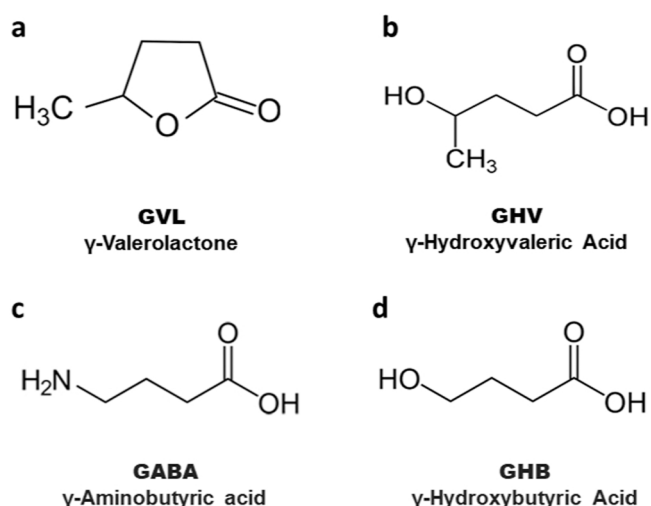


Fig. 1. Chemical structures of GVL (γ -Valerolactone (a)); GHV (γ -Hydroxyvaleric Acid; (b)); GABA (γ -Aminobutyric acid; (c)) and GHB (γ -Hydroxybutyric Acid (d)).

We have recently demonstrated in a preclinical study that urinary excretion of GVL is maximal in the first 5 h after the gavage administration and its progressive undetectability in the range of 24–36 h (Camuto et al., 2022).

GVL is not metabolized to GHB or GABA but is processed by liver and plasma lactonase, which splits the lactone ring to open active form γ -hydroxyvaleric acid (GHV or 4-methyl-GHB; EMCDDA, 19). Few studies on receptor-affinity of GHV revealed that the affinity of GHV for GHB receptors is approximately two-fold lower than that of the endogenous GHB (Andersen-Streichert et al., 2013; Bourguignon et al., 1988; Carter et al., 2005). *In vivo* studies revealed that both GHB and GHV produce ataxia and catalepsy in a dose-dependent manner in rats (Carter et al., 2005; Marinetti et al., 2012). Moreover, the gavage administration of GVL at a dose of 400 mg/kg reduced significantly the visual placing response in the first 4 h after intake, and the effect disappeared from 5 h (Camuto et al., 2022).

The present study is aimed to investigate the *in vivo* effect of GVL gavage administration on neurological (myoclonia, convulsions) signs, motor (bar, drag and accelerod test), sensorimotor (visual, acoustic, and overall tactile) responses, righting reflex, body thermoregulation and cardiorespiratory changes (heart rate, respiratory rate, SpO2 saturation, and pulse distention) in mice. All the effects induced by GVL were compared to those induced by GHB (Arfè et al. 2021). Moreover, we evaluated *in silico* ADMET profile of GVL in comparison to GHB and GHV to provide rapid screening of the toxic potential of these compounds.

2. Materials and methods

2.1. Animals

Male ICR (CD-1®) mice weighing 30–35 g (Centralized Preclinical Research Laboratory, University of Ferrara, Italy) were group housed (5 mice per cage; floor area per animal was 80 cm²; minimum enclosure height was 12 cm), exposed to a 12:12-h light-dark cycle (light period from 6:30 AM to 6:30 PM) at a temperature of 20–22 °C and humidity of 45–55% and were provided ad libitum access to food (Diet 4RF25 GLP; Mucedola, Settimo Milanese, Milan, Italy) and water. The experimental protocols performed in the present study were in accordance with the U. K. Animals (Scientific Procedures) Act of 1986 and associated guidelines and the new European Communities Council Directive of September 2010 (2010/63/EU). Experimental protocols were approved by the Italian Ministry of Health (license n. 223/2021-PR, CBCC2.46. EXT.21) and by the Animal Welfare Body of the University of Ferrara. According

to the ARRIVE guidelines, all possible efforts were made to minimise the number of animals used, to minimise the animals' pain and discomfort.

2.2. Drug preparation and animal dose determination

GVL was purchased from Sigma Aldrich, Milano, Italy, and GHB (sodium salt) was purchased from LCG standards S.r.l. Both compounds were dissolved in saline (0.9% NaCl) solution that was also used as vehicle and administered orally using gavage needles at a volume of 4 µl/g (Arfè et al. 2021). The range of doses (100–3000 mg/kg) of GVL were chosen based on previous studies (Carter et al., 2005; Arfè et al. 2021).

2.3. Behavioral tests

The effects of GVL were investigated using a battery of behavioral tests widely used in studies of "safety-pharmacology" for the preclinical characterization of NPS in rodents (Ossato et al., 2015, 2018; Vigolo et al., 2015; Arfè et al. 2021; Bilel et al., 2022). All experiments were performed between 8:30 AM and 2:00 PM. Experiments were conducted blindly by trained observers working in pairs (Ossato et al., 2015, 2018). Mouse behavior (sensorimotor responses) was videotaped and analyzed offline by a different trained operator who gave test scores.

2.3.1. Evaluation of the visual response

Mouse Visual response was verified by two behavioral tests which evaluated the ability of the animal to capture visual information even when the animal is moving (the visual placing response) or when it is stationary (the visual object response). *Visual Placing response* test is performed using a tail suspension modified apparatus able to bring down the mouse towards the floor at a constant speed of 10 cm/sec (Ossato et al., 2015, 2018). Briefly, CD-1 mice were suspended 20 cm above the floor by an adhesive tape that it was placed approximately 1 cm from the tip of the tail (Marti et al., 2019). The downward movement of the mouse is videotaped by a camera (B/W USB Camera day&night with varifocal lens; Ugo Basile, Italy) placed at the base of the tail suspension apparatus. Videos were analyzed off-line by a trained operator who did not know the drug treatments performed. The analysis frame by frame allows evaluating the beginning of the reaction of the mouse while it is close to the floor. The first movement of the mouse when it perceives the floor is the extension of the front legs. When the mouse starts the reaction, an electronic ruler evaluates the perpendicular distance in millimeters between the eyes of the mice to the floor. The untreated control mouse typically perceives the floor and it prepares to contact at a distance of about 28 ± 4.3 cm.

Visual object response test was used to evaluate the ability of the mouse to see an object approaching from the front (frontal view) or the side (lateral view), thus inducing the animal to shift or turn the head, bring the forelimbs in the position of "defense" or retreat it (Ossato et al., 2015, 2018). For the frontal visual response, a white horizontal bar was moved frontally to the mouse head and the maneuver was repeated 3 times. For the lateral visual response, a small dentist's mirror was moved into the mouse's field of view in a horizontal arc, until the stimulus was between the mouse's eyes. The procedure was conducted bilaterally (Ossato et al., 2015, 2018) and was repeated 3 times. The score assigned was a value of 1 if there was a reflection in the mouse movement or 0 if not. The total value was calculated by adding the scores obtained in the frontal with that obtained in the lateral visual object response (overall score 9).

2.3.2. Evaluation of acoustic response

Acoustic response measures the reflex of the mouse in reply to an acoustic stimulus produced behind the animal. Four acoustic stimuli of different intensities and frequencies were tested (Ossato et al., 2015, 2018). Each sound test was repeated three times, giving a value of 1 if there was a response and 0 if not present, for a total score of 3 for each

sound. The acoustic total score was calculated by adding scores obtained in the four tests (overall score 12). Evaluation of the visual object response was measured at 0, 10, 30, 60, 120, 180, 240 300 min after gavage administration.

2.3.3. Evaluation of overall tactile response

The tactile response in the mouse was verified through vibrissae, pinna and corneal reflexes (Ossato et al., 2015, 2018). *Vibrissae reflex* was evaluated by touching vibrissae (right and left) with a thin hypodermic needle once for side giving a value of 1 if there was a reflex (turning of the head to the side of touch or vibrissae movement) or 0 if not present (overall score 2).

Pinna reflex was assessed by touching pavilions (left and right) with a thin hypodermic needle. First the interior pavilions and then the external. This test was repeated twice for side giving a value of 1 if there was a reflex and 0 if not present (overall score 4). *Corneal reflex* was assessed gently touching the cornea of the mouse with a thin hypodermic needle and evaluating the response, assigning a value of 1 if the mouse moved only the head, 2 if it only closed the eyelid, 3 if it closed the lid and moved the head. The procedure was conducted bilaterally (overall score 6).

2.3.4. Evaluation of core and surface body temperature

To better assess the effects of the ligands on thermoregulation, we measured both changes in the core (rectal) and surface (ventral fur) temperature. The *core temperature* was evaluated by a probe (1 mm diameter) that was gently inserted, after lubrication with liquid vaseline, into the rectum of the mouse (to about 2 cm) and left in position until the stabilization of the temperature (about 10 sec; Vigolo et al., 2015). The probe was connected to a Cole Parmer digital thermometer, model 8402. The *surface temperature* was measured by a Microlife FR 1DZ1 digital infrared thermometer, placed at 1 cm from the surface of the abdomen of the mouse (Vigolo et al., 2015). Core and surface mouse body temperatures were measured at 0, 30, 50, 85, 140, 200, 260 and 320 min after gavage administration.

2.3.5. Evaluation of pain induced by a mechanical and thermal stimulus

Acute mechanical nociception was evaluated using the tail pinch test (Vigolo et al., 2015). A special rigid probe connected to a digital dynamometer (ZP-50 N, IMADA, Japan) was gently placed on the tail of the mouse (in the distal portion) and a progressive pressure was applied. When the mouse flicked its tail, the pressure was stopped, and the digital instrument saved the maximum peak of weight supported (g/force). A cut off (500 g/force) was set to avoid tissue damage. The test was repeated three times and the final value was calculated with the average of 3 obtained scores. *Acute thermal nociception* was evaluated using the tail withdrawal test (Vigolo et al., 2015). The mouse was restrained in a dark plastic cylinder and half of its tail was dipped in water of 48°C and the time latency (in seconds) that the tail was left in water was recorded. A cut off (15 seconds) was set to avoid tissue damage. Acute mechanical and thermal nociception was measured at 0, 35, 55, 90, 145, 205, 265 and 325 min after gavage administration.

2.3.6. Grip strength test

The effect of the administration on muscle strength in mice was evaluated by a grip strength test. Briefly, the muscle strength of the mouse was measured by the use of a special grid on which the operator clings the mouse and subsequently tries to gently remove it (Foti et al., 2019). The grid is connected to a digital dynamometer (ZP-50 N, IMADA, Japan), which measures the tensile muscle in g/strength. The grip strength test was performed at 0, 15, 35, 70, 125, 185, 245 and 305 min post administration. The test was repeated three times and the final value was calculated with the average of 3 obtained scores.

2.3.7. Motor activity assessment

Alterations of motor activity induced by GVL were measured using

the accelerod tests and the drag tests (Ossato et al., 2015, 2018; Vigolo et al., 2015). In the *accelerod test* animals were placed on a rotating cylinder that increases velocity automatically in a constant manner (0–60 rotations/min in 5 min). The time spent on the cylinder was measured. The accelerod test was performed at 0, 40, 65, 95, 150, 210, 270 and 330 min post administration. In the *drag test* the mouse was lifted by the tail, leaving the front paws on the table and dragged backward at a constant speed of about 20 cm/sec for a fixed distance (100 cm). The number of steps performed by each paw was recorded by two different observers. For each animal from five to seven measurements were collected. The drag test was performed at 0, 45, 70, 100, 155, 215, 275 and 335 min after gavage administration.

2.3.8. Bar test and Righting Reflex

These two tests measure the grade of akinesia (Arfè et al. 2021) and hypnosis/unconsciousness (Franks, 2006; Irifune et al., 2007) established in animals following administration of substances. In *Bar test* each animal's forelimbs were placed on a bar made of plastic (block height 6 cm). The time spent on the bar was measured (immobility cut off: 20 sec) and the akinesia was calculated as total time spent on the bar after three consecutive trials (total maximal time of catalepsy: 60 sec).

In *Righting Reflex test*, the animal was placed in a supine position and loss of righting was defined as not placing the plantar surface of any paw on the floor. The time spent on supine position was measured (immobility cut off: 30 sec) and the loss of righting response was calculated as total time spent on supine position after three consecutive trials. For each mouse both these two tests were performed immediately before the drag test at 0, 20, 40, 75, 130, 190, 250 and 310 min after gavage administration.

2.3.9. Evaluation of breath rate

The experimental protocol for the detection of respiratory rate provides that the animal is leaving free in a cage and the respiration patterns of the mice were videotaped by a camera (B/W USB Camera day&night with varifocal lens; Ugo Basile, Italy) placed above observation's cage. A trained operator who does not know the drug treatments performed analyses movies off-line. The analysis frame by frame allows to better evaluate the number of breath rates of the mouse evaluated through the count of about 257 ± 11 breath rates per minutes (brpm). Breath rates were measured at 0,15,40,70, 130, 190, 250 and 310 min after gavage administration.

2.3.10. Cardio-respiratory analysis

As previously reported the experimental protocol for detection of cardio-respiratory parameters used in this study is designed to monitor awake and freely moving animals with no invasive instruments and with minimal handling (Bilel et al., 2020, 2022; Arfè et al. 2021). Around the neck of the animal is applied a collar which detects continuously, through a sensor, heart rate (HR), breath rate (BR), oxygen saturation (SpO₂) and vessel distention (μ M) with a frequency of 15 Hz. While running the experiment, the mouse is kept freely moving in a cage (with no access to food and water) monitored by the sensor collar through the software MouseOx Plus (STARR Life Sciences® Corp. Oakmont, PA). In the first hour, a collar is placed on the animal's neck for simulates the real one used in the test, minimizing the possible effects of stress during the experiment. After, the real collar (with sensor) is replaced and baseline parameters are monitored for 60 minutes. Subsequently, the mice are administered with GVL and GHB (3000 mg/kg) by oral gavage administration and data is recorded for 5 hours.

2.4. Data and statistical analysis

Core and surface temperature values are expressed as the difference between control temperature (before gavage) and temperature following drug administration ($\Delta^\circ\text{C}$). Antinociception (tail withdrawal and tail pinch tests), righting reflex and catalepsy (bar test) are

calculated as the percent of maximal possible effect $\{EMax\% = [(test - control latency) / (cut off time - control)] \times 100$. For the core and surface temperature data are presented in $\Delta^\circ\text{C}$ for each substance while in maximal possible effect $\{EMax\% = [(test T^\circ - control T^\circ) / (cut off T^\circ - control T^\circ)] \times 100$ for dose-response curves (ED₅₀). Emax% (tail withdrawal, tail pinch and bar test) and percentage of basal value (drag test and accelerod test). In sensorimotor response experiments, data are expressed in arbitrary units (visual objects response; acoustic response; vibrissae, corneal and pinna reflex) or percentage of baseline (visual placing response). Data are expressed in percentage of basal value [maximal muscle strength (expressed as gf), breath rate (expressed as breath per minute brpm), heart rate (expressed as heart beats per min bpm), pulse distention (vessel diameter changes expressed as μ m), respiratory rate (expressed as respiratory rate per minute rpm) and SpO₂ saturation (oxygen blood saturation expressed as %)]. The statistical analysis of the effects of the individual substances in different concentrations over time and that of antagonism studies were performed using a two-way ANOVA followed by a Bonferroni test for multiple comparisons. Significance was set at $P < 0.05$. ED₅₀ values were determined using non-linear regression analysis of dose-response curves performed using Prism 8.0 software (GraphPad Prism, San Diego CA). The statistical analysis of ED₅₀ values (Table 1) relative to each test were performed with Unpaired t test.

2.5. In silico ADMET prediction of GHB, GVL and GHV

The *in silico* ADMET prediction of GHB, GVL and GHV (chemical structures, Fig. 1) profiles was conducted through Simulations Plus ADMET Predictor® Version 10.4 ($\times 64$) on a Windows 11 operating system (ADMET Predictor®, Simulations-Plus Inc). The program predicts the physicochemical, pharmacokinetics, and toxicity properties based on the molecular structures of compounds. It uses artificial neural network ensemble (ANNE) models, which were trained to ensemble

Table 1

Overview of computed ADMET parameters with their recommended ranges for Orally active drugs.

Parameter	Recommended Range	Comments
ADMET_Risk	< 7	Includes components of all risk models as well as fraction unbound to plasma and volume of distribution.
Absn_Risk	< 4	Considers size, rotational bonds, hydrogen bonding capacity, polar surface area, permeability, lipophilicity, solubility.
TOX_Risk	< 2	Consists of hERG, acute toxicity in rats, carcinogenicity in chronic rat/mouse studies, hepatotoxicity, mutation.
CYP_Risk	< 2	Includes inhibition of CYPs 1A2, 2C19, 2C9, 2D6, and 3A4, excessive clearance, and inhibition of midazolam or testosterone.
Peff [cm/ s $\times 10^4$]	≥ 0.5	Human effective jejunal permeability S+Peff < [0.40,0.60 cm/s $\times 10^4$]
MDCK [cm/s $\times 10^7$]	≥ 30	S+MDCK > 30 cm/s $\times 10^7$ indicates high permeability
Fup [%]	>10%	hum_fup% and mou_fup% < 10% indicates extensive plasma binding
RBP	< 1.0	hum_RBP and mou_RBP > 1 indicates a partitioning to erythrocytes
Vd [L/kg]	≤ 3.7	V _d > 3.7 indicates high distribution at steady state
BSEP_IC50 [μ M]	≤ 60	BSEP_IC50 > 60 μ M indicates high activation of the bile salt export pump

ADMET_Risk, Absorption, Distribution, Metabolism, Excretion, and Toxicity risk; Absn_Risk, Absorption risk TOX_Risk, Toxicity risk; CYP_Risk, Cytochrome risk, Peff, human effective jejunal permeability; MDCK, apparent permeability through Madin-Darby canine kidney cell layers; Fup%, percent unbound to blood plasma proteins in human; RBP, blood to plasma ratio in human; Vd, volume of distribution; BSEP_IC50, Bile Salt Export Pump IC50;

with data sets that share the same "architecture" (i.e. same inputs and number of neurons) from well-defined drugs, using the 2D structure and the atomic descriptors for data selection. The criteria used to set the ADMET scores are illustrated in Table 1.

3. Results

3.1. Behavioural studies

3.1.1. Major neurological changes

Gavage administration of GVL did not induce neurological changes in mice at the range dose (100–2000 mg/kg). Only the highest dose of GVL (3000 mg/kg), induced myoclonia in the first 3 hours of observation followed by a deep sedative hypnotic state/or possible coma state in 100% of mice and the effect persisted up to 5 h of observation. Moreover, lethality was induced after 24 hours, in the group of animals treated with the highest dose (3000 mg/kg).

3.1.2. Sensorimotor studies

3.1.2.1. Evaluation of the visual response. Visual object (Fig. 2a) and placing (Fig. 2b) responses were not affected by gavage administration of the vehicle in mice over the course of the 5 h of analysis.

Gavage administration of GVL (100–3000 mg/kg) reduced, in a significant ($p < 0.0001$) and dose-dependent manner, both the visual object (Fig. 2a) and the visual placing (Fig. 2b) responses in mice over the 5 h observation. In particular, the gavage administration of the doses 1200 and 2000 mg/kg of GVL induced respectively a decrease of about 20% and 40% in visual object response (Fig. 2a) of mice. These effects appeared 30 min after treatment and persisted up to 5 of measurements. At the highest dose tested (3000 mg/kg), GVL induced a total inhibition of the visual object response at 240 and 300 min after gavage administration [Fig. 2a; effect of treatment ($F_{(7,448)}=518.2$, $p < 0.0001$), time ($F_{(7,448)}=73.31$, $p < 0.0001$) and a significant time x treatment interaction ($F_{(49,448)}=17.99$, $p < 0.0001$].

Comparison of the dose-response curve to GHB in the visual object response revealed that GHB (Table 1; $ED_{50}=356.3$ mg/kg) is about five times more potent than GVL ($ED_{50}=1705$ mg/kg) in reducing the visual object reflexes of mice [Fig. 2c; effect of treatment ($F_{(1,98)}=66984$, $p < 0.0001$), time ($F_{(6,98)}=2256$, $p < 0.0001$) and time x treatment interaction ($F_{(6,98)}=4458$, $p < 0.0001$].

Gavage administration of GVL (100–3000 mg/kg) reduced, significantly and dose-dependently, the visual placing response of mice. In particular, the dose of 1200 mg/kg reduced the visual-motor reflexes of mice to about 40% at 15 min of gavage administration and the effect persisted up to 5 h. The effects of GVL were more profound at higher doses (2000–3000 mg/kg). In particular, at the highest dose tested, GVL

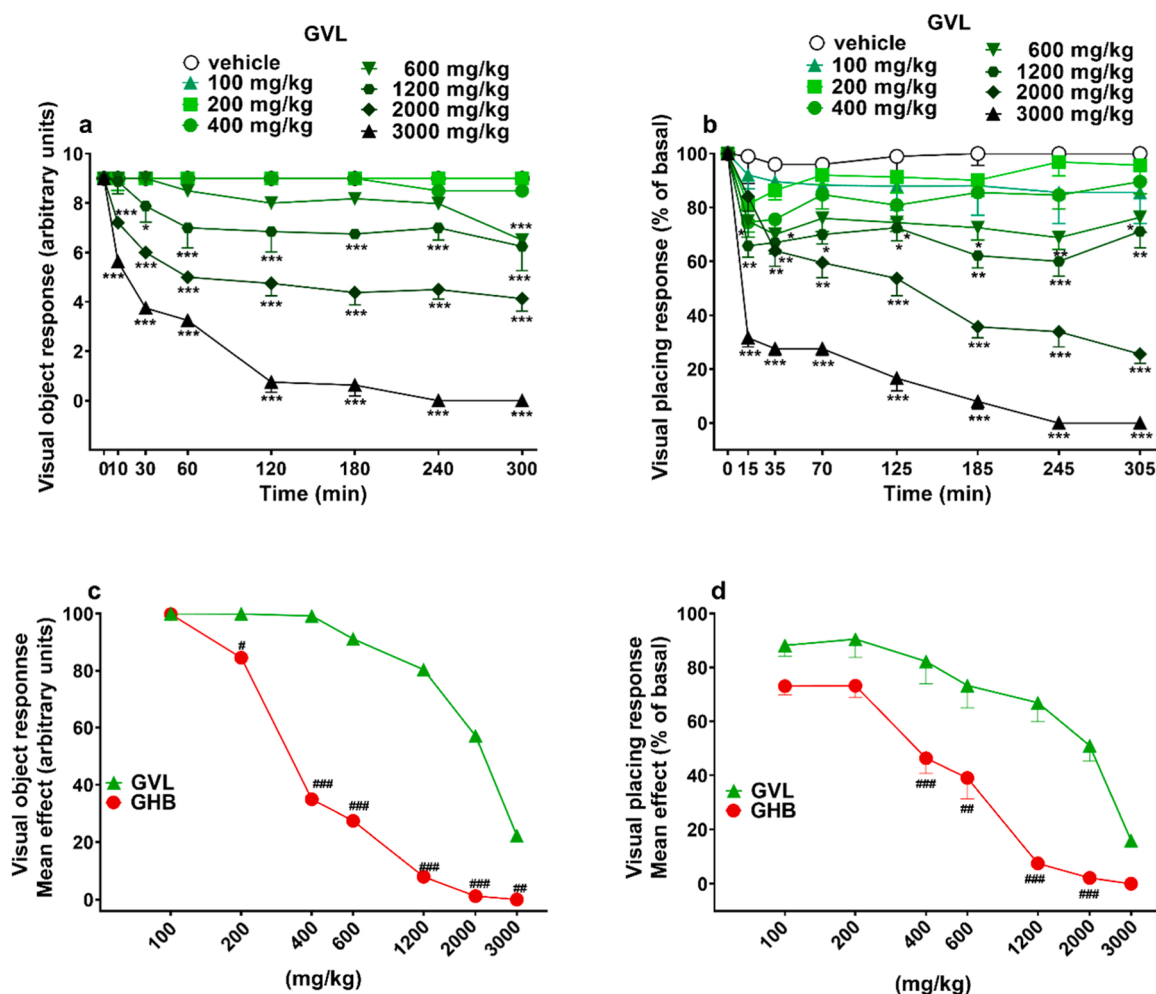


Fig. 2. Effect of GVL (100–3000 mg/kg gavage; panels a, b) on the visual object (left) and placing response (right) test in the mouse. Comparison with GHB (100–3000 mg/kg gavage) of the maximum effect observed at different doses on visual object (panel c) and visual placing (panel d) test. Data are expressed as mean \pm SEM ($n = 8$ / group). Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose-response curve of both compounds at different times (panels a, b) and the maximum effect observed at different doses (panels c, d). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus saline; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus GVL.

induced inhibition of 100% of visual placing in mice at 240 and 300 min after treatment [Fig. 2b; effect of treatment ($F_{(7,448)}=113.2$, $p<0.0001$), time ($F_{(7,448)}=25.44$, $p<0.0001$), and time x treatment interaction ($F_{(49,448)}=5.029$, $p<0.0001$).

Comparison of the dose-response curve to GHB in the visual placing response revealed that GHB (Table 1; $ED_{50}=570.2$ mg/kg) is about three times more potent than GVL ($ED_{50}=1506$ mg/kg) in reducing the visual placing response of mice [Fig. 2d; effect of treatment ($F_{(1,98)}=123.9$, $p<0.0001$), time ($F_{(6,98)}=52.52$, $p<0.0001$) and time x treatment interaction ($F_{(6,98)}=5.102$, $p=0.0001$).

3.1.2.2. Evaluation of acoustic and tactile response. Acoustic and overall tactile responses did not change in vehicle-treated mice over the 5 h observation (Fig. 3a, b).

Gavage administration of GVL (100–3000 mg/kg) decreased significantly ($p<0.0001$) and dose-dependently the acoustic response in mice (Fig. 3a). In particular, the inhibitory effect of GVL at the dose of 3000 mg/kg was more rapid than the other dosages in mice (~16% at 10 min of treatment) and the effect reached the maximum (100%) at 240 min of treatment and the effect persisted up to 300 min [Fig. 3a; effect of treatment ($F_{(7,448)}=266.5$, $p<0.0001$), time ($F_{(7,448)}=65.66$,

$p<0.0001$) and time x treatment interaction ($F_{(49,448)}=17.90$, $p<0.0001$).

Comparison of the dose-response curve to GHB in the acoustic response revealed that GHB (Table 1; $ED_{50}=397.5$ mg/kg) is about four times more potent than GVL ($ED_{50}=1661$ mg/kg) in reducing the acoustic reflexes of mice [Fig. 3c; effect of treatment ($F_{(1,98)}=28407$, $p<0.0001$), time ($F_{(6,98)}=8871$, $p<0.0001$) and time x treatment interaction ($F_{(6,98)}=1885$, $p<0.0001$).

The gavage treatment with GVL decreased the overall tactile response in mice only at higher doses tested (1200–3000 mg/kg). In particular, the highest dose tested (3000 mg/kg) induced a total inhibition of tactile response at 240 min of treatment and the effect persisted up to 300 min [Fig. 3b; effect of treatment ($F_{(7,448)}=109.1$, $p<0.0001$), time ($F_{(7,448)}=48.63$, $p<0.0001$) and time x treatment interaction ($F_{(49,448)}=18.20$, $p<0.0001$).

Comparison of the dose-response curve to GHB in the overall tactile response revealed that GHB (Table 1; $ED_{50}=468.8$ mg/kg) is about four times more potent than GVL ($ED_{50}=1828$ mg/kg) in reducing the overall tactile response of mice [Fig. 3d; effect of treatment ($F_{(1,98)}=22253$, $p<0.0001$), time ($F_{(6,98)}=5642$, $p<0.0001$) and time x treatment interaction ($F_{(6,98)}=1374$, $p<0.0001$).

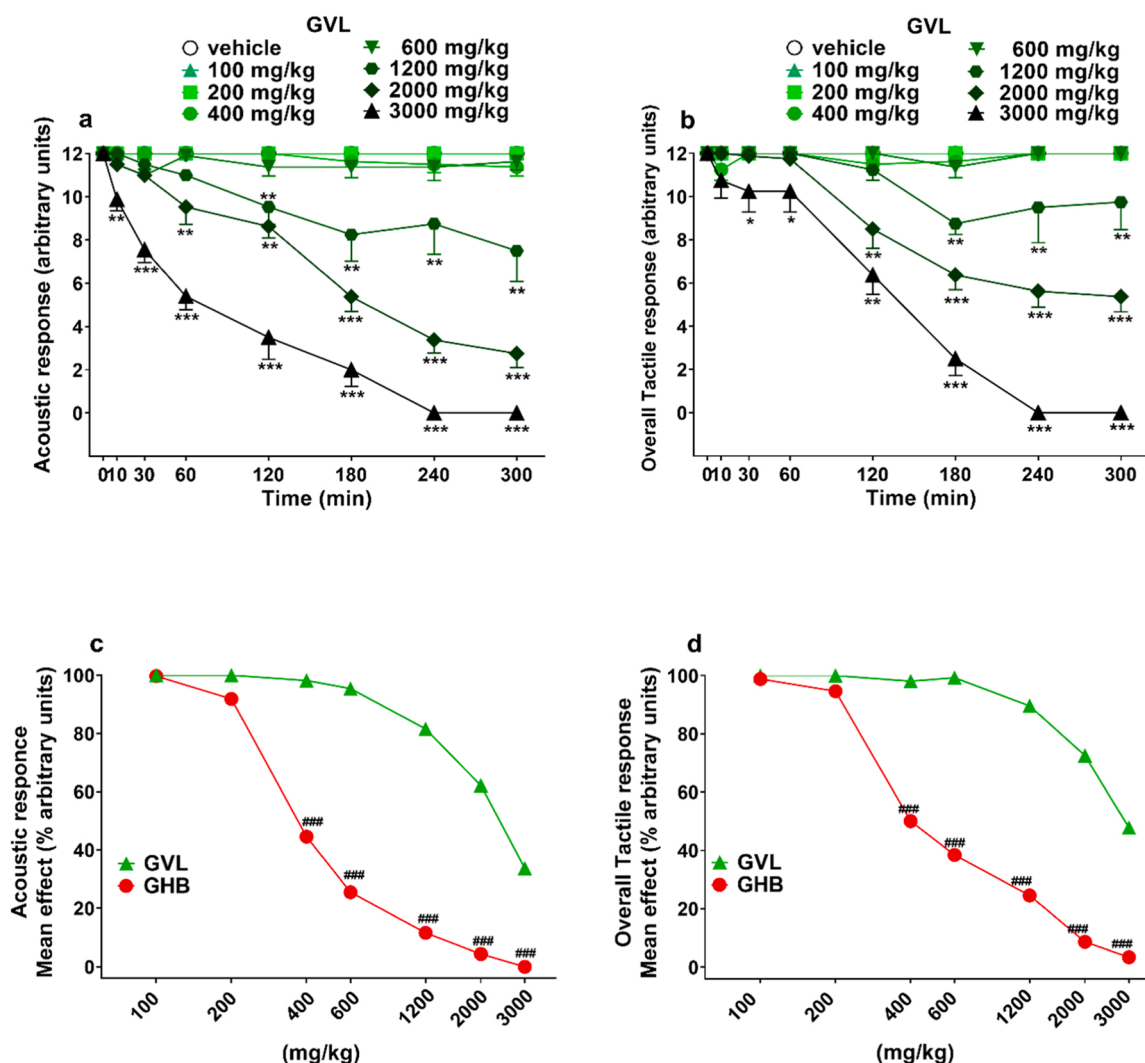


Fig. 3. Effect of GVL (100–3000 mg/kg; gavage; panels a, b) and GHB on the acoustic (left) and the overall tactile (right) response in the mouse and comparison with GHB (100–3000 mg/kg gavage) of the maximum effect observed at different doses acoustic (panel c) and overall tactile (panel d) response. Data are expressed as arbitrary units and represent the mean \pm SEM of 8 mice/group. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose-response curve of each compound at different times (panels a, b) and the maximum effect observed at different doses (panels c, d). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus saline. ### $p<0.001$ versus GVL.

3.1.3. Evaluation of cataleptic and righting reflex responses

Gavage administration of vehicle did not induce any significant effects in mice in the Bar and Righting tests over the 5 h observation (Fig. 4a, b).

The administration of GVL did not induce catalepsy and incapacities of the righting reflexes at 100–2000 mg/kg, but only at the highest dose (3000 mg/kg). In particular, the cataleptic effect of GVL was significantly increased in mice at 75 min and persisted until the end of the test where it reached maximal effects (100%) at 240 min and 310 min [Fig. 4a; effect of treatment ($F_{(7448)}=281.3$, $p<0.0001$), time ($F_{(7448)}=51.42$, $p<0.0001$) and time x treatment interaction ($F_{(49,448)}=51.42$, $p<0.0001$)].

Comparison of the dose-response curve to GHB response in the bar test revealed a major potency of GHB (Table 1; $ED_{50}=376.8$ mg/kg) with respect to GVL ($ED_{50}=ND$) in inducing catalepsy in mice [Fig. 4c; effect of treatment ($F_{(1,90)}=489.2$, $p<0.0001$), time ($F_{(6,90)}=27.19$, $p<0.0001$) and time x treatment interaction ($F_{(6,90)}=18.45$, $p<0.0001$)].

The highest dose of GVL (3000 mg/kg) induced a decrease of the righting reflexes of mice which persisted up to 5 h of measurements (Fig. 4b). Similarly, to the bar test, the inhibition of righting reflexes appeared at 75 min after GVL administration and the maximal effects were reached at 250 and 310 min.

Comparison of the dose-response curve to GHB in the righting response test revealed a major potency of GHB at higher doses (2000 mg/kg and 3000 mg/kg), in comparison to GVL [Fig. 4d; effect of treatment ($F_{(1,90)}=318.5$, $p<0.0001$), time ($F_{(6,90)}=626.1$, $p<0.0001$) and time x treatment interaction ($F_{(6,90)}=112.9$, $p<0.0001$)].

3.1.4. Evaluation of core and surface temperature responses

As shown in Fig. 5, gavage administration of the vehicle did not change body temperature (Fig. 5a, b) responses in mice over the 5 h observation.

Gavage administration of GVL (100–3000 mg/kg) reduced significantly the core temperature in mice [Fig. 5a; effect of treatment ($F_{(7448)}=309.3$, $p<0.0001$), time ($F_{(7448)}=179.3$, $p<0.0001$) and time x treatment interaction ($F_{(49,448)}=20.32$, $p<0.0001$)]. In particular, core temperature was rapidly (30 min after treatment) reduced at the range doses (1200–3000 mg/kg) and the effect persisted up to 320 min after gavage administration. The dose of 3000 mg/kg of GVL induced the highest decrease in core temperature which reached -14°C at 300 min after gavage administration.

Comparison of the dose-response curve to GHB in the core temperature response revealed that GHB (Table 1; $ED_{50}=1219$ mg/kg) is more potent than GVL ($ED_{50}=1959$ mg/kg) in core body temperature in mice [Fig. 5c; effect of treatment ($F_{(1,98)}=870.9$, $p<0.0001$), time

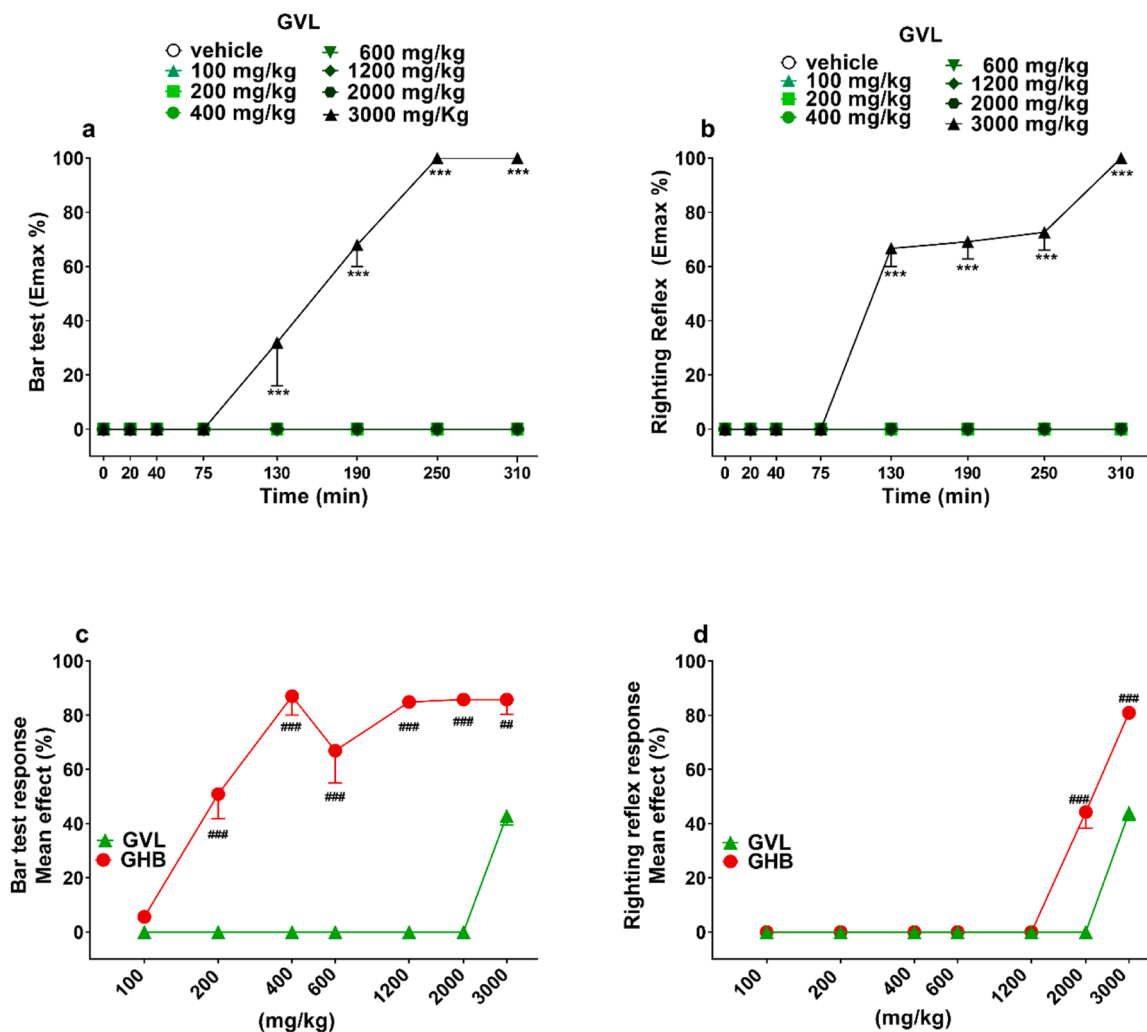


Fig. 4. Effect of GVL (100–3000 mg/kg gavage; panels a, b) on the bar test (left) and righting response (right) test in the mouse and comparison with GHB (100–3000 mg/kg gavage) of the maximum effect observed in 5 h on the bar test (panel c) and righting response (panel d) test. Data are expressed as mean \pm SEM ($n = 8$ / group). Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons for the dose-response curve of each compound at different times (panels a, b) and the maximum effect observed in 5 h (panels c, d). *** $p<0.001$ versus saline; ## $p<0.01$, ### $p<0.001$ versus GVL.

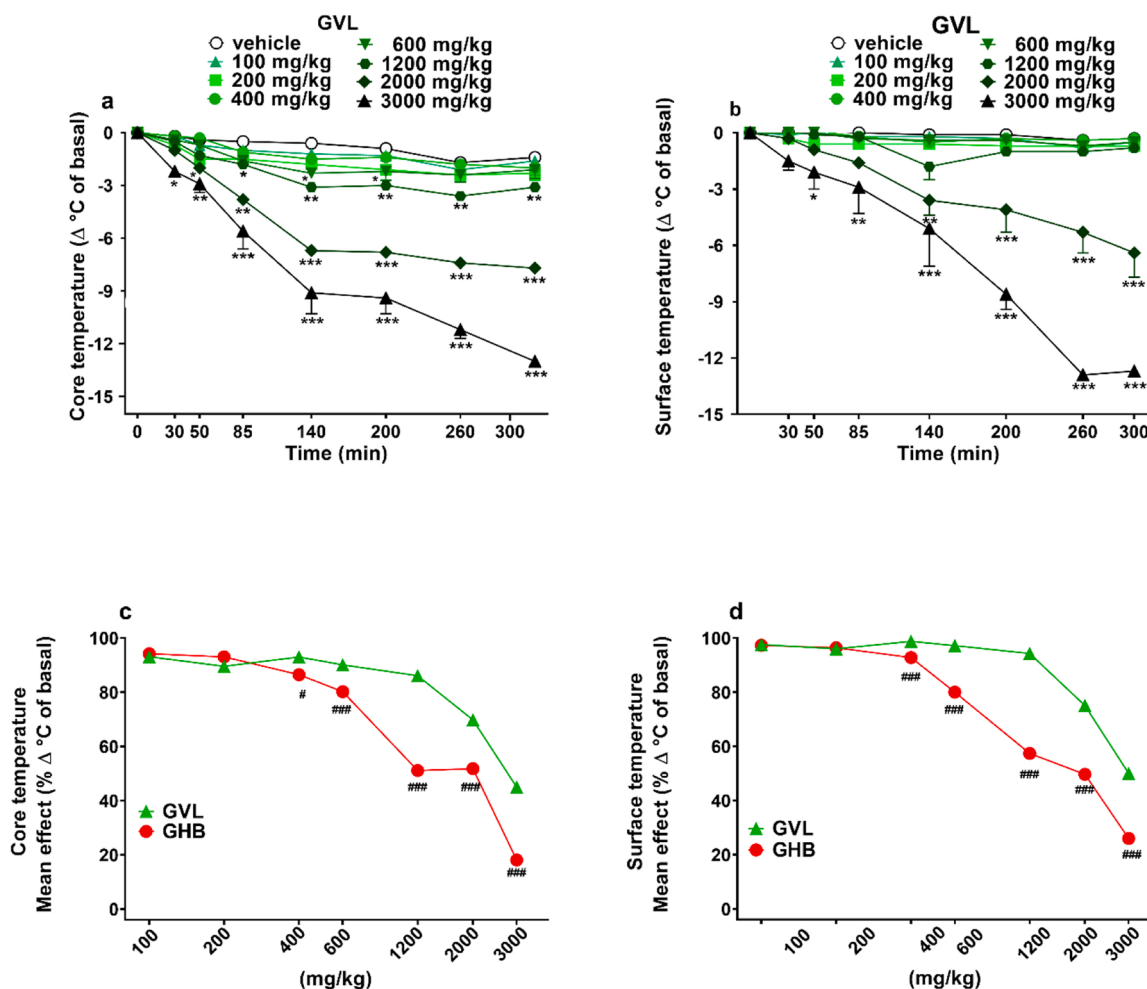


Fig. 5. Effect of GVL (100–3000 mg/kg gavage; panels a, b) on the core temperature (left) and surface temperature (right) test in the mouse and comparison with GHB (100–3000 mg/kg gavage) of the maximum effect observed at different doses core temperature (panel c) and surface temperature (panel d) test. Data are expressed as mean \pm SEM ($n = 8$ / group). Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons for the dose-response curve of each compound at different times (panels a, b) and the maximum effect observed at different doses (panels c, d). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus saline; # $p < 0.05$, ### $p < 0.001$ versus GVL.

($F_{(6,98)}=1480$, $p < 0.0001$) and time \times treatment interaction ($F_{(6,98)}=870.9$, $p < 0.0001$).

Similarly, the gavage administration of GVL (100–3000 mg/kg) reduced the surface temperature in mice [Fig. 5b; main effect of treatment ($F_{(7448)}=138.6$, $p < 0.0001$), time ($F_{(7448)}=43.30$, $p < 0.0001$) and time \times treatment interaction ($F_{(49,448)}=14.10$, $p < 0.0001$)]. In particular, the surface temperature was significantly reduced at the range of doses 2000 (-8 °C) and 3000 mg/kg (-13 °C). The effect is slightly delayed (50 min) with respect to the core temperature (30 min) and persisted up to 320 min after gavage administration.

Comparison of the dose-response curve to GHB in the surface temperature response revealed that GHB (Table 1; $ED_{50}=1123$ mg/kg) is more potent than GVL ($ED_{50}=2001$ mg/kg) in reducing the surface temperature in mice [Fig. 5d; effect of treatment ($F_{(1,98)}=1273$, $p < 0.0001$), time ($F_{(6,98)}=1477$, $p < 0.0001$) and time \times treatment interaction ($F_{(6,98)}=149.6$, $p < 0.0001$)].

3.1.5. Evaluation of pain induced by a mechanical and thermal stimulus

Gavage administration of the vehicle did not change mechanical (Fig. 6a) and thermal (Fig. 6b) pain threshold in mice over the 5 h observation.

Gavage administration of GVL induced a significant increase of the threshold to acute mechanical stimuli only at the highest dose tested (3000 mg/kg); [Fig. 6a; effect of treatment ($F_{(7448)}=87.74$, $p < 0.0001$),

time ($F_{(7,448)}=8.687$, $p < 0.0001$) and time \times treatment interaction ($F_{(49,448)}=12.99$, $p < 0.0001$)]. The mechanical analgesia appeared at 145 min and increased to about 92% at 325 min after treatment.

Comparison of the dose-response curve to GHB in the tail pinch test revealed that GHB (Table 1; $ED_{50}=433.2$ mg/kg) is more potent than GVL ($ED_{50}=ND$) in increasing mechanical analgesia in mice [Fig. 6c; effect of treatment ($F_{(1,90)}=503.4$, $p < 0.0001$), time ($F_{(6,90)}=84.10$, $p < 0.0001$) and time \times treatment interaction ($F_{(6,90)}=38.63$, $p < 0.0001$)].

Gavage administration of GVL induced also a significant increase of the threshold to acute thermal pain stimuli only at higher doses tested (2000 and 3000 mg/kg); [Fig. 6b; main effect of treatment ($F_{(7448)}=191.5$, $p < 0.0001$), time ($F_{(7448)}=31.75$, $p < 0.0001$) and time \times treatment interaction ($F_{(49,448)}=25.63$, $p < 0.0001$)]. The analgesic effect of higher doses appeared 90 min after treatment. The maximal effect induced by the dose of 2000 mg/kg was observed at 140 min where thermal analgesia threshold increased to about 20%. The maximal analgesic effect induced by the dose of 3000 mg/kg was at the end of the test (325 min) where thermal analgesia threshold increased to 89%.

Comparison of the dose-response curve to GHB in the tail withdrawal test revealed that GHB (Table 1; $ED_{50}=626.6$ mg/kg) is more potent than GVL ($ED_{50}=ND$) in increasing thermal analgesia in mice [Fig. 6d; effect of treatment ($F_{(1,90)}=681.3$, $p < 0.0001$), time ($F_{(6,90)}=117.1$, $p < 0.0001$) and time \times treatment interaction ($F_{(6,90)}=52.56$, $p < 0.0001$)].

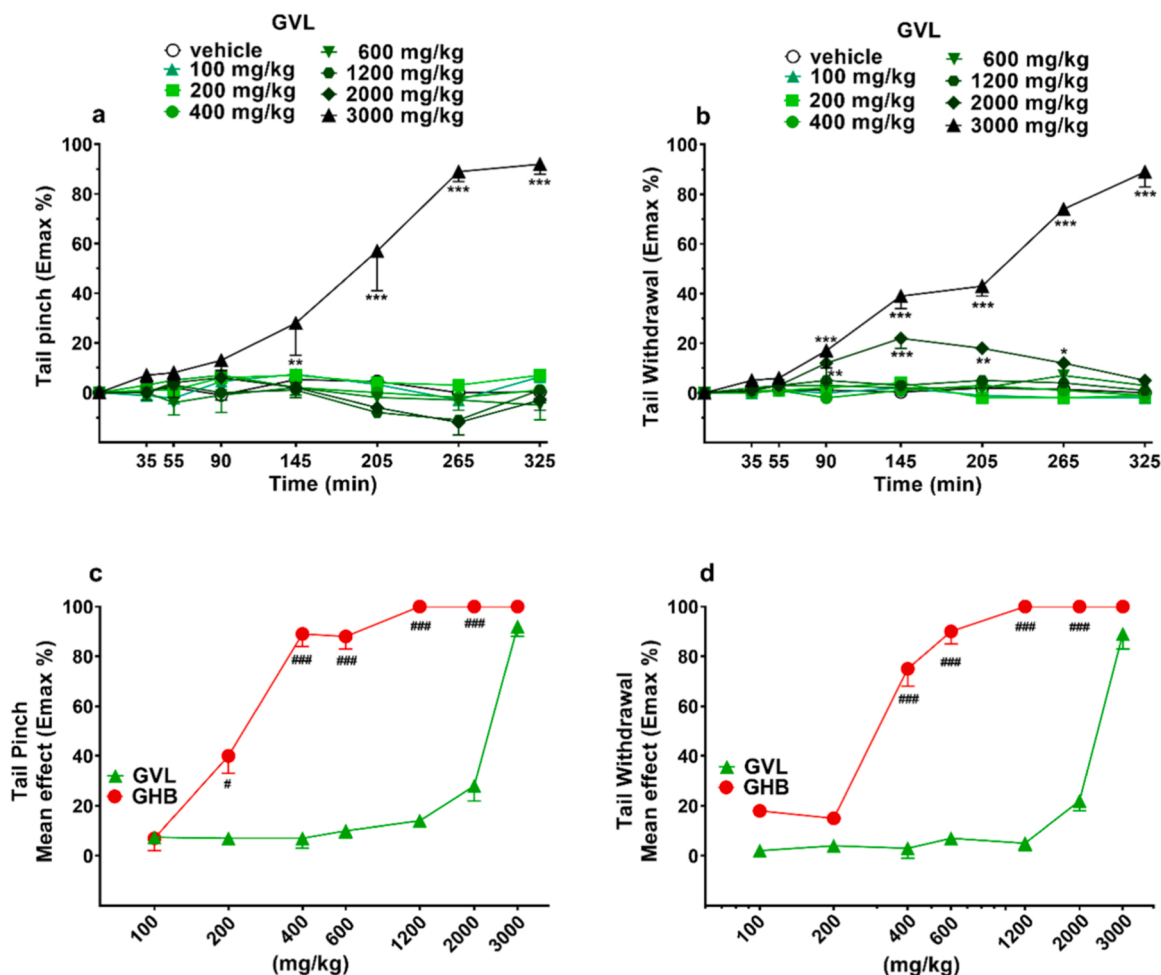


Fig. 6. Effect of GVL (100–3000 mg/kg gavage; panels a, b) on the tail pinch (left) and tail withdrawal (right) test in the mouse and comparison with GHB (100–3000 mg/kg gavage) of the maximum effect observed at different doses, on mechanical (panel c) and thermal (panel d) analgesia. Data are expressed as percentage of maximum effect and represent the mean \pm SEM of 8 mice/group. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons for the dose-response curve of each compound at different times (panels a, b) and the maximum effect observed at different doses (panels c, d). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. # $p < 0.05$, ### $p < 0.001$ versus GVL.

3.1.6. Motor activity assessment

Gavage administration of the vehicle did not change motor activity in the accelerod (Fig. 7a) and drag (Fig. 7b) tests in mice over the 5 h observation. Gavage administration of GVL induced a biphasic effect in both tests.

In particular, the dose of 100 and 200 mg/kg facilitated the motor performance of mice in the accelerod test (Fig. 7a). The motor performance in the accelerod test was not significantly affected in the group of mice treated by the range dose of 400–1200 mg/kg. The highest doses tested (2000–3000 mg/kg) of GVL inhibited motor performance, and the effect appeared at 150 min with the dose of 2000 mg/kg and persisted until the end of the test. The dose of 3000 mg/kg induced a significant reduction of mice performance at 95 min after treatment, the inhibitory effect was total (100%) at 270 min and 330 of the treatment [Fig. 7a; main effect of treatment [($F_{(7,448)} = 238.6, p < 0.0001$), time ($F_{(7,448)} = 10.83, p < 0.0001$) and time \times treatment interaction ($F_{(49,448)} = 11.69, p < 0.0001$]].

Comparison of the dose-response curve to GHB in the accelerod test revealed a major potency and efficacy of GHB at the range dose (400–2000 mg/kg) in comparison to GVL [Fig. 7c; effect of treatment ($F_{(1,90)} = 79.46, p < 0.0001$), time ($F_{(6,90)} = 39.36, p < 0.0001$) and time \times treatment interaction ($F_{(6,90)} = 8.077, p < 0.0001$]].

On the other hand, only the lowest dosage of GVL (100 mg/kg) increased the number of the steps of mice in the drag test but the effect

was not statistically significant (Fig. 7b). Despite, gavage administration of the dose of 1200 mg/kg of GVL induced a significant decrease of the number of steps only at 65 min after the treatment and the effect tend to decrease in the rest of hours of measurements. The effect was more profound with the higher dose tested (2000 and 3000 mg/kg). In particular, the dose of 3000 mg/kg significantly reduced the number of steps of mice at 65 min and the inhibitory effect was total (100%) at 270 and 330 min after the treatment [Fig. 7b; effect of treatment [($F_{(7,448)} = 82.51, p < 0.0001$), time ($F_{(7,448)} = 11.95, p = 0.0024$) and time \times treatment interaction ($F_{(49,448)} = 4.290, p < 0.0001$]].

Comparison of the dose-response curve to GHB in the drag test revealed that GHB (Table 1; $ED_{50} = 445.5$ mg/kg) is about four times more potent than GVL ($ED_{50} = 1712$ mg/kg) in reducing the number of the steps of mice [Fig. 7d; effect of treatment ($F_{(1,98)} = 131.0, p < 0.0001$), time ($F_{(6,98)} = 43.17, p < 0.0001$) and time \times treatment interaction ($F_{(6,98)} = 6.469, p < 0.0001$]].

3.1.7. Evaluation of grip strength test

Gavage administration of the vehicle did not affect the grip strength of mice over the 5 h observation (Fig. 8a).

Gavage administration of GVL reduced the grip force of mice only at higher doses (2000–3000). In particular, GVL slightly reduced (20%) the grip strength at the dose of 2000 mg/kg, the effect appeared at 125 min after treatment and disappeared at 325 min after treatment. The dose of

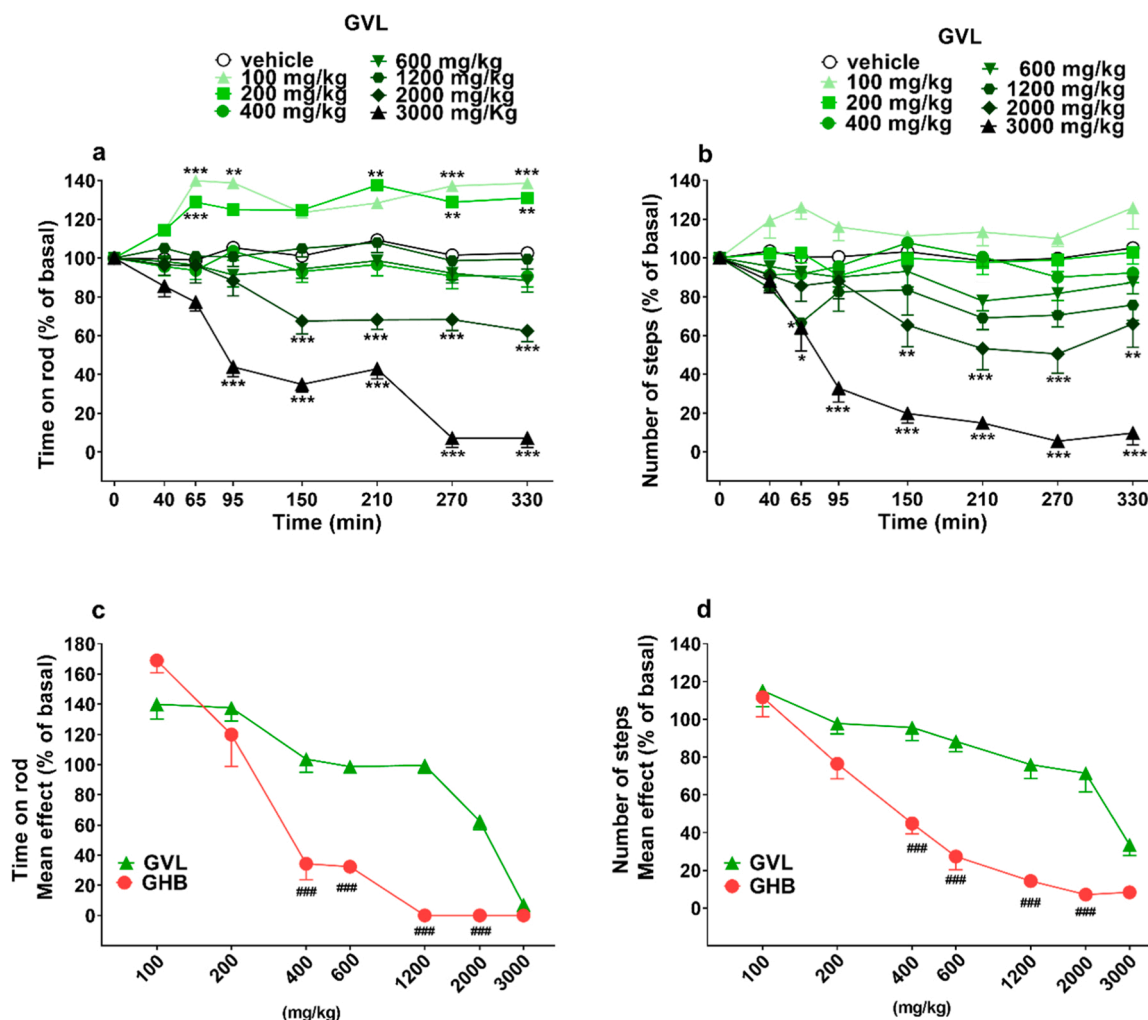


Fig. 7. Effect of GVL (100–3000 mg/kg gavage; panels a, b) on the accelerod (left) and drag test (right) in the mouse and comparison with GHB (100–3000 mg/kg gavage) of the maximum effect observed at different doses on accelerod (GVL and GHB 100–3000 mg/kg gavage) and drag test (panel c) and drag test (panel d). Data are expressed as percentage of baseline and represent the mean \pm SEM of 8 mice/group. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons for the dose-response curve of each compound at different times (panels a, b) and the maximum effect observed in different doses (panels c, d). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus saline; ### $p < 0.001$ versus GVL.

3000 mg/kg induced a long-lasting inhibition of the grip force that appeared at about 70 min after treatment and persisted until the end of the test with a total (100%) inhibition at 325 min of the test [Fig. 8a; main effect of treatment ($F_{(7,448)}=67.77$, $p < 0.0001$), time ($F_{(7,448)}=20.02$, $p < 0.0001$) and time \times treatment interaction ($F_{(49,448)}=8.724$, $p=0.0001$)].

Comparison of the dose-response curve to GHB in the grip strength test revealed a major potency of GHB (Table 1; $ED_{50}=1073$ mg/kg) in reducing the grip pulling strength of mice in comparison to GVL ($ED_{50}=ND$) [Fig. 9b; effect of treatment ($F_{(1,90)}=81.34$, $p < 0.0001$), time ($F_{(6,90)}=33.68$, $p < 0.0001$) and time \times treatment interaction ($F_{(6,90)}=14.01$, $p < 0.0001$)].

3.1.8. Evaluation of breath rate

Breath rate was unvaried in vehicle-treated mice over the 5 h observation (Fig. 9a).

Gavage administration of GVL (100–3000 mg/kg) induced a significant reduction of breath rate at higher doses (2000 and 3000 mg/kg). In particular, GVL induced a delayed decrease of breath rate at that appeared at 190 min at the dose of 2000 mg/kg with maximal inhibition of about 31% registered at 250 min after the gavage administration and the effect persisted until the end of the test. While, the dose of 3000 mg/kg of GVL induced a significant bradypnea at 70 min after

administration and the maximal effects reached 90% and 98%, respectively, at 250 min and 310 min of measurements [Fig. 9a; effect of treatment ($F_{(7,448)}=85.63$, $p < 0.0001$), time ($F_{(7,448)}=87.04$, $p < 0.0001$) and time \times treatment interaction ($F_{(49,448)}=9.265$, $p < 0.0001$)].

Comparison of the dose-response curve to GHB in the breath rate revealed a major potency of GHB (Table 1; $ED_{50}=774.9$ mg/kg) in inducing respiratory depression in comparison to GVL ($ED_{50}=ND$) [Fig. 10b; effect of treatment ($F_{(1,90)}=107.6$, $p < 0.0001$), time ($F_{(6,90)}=51.85$, $p < 0.0001$) and time \times treatment interaction ($F_{(6,90)}=13.29$, $p < 0.0001$)] (Table 2).

3.2. Cardio-respiratory analysis

After gavage administration of the highest dose tested of GVL (3000 mg/kg), we observed a deep state of coma, especially during the end of the behavioral tests. Indeed, we chose to investigate its possible cardiorespiratory adverse effects in mice and compare its effects with GHB (Arfê et al. 2021).

Vehicle administration did not change basal heart rate (684 ± 10 bpm; Fig. 10a), breath rate (190 ± 12 brpm; Fig. 10b), pulse distension (vessel diameter, 244 ± 32 μ m; Fig. 10c) and SpO₂ ($99 \pm 1\%$; Fig. 10c) in mice. After 10 min of gavage administration of the highest dose of GVL (3000 mg/kg), the heart rate modestly but not significantly

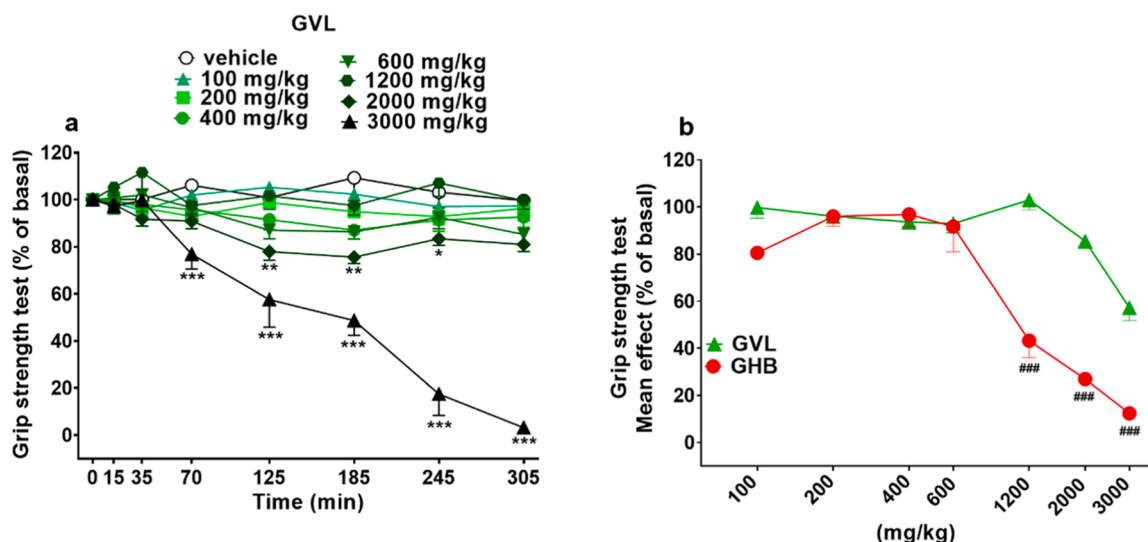


Fig. 8. Effect of GVL (100–3000 mg/kg gavage; panel a) on the grip strength test; GVL and GHB (100–3000 mg/kg gavage; panel b) comparison of the maximum effect observed in different doses muscle strength of the mouse. Data are expressed as percentage of maximum effect and represent the mean \pm SEM of 8 mice/group. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose-response curve of each compound at different times (a) and the maximum effect observed in different doses (panel b). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus saline; ### $p < 0.001$ versus GVL.

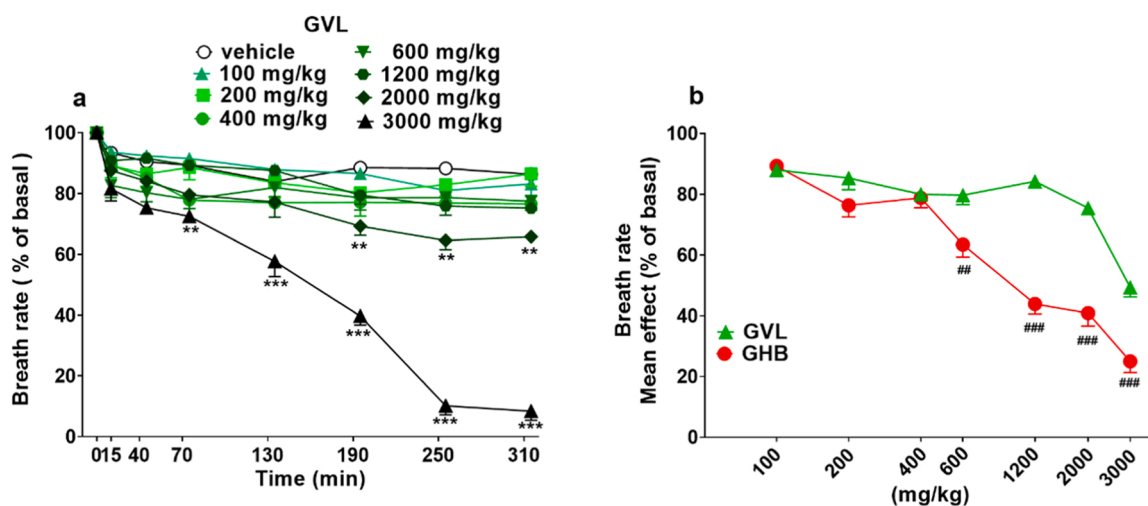


Fig. 9. Effect of GVL (100–3000 mg/kg gavage; panel a) on the breath rate; GVL and GHB (100–3000 mg/kg gavage; panel b) the maximum effect observed in different doses in mouse. Data are expressed as percentage of maximum effect and represent the mean \pm SEM of 8 mice/group. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose-response curve of each compound at different times (panel a) and the maximum effect observed in 5 h (panel b). ** $p < 0.01$, *** $p < 0.001$ versus saline; ## $p < 0.01$, ### $p < 0.001$ versus GVL.

decreased to about 13%. The effect tended to increase at 200 min after the treatment and a maximal decrease (about 30%) of the heart rate was registered in the last two hours of measurements [Fig. 10a; significant effect of treatment ($F_{(2,1080)} = 390.4$, $p < 0.0001$, time ($F_{(71,1080)} = 0.6682$, $p = 0.9839$) and time \times treatment interaction ($F_{(142,1080)} = 1.798$, $p < 0.0001$]. GVL reduced to about 13% the breath rate, immediately after administration and the bradypnea episodes increased (~30%) after the second hour of the treatment. A maximal decrease (about 80%) in the breath rate was registered between 140 min and 240 min after treatment and the effect persisted until the end of measurements [Fig. 10b; significant effect of treatment ($F_{(2,1164)} = 3476$, $p < 0.0001$), time ($F_{(71,1164)} = 32.78$, $p < 0.0001$) and time \times treatment interaction ($F_{(142,1164)} = 12.73$, $p < 0.0001$)]. The pulse distention was not affected after gavage administration of the highest dose of GVL (3000 mg/kg) [Fig. 10c; significant effect of treatment ($F_{(2,1080)} = 254.9$, $p < 0.0001$), time ($F_{(71,1080)} = 3.146$, $p < 0.0001$) and time \times treatment interaction ($F_{(142,1080)} = 2.273$, $p < 0.0001$)]. The SpO₂ was also reduced

to about 15% after 2 hours of treatment and the effect seemed to display transitional recovery periods alternating to moments where SPO₂ shows a reduction to about 30% and the effect persisted up to 5 hours after registration [Fig. 10d; effect of treatment ($F_{(2,1080)} = 1342$, $p < 0.0001$), time ($F_{(71,1080)} = 5.027$, $p < 0.0001$) and time \times treatment interaction ($F_{(142,1080)} = 4.5251$, $p < 0.0001$)].

At the end of the test, all the animals survived, even if they remained in a state of deep coma that led to their death within 24 h after measurements. However, the highest dose of GHB (3000 mg/kg) induced death in 50% of mice and induced potent alterations of the cardio-respiratory parameters in surviving ones (Arfè et al. 2021). In particular, the heart rate was reduced immediately after the treatment and the maximal reduction (~33%) was registered at 100 min. After a brief recovery, the bradycardia episodes increased again (~26%) and persisted up to 5 hours of measurement. At the same time, the pulse distention was slightly reduced during the first hour after GHB administration, but the effect was significant and persistent during the last three hours

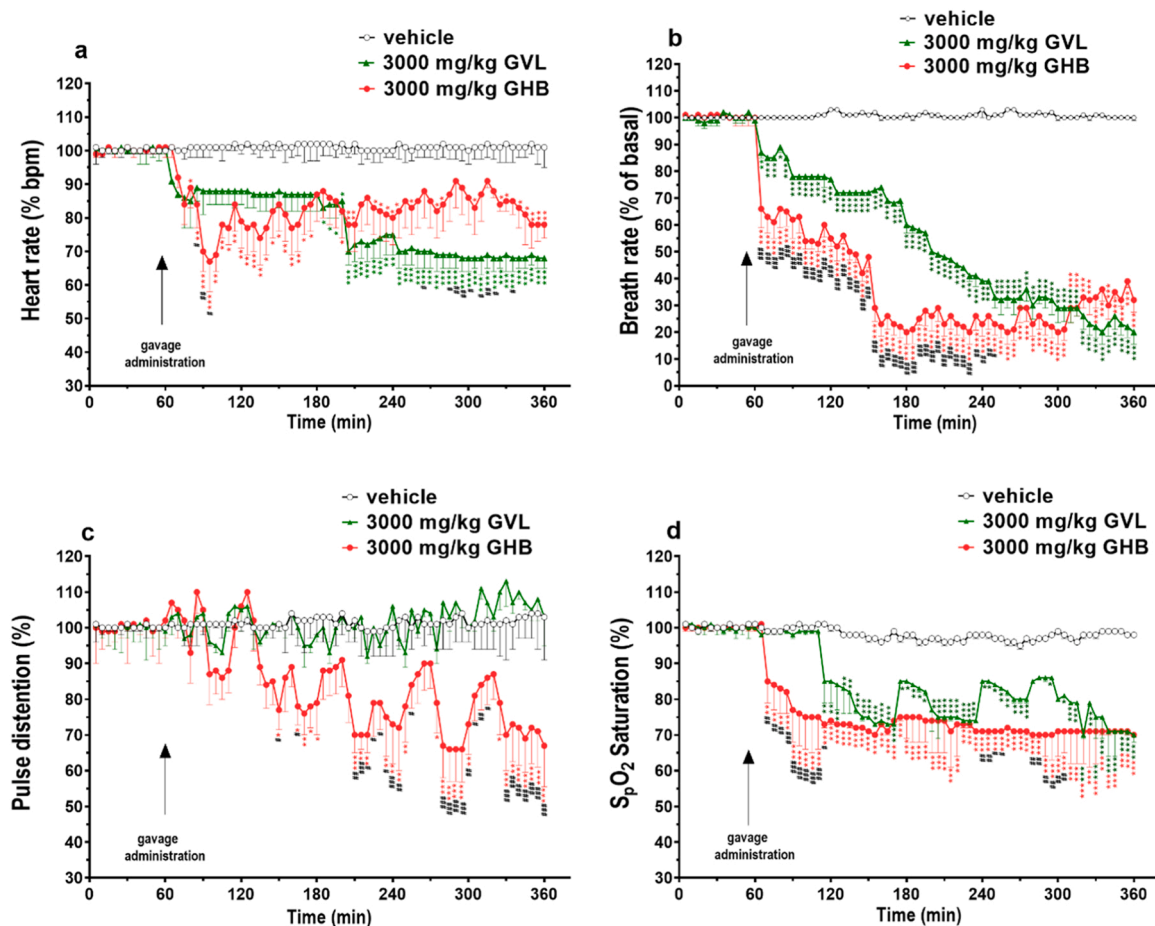


Fig. 10. Effect of GVL (3000 mg/kg gavage) and GHB (3000 mg/kg gavage) on heart rate (panel a), the breath rate (panel b), the pulse distention (panel c) and the oxygen arterial saturation (panel d). Data are expressed as percentage of basal value (heart rate, breath rate, the pulse distention) as percentage of oxygen blood saturation (%SpO₂ saturation) and represent, the mean \pm SEM of 6 mice/group. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose-response curve of each compound at different times. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus saline; ## $p < 0.01$, ### $p < 0.001$ versus GVL.

where the pulse distention was reduced to about 34% at 300 min of the registration [Fig. 10c; significant effect of treatment ($F_{(2,1080)} = 390.4$, ($p < 0.0001$), time ($F_{(71,1080)} = 0.6682$, $p = 0.9839$) and time \times treatment interaction ($F_{(142,1080)} = 1.798$, ($p < 0.0001$)]. The breath rate was also reduced to about 35% immediately after GHB and persisted up until the second hour of the treatment. In particular, the breath rate was inhibited to about 75% and the effect persisted until the end of the experiment [Fig. 10b; significant effect of treatment ($F_{(2,1164)} = 3476$, ($p < 0.0001$), time ($F_{(71,1164)} = 32.78$, $p < 0.0001$) and time \times treatment interaction ($F_{(142,1164)} = 12.73$, ($p < 0.0001$)]. The SpO₂ was also reduced to about 15% immediately after gavage administration of GHB and the effect persisted up to 5 hours after registration [Fig. 10d; effect of treatment ($F_{(2,1080)} = 1342$, $p < 0.0001$), time ($F_{(71,1080)} = 5.027$, $p < 0.0001$) and time \times treatment interaction ($F_{(142,1080)} = 4.5251$, $p < 0.0001$)].

The statistical comparison of the cardiorespiratory effects induced by 3000 mg/kg of GVL to GHB revealed the major effectiveness of GHB (***) ($p < 0.001$) in reducing the breath rate, oxygen saturation, and pulse distention but not the heart rate. GVL was more effective than GHB in reducing the heart rate in the last two hours of measurements (** $p < 0.01$).

3.3. ADMET prediction of GHB, GVL, and GHV

The risk scores for GHB, GVL, and the active metabolite of GVL (GHV) were calculated (Table 3). All risk score thresholds are illustrated in Table 1. GHB and its derivatives were below the ADMET risk

threshold of 7. No absorption risk was predicted for the three compounds, Toxicity risk of the three compounds was below the risk threshold of 2, however, ADMET predicts potential risk codes related to hepatotoxicity (HEPX) and cardiotoxicity (hERG) for GHB and GVL. GHV toxicity risk code was predicted to be related to hepatotoxicity only. The hepatotoxicity rule "HEPX" of ADMET reflects the way actual blood test results are interpreted. In particular, hepatotoxicity is indicated if aspartic acid transaminase (Ser AST) and alanine transaminase (Ser ALT) are both elevated in serum. The liver injury also usually elevates serum levels of lactate dehydrogenase and including Ser LDH in the rule reduces the number of false positives. Concomitant elevation of the other serum enzyme model predictions, Ser AlkPhos or Ser GGT, is indicative of even more severe liver injury. As shown in Table 4 the three compounds were predicted to induce elevated levels of Ser AST, Ser ALT, Ser LDH, Ser AlkPhos, and Ser GGT.

GHB and GVL were below the threshold of the cytochrome risk (<2). ADMET predictor revealed no cytochrome risk for GHV. Cytochrome risk code of clearance (CL) was predicted for GHB and GVL. Only GVL was predicted to have a potential mutation risk (yellow flag plot, Table 3), however, this risk is only mentioned but it is not considered in our study.

The absorption profile of GHB, GVL, and GHV was also predicted (Table 5). The three compounds were predicted to have high gastrointestinal permeability (S+Peff). The human jejunal permeability of GVL was ± 2 times higher than GHB and GHV. In addition, GVL was predicted to have a very high kidney permeability predicted in Madin-Darby

Table 2

ED₅₀ values of GVL and GHB based on in vivo performed behavioral tests. Data are expressed as mean±SEM. ED₅₀ has been calculated by non-linear regression curve fitting of the dose-response curves determined using Prism 8.0 software (GraphPad Prism, San Diego CA). ED₅₀ values relative to each test were statistically compared performing Unpaired t test. ***p<0.001 versus GHB.




Test	GVL ED ₅₀ (mg/kg)	GHB ED ₅₀ (mg/kg)
Visual object	1705±0.010 (1625–1787)	356.3±0.008*** (342.8–370.3)
Visual placing	1506±0.047 (1176–1875)	570.2±0.028*** (501.1–650.7)
Startle reflex	1661±0.009 (1591–1732)	397.5±0.008*** (384.1–411.3)
Overall tactile	1828±0.008 (1758–1900)	468.8±0.013*** (440.4–499.5)
Bar	N.D.	376.8±0.031 (325.1–431.9)
Righting reflex	N.D.	N.D.
Core temperature	1738±0.011 (1648–1831)	1219±0.019*** (1116–1330)
Surface temperature	2023±0.004 (1977–2067)	1123±0.014*** (1052–1198)
Tail pinch	N.D.	433.2±0.029*** (562.6–703.3)
Tail withdrawal	N.D.	626.6±0.023*** (562.6–703.3)
accelerod	N.D.	N.D.
Drag	1712±0.053 (1286–2180)	445.5±0.034*** (378.8–526.3)
Grip strength	N.D.	1073±0.032 (992.7–1242)
Breath rate	N.D.	774.9±0.035 (662.0–911.9)

canine kidney cells (MDCK) with respect to GHB and GHV that show a low permeability (<30 cm/s x10⁷). GVL was also predicted to have a high blood-brain barrier (BBB) penetration with respect to the other compounds. GVL was neither a substrate or inhibitor of P-glycoprotein (P-gp); however, GHB and GHV resulted to be substrates of P-gp. The three compounds resulted to be substrates of the organic anion transporting 1 (OAT1). Only GVL was predicted to be not a substrate of organic anion transporting 3(OAT3). GHB and GHV were both predicted to be substrates of OAT3.

Some pharmacokinetic properties were also predicted (Table 6). In relation to absorption, the predicted percent unbound to blood plasma proteins in human (hum_fup%) was higher than 10% for the three drugs indicative of low plasm binding of the three drugs (see Table 1 for

Table 3

Risk scores of GHB, GVL and GHB by ADMET Predictor®. The wedge colors in the star plots correspond to the following: green for ADMET_Risk, blue for Absn_Risk, red for TOX_Risk, purple for CYP_Risk, and yellow for MUT_Risk.

Drug	Overall Risks	ADMET_Risk	Absn_Risk	TOX_Risk	CYP_Risk
GHB		2 hERG; HEPX,CL	0	1.5 hERG; HEPX	0.5 CL
GVL		2 hERG; HEPX, CL, MUT	0	1.5 hERG; HEPX	0.5 CL
GHV		1 HEPX	0	1 HEPX	0

hERG, human Ether-a-go-go Related Gene, a qualitative estimation of the likelihood of hERG potassium channel inhibition in human; HEPX, hepatotoxicity; CL high microsomal clearance; MUT mutation.

thresholds). The predicted values of fup% in human were very similar to the value predicted for mice (mou_fup%). GVL was predicted to have a blood to plasma ratio (hum_RBP%) in human higher than GHB and GHV however the predicted mouse RBP (mou_RBP) was lower than 1 for GVL and other compounds.

The predicted volumes of distribution (Vd) of the three compounds were generally low, indicating that their distribution will likely be limited to plasma. The Vd of GVL was predicted to be ±5 times higher than GHB and GHV.

The inhibition of the bile salt export pump (BSEP) is one of the factors that cause liver dysfunction. The tested compounds exceed the threshold of 60µM which refers to BSEP inhibitors (Table 6). The predicted BSEP_IC50 of GVL was very high with respect to GHB and GVL. In contrast to GHB and GHV, the primary mechanism predicted for GVL clearance was metabolism; while for GHB and GHV the primary clearance route was predicted to be renal. Indeed, GHB and GHV are classified as class_1A according to ECCS (Extended Clearance Classification System) while GVL is class_2.

The metabolic profile of GHB, GVL, and GHV was predicted (Table 7). GHB and GHV are not expected to be inhibitors or substrates for CYP3A4, CYP1A2, CYP2C9, CP2A6, CYP2B6, CYP2D6, and CYP2E1. GVL was predicted to be only a substrate of CP2A6 and CYP2B6.

4. Discussion

The present study reveals the in vivo effects of acute gavage administration of GVL in comparison to GHB (Arfè et al. 2021) at the range dose (100–3000 mg/kg) on neurological, sensorimotor, motor,

Table 4

Predicted effects of GHB, GVL and GHV and their percentage of accuracy (%) by ADMET Predictor® on hepatic enzyme levels in human serum.

Drug	Ser_AlkPhos	Ser_GGT	Ser_LDH	Ser_AST	Ser_ALT
GHB	Elevated (59%)	Elevated (93%)	Elevated (78%)	Elevated (96%)	Elevated (69%)
GVL	Elevated (72%)	Elevated (57%)	Elevated (78%)	Elevated (96%)	Elevated (86%)
GHV	Elevated (75%)	Elevated (93%)	Elevated (68%)	Elevated (46%)	Elevated (41%)

Ser_AlkPhos, serum alkaline phosphatase levels; Ser_GGT, serum Gamma-glutamyl transferase levels.

Ser_LDH, serum lactate dehydrogenase levels; Ser_AST serum aspartic acid transaminase, Ser_ALT serum alanine transaminase.

Table 5
Predicted permeability and transporting properties of GHB, GVL and GHV and their percentage of accuracy (%) by ADMET Predictor®.

Drug	S+Peff [cm/s x 10 ⁴]	S+MDCK [cm/s x10 ⁷]	BBB Penetration	Pgp_Sub	Pgp_Inh	OAT1_Sub	OAT3_Sub
GHB	2.634	11.832	Low (48%)	Yes	No (93%)	Yes (95%)	Yes (99%)
GVL	5.274	682.461	High (99%)	No	No (72%)	Yes (87%)	No (60%)
GHV	2.812	13.763	Low (59%)	Yes (99%)	No (78%)	Yes (95%)	Yes (99%)

S+Peff, human effective jejunal permeability; S+MDCK, apparent permeability through Madin-Darby canine kidney cell layers; BBB, Blood Brain Barrier; P-gp_Sub, P-glycoprotein substrate; P-gp_Inh, P-glycoprotein inhibitor; OAT1_Sub, Organic Anion Transporting 1 substrate; OAT3_Sub, Organic Anion Transporting 3 substrate.

Table 6
Predicted pharmacokinetic properties of GHB, GVL and GHV by ADMET Predictor®.

Drug	hum_fup [%]	mou_fup [%]	hum_RBP	mou_RBP	Vd [L/kg]	BSEP_IC50 [uM]	S+CL_Mech	ECCS_Class
GHB	57.896	77.896	0.981	0.737	0.278	73.98	Renal	Class_1A
GVL	86.085	82.391	1.29	0.851	0.94	273.305	Metabolism	Class_2
GHV	51.204	75.064	0.925	0.733	0.286	155.104	Renal	Class_1A

hum_fup%, percent unbound to blood plasma proteins in human; mou_fup%, percent unbound to blood plasma proteins in mouse; hum_RBP, blood to plasma ratio in human; mou_RBP; blood to plasma ratio in mouse; Vd, volume of distribution; BSEP_IC50, Bile Salt Export Pump IC50; S+CL_Mech, predicts clearance mechanism as primarily metabolism, renal or hepatic uptake; ECCS_class: Extended Clearance Classification System (based on Varma et al., 2015).

Table 7
Predicted cytochrome enzymes involved in GHB, GVL and GHV metabolism and their percentage of accuracy (%)by ADMET Predictor®.

DRUG	CYP 3A4		CYP 2C9		CYP 2A6		CYP 2B6		CYP 2D6		CYP2E1			
	Inh.	Subs.	Inh.	Subs.	Inh.	Subs.	Inh.	Subs.	Inh.	Subs.	Inh.	Subs.		
GHB	No (78%)	No (84%)	No (97%)	No (85%)	No (95%)	No (95%)	-	No (98%)	-	No (98%)	No (95%)	No (95%)	-	No (82%)
GVL	No (81%)	No (54%)	No (76%)	No (70%)	No (95%)	No (85%)	-	Yes (44%)	-	Yes (51%)	No (95%)	No (85%)	-	Yes (91%)
GHV	No (81%)	No (84%)	No (97%)	No (97%)	No (95%)	No (95%)	-	No (98%)	-	No (98%)	No (95%)	No (95%)	-	No (94%)

Inh., inhibitor; Subs., substrate.

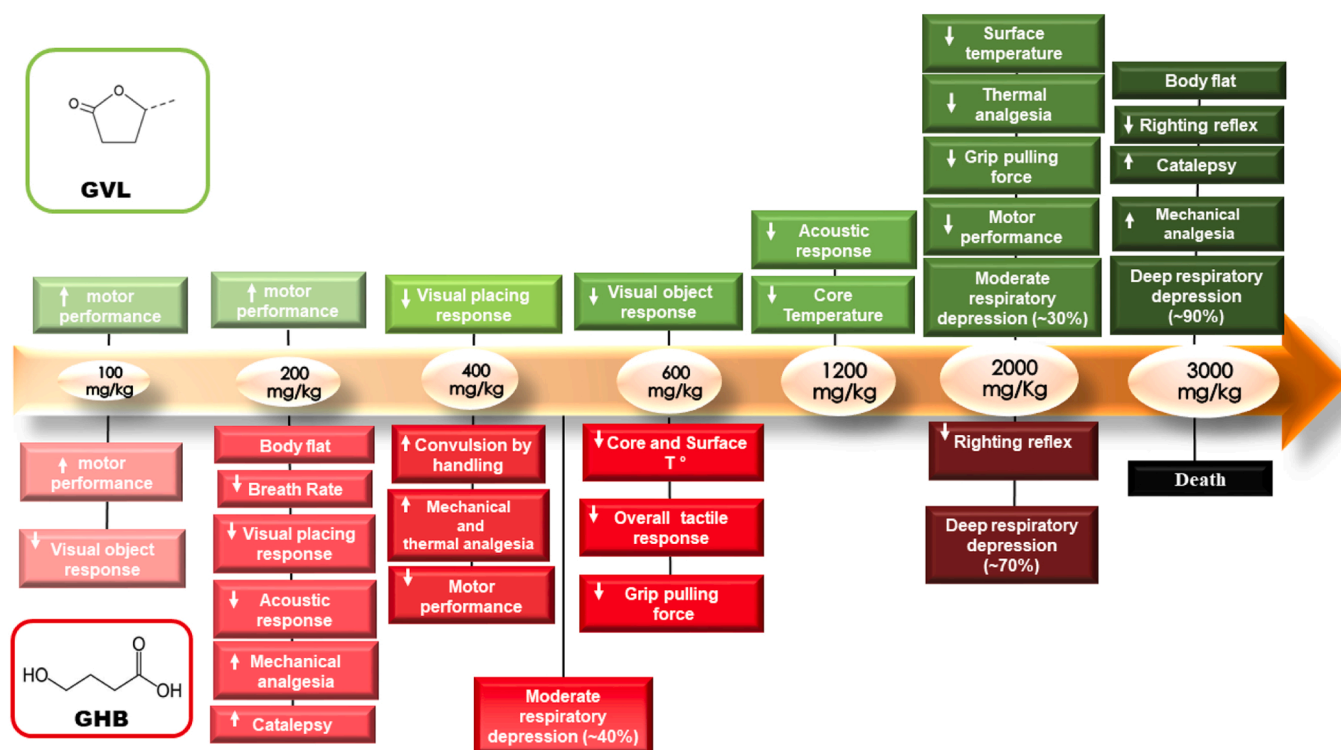


Fig. 11. Schematic comparison of the progressive appearance of pharmacological and behavioral effects of the gavage administration of the increasing doses of GVL (100–3000 mg/kg) in respect to GHB (100–3000 mg/kg; Arfê et al., 2021) in CD-1 adult male mice.

and cardiorespiratory parameters in mice. GVL mimicked the pharmacological effects of GHB in terms of sedative and hypnotic but with a lower potency with respect to GHB (Fig. 11).

4.1. Behavioral responses

4.1.1. Visual object and placing responses

We have first demonstrated that GVL inhibited in a dose-dependent manner the visual object and placing responses, and the effect persisted up to 5 h at higher doses (Fig. 2). However, the effect of GHB was more potent, and the inhibition of visual reflexes reached the maximum at lower doses (400–3000 mg/kg) in respect to GVL (3000 mg/kg). The effect of GVL and GHB on visual impairments was reported in our recent studies (Camuto et al., 2022; Arfè et al. 2021). There is no clinical evidence regarding the effect of GVL on visual responses. However, one of the anecdotal affirmations of “psychonaut” who compared his feelings after GVL intake in comparison to GHB, stated. “At the half-hour point, a sedation can be felt, but the feeling is in part unlike that of GHB itself, this feeling is more like natural sleepiness than the feeling gotten from GHB and other sedating pharmaceuticals” (<http://www.erowid.org/archive/rhodium/chemistry/4-methyl-ghb.html>). The effects of GHB on human visual responses have been widely reported. Specifically, GHB is included in the list of the drugs that have an influence on driving (DUID) (Stephens and Baselt, 1994; Centola et al., 2018; Bosman and Lusthof, 2003; Al-Samarraie et al., 2010). The reported effects of GHB in cases of DUID are: incapacity, decreased consciousness, sleepiness, and decreased driving performance. Moreover, electroencephalogram (EEG) and behavioral studies in humans revealed that GHB enhances sleep by lengthening slow-wave/delta sleep (Mamelak et al., 1977). Preclinical studies revealed that GHB could induce sleep in rabbits and dogs using 1 g/kg (i.v.) and rats using 0.5 g/kg (i.p.) (Laborit, 1964). Thus, similarly to GHB, the effect of GVL on visual responses in mice could be related to its inhibitory effects on the CNS (Carter et al., 2005). The mechanism by which GHB and its related substances could impair visual responses was discussed in our previous study (Arfè et al. 2021).

4.1.2. Acoustic and tactile response

GVL reduced also the acoustic and tactile responses in mice in a similar but lower potency in comparison to GHB (Fig. 3). This finding is consistent with the study by Marinetti and his colleagues, where it has been demonstrated the inhibitory effects of GVL on the acoustic startle response at the dose of 1600 mg/kg. Higher doses of GVL induced an almost total inhibition of noise alone to startle amplitude. In the same study, the correlation between the concentration in the brain of the active metabolite of GVL (4-methyl-GHB) and the GVL-reduced startle amplitude could reveal the role of GHB receptors in the inhibition of the acoustic response in mice after GVL administration, in a similar but lower efficacy with respect to GHB (Marinetti et al., 2012).

4.1.3. Analgesic response

We have demonstrated in our study that only higher doses of GVL induced mechanical and thermal analgesia. GHB instead, dose-dependently, increased mechanical and thermal analgesia. Our data reveals the lower potency of GVL and the delayed mechanical and thermal analgesia in comparison to GHB. Of note, the dose of 3000 mg/kg of GVL induced body flat, loss of righting reflex, inhibition of motor activity (bar test, accelerod test, and drag test), and muscle relaxation (Grip strength test). Thus, the analgesic effects induced by GVL could be related to its anesthetic and sedative action at higher doses (Carter et al., 2005). The analgesic effects of GHB and the possible mechanisms involved in pain perception were discussed in our previous study (Arfè et al. 2021). Based on the data obtained in our previous study on GVL metabolism and its time of excretion (Camuto et al., 2022), we can hypothesize that the metabolite of GVL (GHV) is responsible for the analgesic/anesthetic effect that appears at 90 min with higher doses (2000 and 3000 mg/kg) since this active metabolite acts on GHB

receptors and induce similar anesthetic effects to GHB at high doses (Carter et al., 2005).

4.1.4. Motor activity

Motor impairments and muscle relaxation are typical effects of sedative hypnotics. We have demonstrated that GVL facilitated the motor performance of mice at low doses (100 and 200 mg/kg), while higher doses (2000 and 3000 mg/kg) induced catalepsy (Fig. 7). Moreover, the highest dose of GVL (3000 mg/kg) induced body flat posture and loss of righting. Our results are in accordance with the previous publications on GHV and GHB-induced motor impairments in mice (Carter et al., 2005; Arfè et al. 2021). The increase of motor activity induced by lower doses (100 and 200 mg/kg) of GVL could be related to the activation of the GHB receptors, which have been linked to an inhibition of GABAergic interneurons and consequent increase in dopamine levels (Bay et al., 2014). Moreover, Castelli and his colleagues demonstrated in their elegant study that GHB and its analogs decrease at low concentrations, the glutamate extracellular levels, and this effect is mediated by GHB receptors (Castelli et al., 2003). In humans, these effects could be translated as anxiolytic effects of GVL. Conversely, high doses of GVL inhibited motor activity in mice and at the highest dose tested, mice showed catalepsy and coma-like states. In case reports of DFSA suspecting GVL intake, the victims reported the feeling of “blackouts” during the time (Andresen-Streichert et al., 2013). These “blackouts”, “knockouts” or loss of consciousness are typical of sedative hypnotics (Raposo Pereira et al., 2018). Yet, as reported with GHB, high doses of GVL could inhibit motor activity acting on GHB receptors (Bay et al., 2014) and GABA_B receptors (Ferraro et al., 2001) thus determining the catalytic and hypokinetic state in animals (Arfè et al. 2021), and humans (Andresen-Streichert et al., 2013). Moreover, we have also demonstrated in the grip strength test that GVL reduced the grip muscle strength of mice at higher doses and GHB showed a major potency with respect to GVL. These results confirm the sedative and cataleptic states obtained in the motor activity tests. This finding is consistent with the study of Carter and colleagues, where GHV produced ataxia in mice at higher doses (3200 mg/kg) in the inverted screen test (Carter et al., 2005).

4.1.5. Thermoregulation effects

In addition to sensorimotor and motor impairments, we have demonstrated that GVL can modulate physiological parameters. GVL-induced hypothermia at the range dose (1200–3000 mg/kg). This is the first study reporting the effect of GVL on thermoregulation, however, this effect has already been studied on GHB (Carai et al., 2008; Arfè et al. 2021). The stimulation of GABA_B receptors seemed to play an important role in inducing hypothermia in mice in parallel to behavioral changes (Gray et al., 1987; Quèva et al., 2003). Moreover, it has been suggested that GABA_B receptors alter body temperature by acting at the hypothalamic thermoregulation center (Gray et al., 1987; Yakimova et al., 1996). Based on these findings it is possible to suggest that GVL could stimulate at high doses GABA_B receptors in a similar but lower potency with respect to GHB, which results in a progressive decrease of body temperature in mice (Carai et al., 2008; Quèva et al., 2003; Arfè et al. 2021).

4.1.6. Cardiorespiratory effects

We have demonstrated in our study that the gavage administration of GVL and GHB induced respiratory and cardiorespiratory changes. In fact, both GHB and GVL caused respiratory depression that is observed at higher doses (2000 and 3000 mg/kg) for GVL, while significant effects appeared already at a low dose of 200 mg/kg of GHB (Fig. 8). Moreover, using the MouseOX instrument, the evaluation of the highest dose of GVL on the cardio-respiratory function reveals a deep and persistent bradycardia and a bradypnea with a fluctuation of the SpO₂ saturation; however, the pulse distention was not affected by GVL administration. Due to its large commercial application like other aliphatic lactones,

GVL cardiotoxicity was evaluated in 1945 by Deichmann and his group. This article revealed that the gavage administration of GVL in rabbits and rats induce an increase in the respiratory rate followed by dyspnea and mild asphyxial convulsions and death results from the combined effects on circulatory and respiratory apparatus (Deichmann et al., 1945). In difference to the results obtained by Deichmann, we didn't observe a biphasic effect in respiration or convulsion and this could be related to the difference of the species, the doses, and the test used. GHB (3000 mg/kg) induced long-lasting bradycardia and a significant decrease in SpO₂ saturation and pulse distention (Arfè et al. 2021). These data are in accordance with the study of Laborit demonstrating that high doses of GHB induced bradycardia, periods of apnea, and an increase in carbon dioxide (pCO₂) (Laborit, 1964). 50% of the animals treated with 3000 mg/kg of GHB died at the end of the MouseOx test, showing severe respiratory depression (Arfè et al. 2021). While the animals treated with GVL died after 24 hours of the test. The median lethal dose (LD₅₀) for GVL is 2640 mg/kg in rabbits (Deichmann et al., 1945) and 5000 mg/kg in rats (Moreno, 1978). In mice the LD₅₀ is not yet determined, however, it was evaluated for its metabolite (GHV LD₅₀= 5600 mg/kg; Carter et al., 2004). The median lethality of GHB is 3.3 g/kg in dogs and 1.7 g/kg in rats where respiratory depression is the main reason for death (Laborit, 1964). These data highlight the major potency of GHB compared to GVL.

4.2. Forensic implications

As mentioned, GHB analogs, including GVL, possess very similar chemical structures to the parent compound and are often used as substitutes for GHB, or to produce GHB. Despite being legally marketed as chemical/industrial solvents, when taken through human consumption, these compounds can produce effects similar to those of GHB intake, such as relaxation, euphoria, agitation, and drowsiness; for this reason, their potential as drugs taken for recreative purpose should not be disregarded.

Nonetheless, as opposed to GHB whose role in DFSA (drug-facilitated sexual assault) or chemical submission cases has been known for years, there are only three cases of DFSA, including one hypothesized as DFSA, related to GVL use reported in the literature (Andresen-Streichert et al., 2013). Despite the lack of data, some information regarding the effects of this compound can be found on dedicated websites in which individuals share their experiences. Some of the latter experiences have been documented in a study by Andresen-Streichert et al. (2013), for example some people have described the sedative effect of GVL as more similar to natural sleepiness, with greater muscle relaxation, compared to the more potent effect experienced with GHB; again, others claim that GVL effects last longer than those of GHB; in addition, some assert that the typical "knock-out" effect of GHB is absent with GVL, while others believe that the sedative and anxiolytic effects of GVL are more potent.

These substances undoubtedly provide a significant forensic challenge. With regard to GVL, there are only few studies in the literature on the subject and, to date, little is known about its effects on humans. The limited number of cases reported in the literature is probably attributable to the fact that many of the routine pharmacological tests performed in hospitals are not predisposed for the research for this substance (García al., 2021); indeed, these compounds require specific field and laboratory testing. Additionally, there are relatively few pharmaco-toxicological studies on GVL.

4.3. *In silico* ADMET prediction as a promising tool for NPS screening

The prediction of ADMET parameters is one of the most reliable tools that has increasingly been included in the research of quantitative structure–activity relationship (QSAR) models for early drug discovery (Kar and Leszczynski, 2020). Due to the lack of knowledge of the pharmaco-toxicological effects of GVL, we applied *in silico* ADMET prediction to compare the toxicokinetic effects of GVL in comparison to

GHB and also GHV. The ADMET risk scores of GHB and GVL were similar however, the prediction of some ADMET parameters revealed differences in the toxicokinetics of the drugs. In particular, GVL appeared to have very high permeability and capacity to cross the BBB in comparison to GHB and GHV. These differences could be related to different interactions played by these drugs with the transporting proteins expressed in BBB such as P-gp, OAT1, and OAT3. Indeed, GVL was predicted to be not a substrate or inhibitor of P-gp and OAT3 while GHB and GHV resulted to be substrates for P-gp, OAT1, and OAT3. Since P-gp, OAT1, and OAT3 play an important role in the bioavailability of the drugs in the brain, these findings could partially explain the differences observed in *in vivo* pharmacokinetics of GVL in comparison to GHB (Bhattacharya and Boje., 2004; Dalla et al., 2022). The ADMET prediction of some distribution parameters of the three drugs reveals a low binding to blood plasma proteins, these data reveal the distribution of the drugs to their tissue targets (Mazarr-Proo and Kerrigan. 2005).

The low V_d predicted for the three compounds might be related to possibly increasing renal filtration (Palatini et al., 1993). Indeed, ADMET prediction reveals that the primary clearance mechanism of GHB and GHV is renal uptake, however, GVL was predicted to undergo hepatic metabolism as the first route of clearance (Morris et al., 2005). Moreover, only GVL was predicted to be a substrate of CYP2A6 and CYP2B6. These data confirm the previous findings on GVL hepatic transformation to the active metabolite GHV (4-methyl-GHB) that acts similarly to GHB (Camuto et al., 2022). ADMET prediction allowed us to evaluate the possible organ injuries that these drugs may cause. Indeed, the three compounds were predicted to induce elevated levels of Ser_AST, Ser_ALT, Ser_LDH, Ser_AlkPhos, and Ser_GGT which are indicative of hepatotoxicity. Moreover, the estimation of the bile Salt Export Pump inhibition (BSEP_IC50) reveals an increasing hepatotoxic risk for GHB as suggested in the literature (Busardò et al., 2015). Finally, the ADMET prediction confirms the toxic risk related to GHB and predicts important toxicokinetic parameters related to GHB, GVL, and GHV. This *in silico* tool should be considered for rapid NPS ADMET screening.

5. Conclusion

The present study demonstrates that GVL inhibited, in a dose-dependent manner, the sensorimotor and motor responses and induced cardiorespiratory depression (at a dose of 3000 mg/kg) in mice. In particular, this study aimed to highlight that the use of GVL (excellent Valium alternative) especially at the tested higher doses (1200–3000 mg/kg), induced similar effects to GHB. While it is known that GHB is implicated in DFSA cases, pharmaco-toxicological knowledge and information about GVL are poorly studied. *In silico* ADMET prediction revealed a risk of hepatotoxicity and cardiotoxicity of GVL. Further *in vivo* investigations are needed in order to define a complete pharmaco-toxicological profile of this dangerous substance.

Ethical statements

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures performed in the studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. The project is activated in collaboration with the Presidency of the Council of Ministers-DPA Anti-Drug Policies (Italy).

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Author contributions

Authors declare that RA, SB and MM designed the studies, with refinements contributed by FDG. MT, GC, MBa, MBO, FB, TB, LC, EB and SB performed the research, conducted initial data analysis, created figures and conducted statistical analysis of data. MM, SB, RA wrote the major drafts of the paper. FDG, GS, AMA, OR and FB edited sections on the manuscript. All authors have approved the submitted version of the manuscript.

Declaration of Competing Interest

Declarations of interest: none.

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