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ORIGINAL ARTICLE

Pharmacological profile of three different γ-butyrolactone derivatives in mice

Kinga Sałat, Tadeusz Librowski, Barbara Filipek

Department of Pharmacodynamics, Jagiellonian University Medical College, Medyczna 9, 30- 688 Cracow, Poland

Abstract: The paper presents the pharmacological profile of the analgesic activity of 3 derivatives of γ-butyrolactone (GBL), marked with the symbols: LMOR, LHEL and L8. In view of the available data indicating potent antinociceptive activity of some GBL, analgesic activity of these compounds was investigated in a few screening models, namely the hot plate, writhing and capsaicin tests. Moreover, spontaneous locomotor activity, local anesthetic activity in modified tail immersion test and acute toxicity were also evaluated. The results of the experiments confirm antinociceptive activity in a vast range of rodent models of pain, especially pain induced by thermal (the hot plate and modified tail immersion tests) or chemical (phenylbenzoquinone but not capsaicin) stimuli.

Key words: hot plate, phenylbenzoquinone-induced writhing, capsaicin, tail immersion, toxicity, mice

INTRODUCTION

The complex process of detection and sensation of pain, namely the nociception consists of 4 phases: transduction, transmission, modulation and perception [1-3]. Transduction is the process of detection of a painful stimulus, transmission of which enables the modulation (either enhancement or inhibition) of the stimulus within the central nervous system. Perception, in turn, involves the limbic system and the cortex.

Although medical intervention (i.e. the use of analgesic drugs) is possible at almost every stage of this process, treatment of pain that accompanies many diseases, especially neuropathic pain (which is a consequence of nerve injury) still remains an important medical problem. Currently available drugs that are able to diminish pain can be divided into 3 groups: nonsteroidal anti-inflammatory drugs (NSAIDs, cyclooxygenase inhibitors) which possess anti-inflammatory, antipyretic and antinociceptive properties; opioids which are ligands for opioid receptors (μ, δ, κ) and a group of analgesic adjuvants. This last group is of special interest for the contemporary and future therapy of pain – not only because of its variety (antidepressants, antiepileptics, hormones, α_2-adrenomimetic agents belong to it), but also in view of heterogenous mechanisms which may, as a final result, heal the pain of different origins and diminish the patient's suffering [1-7].

There are some reports indicating that anticonvulsants are particularly effective in some kinds of pain [8, 9]. Moreover, some antiepileptic agents (e.g. gabapentin, pregabalin, lamotrigine, tiagabine, zonisamide) have been shown to be effective in animal models of pain [10, 11], and their mechanism of action (voltage-gated ion channel blockade) seems to be a potential and very promising target for upcoming drugs. Of note is the fact that several groups of antidepressants (e.g. tricyclic antidepressants), similarly to certain opioids (tramadol), can modulate pain transmission influencing descending antinociceptive pathways deriving from the periaqueductal grey and rostroventral medulla.

Previously, several derivatives of α-substituted *N*-benzylamides of γ-hydroxybutyric acid (GHB) were reported to display anticonvulsant activity [12-15]. On the other hand, some derivatives of γ-butyrolactone (GBL) – a cyclic precursor of GHB [16] – possess anticonvulsant and analgesic activity [17-19].

Currently, we have extended our investigation to a group of new 3-mono-substituted derivatives of GBL with potential analgesic activity. Structures containing pharmacophoric γ-butyrolactone moiety with heterocyclic (isochinolinyl, morpholinyl or arylpiperazine) group were designed. The latter is a very well-known fragment constituting a numerous group of serotonin $(5HT)_{1A}$ receptor ligands [20], and arylpiperazine derivatives have been reported to exert potent and efficacious analgesic activity [21].

MATERIALS AND METHODS

Chemicals. Three derivatives of γ-butyrolactone (LMOR, LHEL and L8) were synthesized for the experiments, suspended in a 0.5% methylcellulose solution (Loba Chemie, Germany) and administered by the intraperitoneal (*ip*) route 30 min before the experiments, excepting tail immersion and acute toxicity tests. Control animals were given an appropriate amount of vehicle (0.5% methylcellulose suspension). Phenylbenzoquinone (INC Pharmaceuticals, Inc. NY) was prepared as a 0.02% solution. Morphine (Morphinum hydrochloricum, Polfa Kutno), acetylsalicylic acid (ASA, Polpharma), lignocaine (Lignocainum hydrochloricum 1%, WZF Polfa Warsaw) and mepivacaine (Maverin 2%, Rhone-Poulenc Rorer) were used as reference drugs. Capsaicin was purchased from Sigma-Aldrich and administered intraplantarly (*i pl*) to the mouse paw.

Animals. For the behavioural experiments, adult male Albino Swiss mice weighing 18-30 g were used. The animals were kept in groups of 15 mice in cages at a room temperature of 22±2°C, under a light/dark cycle and had free access to food

Corresponding author: Kinga Sałat, Department of Pharmacodynamics, Medical College, Jagiellonian University, Medyczna 9, 30-688 Cracow, Poland.
E-mail: salat.kinga@gmail.com

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and water before the experiments. Each experimental group consisted of 6-12 animals/dose and all the animals were used only once. For all the experiments the mice were habituated to the vivarium for a minimum of 72 h before experimentation. The experiments were performed between 08:00-15:00. The procedures were approved by the Local Ethics Committee in Cracow.

The hot plate test. In the hot plate test the mice were treated intraperitoneally either with the compound or the vehicle 30 min before being placed on a hot plate apparatus (Hot Plate 2A Type Omega) with the temperature controlled for 55-56°C. The time until the animal licked its back paws or jumps was recorded by means of a stop-watch [22]. Centrally-acting analgesics such as morphine prolonged the reaction time, whereas those acting peripherally (NSAIDs) showed no or minimal activity in this test [23].

The writhing test. Conversely, NSAIDs are highly antinociceptive in the writhing test in which mice are treated with 0.25 ml of 0.02% phenylbenzoquinone solution 30 min after *ip* administration of the investigated compound or vehicle. The mice we then placed individually into glass beakers and 5 min allowed to elapse. After that period of time, a 10-min observation was conducted on each animal – the number of characteristic writhes was counted. The analgesic effect of the tested substances consisted in diminishing the number of writhes observed [24].

Spontaneous locomotor activity. The locomotor activity was assessed by means of cages supplied with photocells, counting the number of laps made by the animal. In the present study, only one dose was tested: the ED_{50} from the hot plate test. In the statistical analysis data obtained 60 min after *ip* administration of the investigated compound were presented.

Capsaicin-induced pain. After an adaptation period (20 min), 20 µl of capsaicin solution prepared in saline (1.6 µg capsaicin per mouse paw) was injected intraplantarly (*i. pl*) in the ventral surface of the right hind paw 30 min after the tested compound had been administered *ip*.

The animals were observed individually for 5 min following capsaicin injection. The amount of time spent licking the injected paw was recorded with a chronometer, and was considered as indicative of nociception [25].

The tail immersion test (modification). The heat method which is used for evaluating the systemic analgesic activity can also be used with a slight modification to determine whether a compound possesses local anaesthetic activity. The method was conducted by injecting subcutaneously (*sc*) the investigated substance in a constant volume of 0.2 ml about 1 cm from the root of the mouse tail. 15 min later the 3 cm distal part of the tail was immersed in water at a controlled temperature of 50 ± 0.5° C. The reaction time (i.e. time at which the tail was pulled away) was measured by means of a chronometer. The whole observation time was limited to 20 s [26].

Acute toxicity. Acute toxicity was investigated in mice according to the method described by Litchfield and Wilcoxon [27]. In the experiment, each group of mice consisted of 6 animals. Behavioural observations were conducted and the total mortality rate assessed during a 72 h period. Finally, the LD_{50} value was established.

Statistical analysis. The data were expressed as mean ± SEM (standard error). To compare the results between the 2 different groups of animals (investigated compound group *vs.* the control group) in the writhing, hot plate and tail immersion tests, the t-Student test was used. In the capsaicin induced nociception the statistical significance was assessed by means of one-way ANOVA, followed by the Newman-Keul's test. The difference of means was statistically significant if $p < 0.05$.

RESULTS

The hot plate test. From all the investigated compounds, LMOR proved to possess the strongest, dose-dependent analgesic efficacy reducing the nociceptive response to the thermal stimulus applied to the mouse paw in the hot plate test. The ED_{50} value for LMOR was 9.92 mg/kg, which was only 3 times higher than this value obtained for morphine (3.39 mg/kg) used as the drug of reference. LHEL and L8 in a dose dependent manner also diminished the animals' reaction. The results are shown in Table 1.

Table 1 The antinociceptive activity of the compounds in the hot plate test.

Compound	Dose [mg/kg]	Latency [s]±SEM	Effect (%)	ED_{50} [mg/kg]
Control	0.5%MC	10.85 ± 0.78	-	-
LMOR	3.75	13.24 ± 1.09	22.03	9.92 (4.88-20.13)
	15	17.61 ± 2.36[c]	62.30	
	30	19.69 ± 2.41[d]	81.47	
L8	30	13.83 ± 2.16	27.47	32.48 (29.89-35.28)
	33.75	18.36 ± 1.51[d]	69.22	
	37.5	19.09 ± 1.67[d]	75.94	
	45	26.29 ± 3.66[d]	142.30	
LHEL	30	12.87 ± 1.88	18.62	39.81 (33.50-47.31)
	45	17.63 ± 2.31[c]	62.49	
	60	20.81 ± 2.99[d]	91.80	
Control	0.5%MC	18.40 ± 1.00	-	-
MORPHINE	1	19.4 ± 2.1	5.4	3.39 (2.24-5.12)
	3	29.9 ± 6.0[a]	60.9	
	6	30.6 ± 3.9[b]	66.3	

Significant difference compared to the vehicle-treated group (methylcellulose):
[a]$p<0.05$, [b]$p<0.02$, [c]$p<0.01$, [d]$p<0.001$.
Each value represents the mean ± SEM obtained from 8 animals.
Route: *ip*.
MC: methylcellulose.

Writhing test. All 3 tested GBL derivatives were potent analgesics in the writhing test, reducing the number of phenylbenzoquinone-induced body stretches. The calculated ED_{50} values for LMOR, L8 and LHEL were: 5.39, 9.57 and 6.12 mg/kg, respectively. They were significantly lower than the ED_{50} calculated for acetylsalicylic acid (39.15 mg/kg) (Table 2).

Spontaneous locomotor activity. In this test, 3 GBL derivatives administered at their calculated ED_{50} values in the hot plate test were investigated. LHEL had no influence on the animals' locomotor activity, whereas LMOR and L8

Table 2 The antinociceptive activity of the compounds in the writhing test.

Compound	Dose [mg/kg]	Number of stretches ±SEM	Effect (%)	ED_{50} [mg/kg]
Control	0.5%MC	31.70 ± 1.38	-	-
LMOR	1.8	21.83 ± 1.99[c]	31.14	5.39 (1.83-15.89)
	7.5	14.00 ± 2.58[d]	55.84	
	15	10.17 ± 1.10[d]	67.92	
	30	6.40 ± 2.50[d]	79.81	
L8	7.5	18.00 ± 2.77[d]	43.22	9.57 (2.87-31.90)
	15	11.83 ± 2.30[d]	62.68	
	30	10.00 ± 2.53[d]	68.45	
LHEL	3.75	19.50 ± 1.84[d]	38.49	6.12 (1.93-19.43)
	7.5	13.60 ± 2.38[d]	57.10	
	30	7.17 ± 1.83[d]	77.38	
Control	0.5%MC	19.20 ± 3.20	-	-
ASA	30	11.20 ± 2.10	41.70	39.15 (29.10-48.40)
	50	8.50 ± 1.30[b]	55.70	
	100	3.20 ± 1.20[d]	83.30	

Significant difference compared to the vehicle-treated group (methylcellulose):
[b]p<0.02, [c]p<0.01, [d]p<0.001.
Each value represents the mean ± SEM obtained from 8 animals.
Route: *ip*.
ASA: acetylsalicylic acid.
MC: methylcellulose.

diminished it, but the results were statistically insignificant (Table 3).

Table 3 Influence of the compounds on spontaneous locomotor activity in mice.

Compound	$ED_{50\ HP}$ [mg/kg]	Number of impulses ±SEM	Effect (%)
Control	0.5%MC	411 ± 36	-
LMOR	9.9	326 ± 48	-20.68
L8	32.00	309 ± 44	-25.06
LHEL	40.00	397 ± 49	-3.41

Each value represents the mean ± SEM obtained from 8 animals.
Route: *ip*.
$ED_{50\ HP}$: ED_{50} values calculated in the hot plate test (for explanation see Methods).

Capsaicin model of nociception. None of the tested compounds was antinociceptive in the neurogenic model of pain when administered intraperitoneally in a dose of 30 mg/kg. None of them was able to diminish the nocifensive reaction (licking or biting the injected paw) (Table 4).

Table 4 Antinociceptive activity of the compounds in the capsaicin test.

Compound	Dose [mg/kg]	Time [s]± SEM	Effect (%)
Control	0.5% MC	45.90 ± 1.12	-
LMOR	30	47.40 ± 5.07	+ 3.27
L8	30	44.54 ± 3.12	2.96
LHEL	30	40.94 ± 5.34	10.81

Each value represents the mean ± SEM obtained from 8 animals.
Route: *ip*.
MC: methylcellulose.

The tail immersion test (modified). In this test only 2% solutions of the GBL derivatives exerted local anaesthetic activity. LHEL was the most potent compound in this respect (75% activity in comparison to the vehicle-treated mice). The drugs of reference (mepivacaine, lignocaine) were much more active as local anaesthetics, both as 1% and as 2% solutions (Table 5).

Table 5 Local anesthetic activity of the compounds in the modified tail immersion test.

Compound	Concentration (%)	Latency [s] ±SEM	Effect (%)
Control	0.5 (MC)	7.26 ± 2.17	-
LMOR	1.0	7.30 ± 1.51	0.55
	2.0	11.88 ± 2.44	63.64
L8	1.0	7.05 ± 2.17	-2.89
	2.0	10.82 ± 2.38	49.04
LHEL	1.0	7.66 ± 1.56	5.51
	2.0	12.73 ± 2.20	75.34
MEPIVACAINE	1.0	14.13 ± 2.67	94.63
	2.0	15.73 ± 2.09[b]	116.67
LIGNOCAINE	1.0	16.82 ± 2.11[c]	131.68
	2.0	19.84 ± 0.16[d]	173.28

Significant difference compared to vehicle-treated group (methylcellulose):
[b]p<0.02, [c]p<0.01, [d]p<0.001.
Each value represents the mean ± SEM obtained from 8 animals.
Route: *ip*.
MC: methylcellulose.

Acute toxicity. Table 6 presents the safety profile of the investigated compounds obtained in the acute toxicity test.

Table 6 Acute toxicity of the compounds.

Compound	Dose [mg/kg]	Behavioral effect	Total mortality (X/Y)	Mortality (%)	LD_{50} [mg/kg]	Therapeutic index (LD_{50}/ED_{50})*
LMOR	1,250	Sedative	2/6	33	1485.980 (1,156.97-1,908.55)	275.69
	1,500	Sedative	3/6	50		
	1,750	Sedative	4/6	67		
	2,000	Sedative	6/6	100		
L8	625	Seizures	1/6	17	747.82 (631.33- 885.80)	78.14
	750	Seizures	4/6	67		
	1,000	Seizures	5/6	83		
LHEL	875	Seizures	1/6	17	975.89 (896.13-1,062.76)	159.48
	1,000	Seizures	4/6	67		
	1,125	Seizures	5/6	83		

Mortality rate evaluated 72 h after intraperitoneal injection.
X: number of mice that died during 72-h observation.
Y: number of mice in each group.
*To calculate the therapeutic index, ED_{50} from the writhing test was used.

LMOR was the only compound that acted as a sedative to mice in the range of doses tested (1,250-2,000 mg/kg). Its LD_{50} value was 1486 mg/kg. Two other GBL derivatives lowered the convulsive threshold at doses tested, and seizures were observed almost immediately after intraperitoneal administration. The LD_{50} values for L8 and LHEL were 747.82 and 975.89 mg/kg, respectively.

DISCUSSION

Although modern medicine of pain comprises variable therapeutic methods for alleviating it (e.g. pharmacotherapy, physiotherapy, surgical methods and acupuncture), the treatment of pain still remains a serious challenge. Contemporarily available analgesic drugs have serious side effects: opioids have drug-addictive properties. Anti-inflammatory drugs in turn may be ulcerogenic, nephro-, hepato- or myelotoxic. Besides, drug abuse and tolerance may occur as side-effects after long-term therapy, therefore the proper treatment of pain is still a relevant medical and toxicological problem which makes researchers seek new active compounds.

During our long-term investigations concerning the pharmacological activity of GBL derivatives we managed to distinguish a group of compounds possessing potential antinociceptive and anticonvulsant properties [6]. Some of these structures proved to have strong analgesic and local anaesthetic activity in screening models in rodents.

Three investigated derivatives: LMOR, L8 and LHEL, acted as anti-nociceptive agents in the hot plate test. As this test is considered for distinguishing centrally-acting analgesics, the obtained ED_{50} values may suggest that the analgesic activity of these compounds – LMOR in particular – is at least to some extent a consequence of their influence on the central nervous system at the supraspinal level [28]. It is of interest that all the compounds were also effective in the writhing test that detects peripherally acting analgesics [23]. However, comparing the ED_{50} values from the hot plate and writhing tests, one fact emerges: as the calculated ED_{50} from the writhing test for all 3 compounds is much lower than the same value from the hot plate model, it must be peripheral anti-nociceptive activity that is mainly responsible for their pharmacological effects. It seems to be a very important fact, as taking the chemical structure of the investigated compounds into account (i.e. their similarity to GABA) their central nervous system affinity is suggested. GABA itself is thought to be a crucial component of the 'pain gate' and its role in analgesia has been well established since 1965 when Melzack and Wall published their 'gate-control theory of pain' first [29]. Recent reports also emphasize the significance of different GABA-ergic drugs (e.g. tiagabine) in the treatment of intractable, especially neuropathic pain [5, 30, 31].

For this reason another experiment was carried out to evaluate whether the spontaneous locomotor activity is not disturbed by the compounds' administration. Since there was no significant effect obtained in this test, it may be concluded that the prolongation of nociceptive reaction latency in the hot plate test and reduced number of stretches in compound-treated mice in the writhing test are not a consequence of their sedative properties.

Analgesic activity observed in the 2 basic screening tests was not confirmed in the capsaicin model of neurogenic pain and was weak in the modified tail immersion test. Therefore, the GBL derivatives' effect is thought not to include the peripheral nerves. It is known that capsaicin, a pungent ingredient of chili pepper, exerts its potent biological activity through binding to thermosensitivie receptors, termed transient receptor potential vanilloid type 1 (TRPV1), present on the surface of the nerve fibres involved in the transmission of pain [32, 33, 34, 35]. If, as we assume, GBL's effect is of peripheral origin, rather it does not derive from direct influence on the nerves involved in the transmission of pain sensations (i.e. unmyelinated C and myelinated Aδ fibres). This activity may be a consequence of their influence on the inflammatory state in peripheral tissues. At this stage of research the precise mechanism of antinociceptive action of LMOR, L8 and LHEL is not clear, although some data suggest their anti-inflammatory effect. Further tests are necessary to elucidate whether the investigated compounds, similarly to NSAIDs, inhibit cyclooxygenase and enzyme-linked immunosorbent assay is suggested as a first step. The fact should also be noted that antinociceptive activity of the GBL derivatives is accompanied by their high LD_{50} values. Therefore, the investigated compounds possess a beneficial therapeutic index (between 78.14 for L8 and 275.69 for LMOR).

In conclusion, the derivatives of GBL proved to exert an anti-nociceptive effect in screening models in mice (hot plate test, writhing test). Central neurotropic and peripheral analgesic activities should be taken into account as far as their mechanism of action is concerned. The compounds' high pharmacological activity is accompanied by their low acute toxicity.

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