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Investigation into Stereoselective Pharmacological Activity of Phenotropil

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Abstract: Phenotropil [*N*-carbamoylmethyl-4-aryl-2-pyrrolidone (2-(2-oxo-4-phenyl-pyrrolidin-1-yl) acetamide; carphedon)] is clinically used in its racemic form as a nootropic drug that improves physical condition and cognition. The aim of this study was to compare the stereoselective pharmacological activity of R- and S-enantiomers of phenotropil in different behavioural tests. Racemic phenotropil and its enantiomers were tested for locomotor, antidepressant and memory-improving activity and influence on the central nervous system (CNS) using general pharmacological tests in mice. After a single administration, the amount of compound in brain tissue extracts was determined using an ultra performance liquid chromatography–tandem mass spectrometry (UPLC/MS/MS) method in a positive ion electrospray mode. In the open-field test, a significant increase in locomotor activity was observed after a single administration of R-phenotropil at doses of 10 and 50 mg/kg and S-phenotropil at a dose of 50 mg/kg. In the forced swim test, R-phenotropil induced an antidepressant effect at doses of 100 and 50 mg/kg, and S-phenotropil was active at a dose of 100 mg/kg. R-phenotropil significantly enhanced memory function in a passive avoidance response test at a dose of 1 mg/kg; the S-enantiomer did not show any activity in this test. However, the concentrations of R- and S-phenotropils in brain tissue were similar. In conclusion, the antidepressant and increased locomotor activity relies on both R- and S-phenotropils, but the memory-improving activity is only characteristic of R-phenotropil. These results may be important for the clinical use of optically pure isomers of phenotropil.

Phenotropil [*N*-carbamoylmethyl-4-aryl-2-pyrrolidone (2-(2-oxo-4-phenyl-pyrrolidin-1-yl) acetamide; carphedon)] was discovered in Russia several decades ago as a nootropic drug that improves physical condition and cognition [1,2]. Initially, it was used on spaceships to increase the working capacities of cosmonauts, but during the last 10 years, phenotropil has been introduced into clinical practice. The phenotropil molecule contains one chiral centre in the 4th position of the pyrrolidone ring. Therefore, phenotropil can exist in two forms that share an enantiomeric relationship. The configuration of these two enantiomers has been designated the 'R' and 'S' forms (fig. 1A,B).

Phenotropil is a phenyl derivative of a known drug, piracetam, which was the first representative of the nootropic drugs [1,3]. Piracetam is used for treatment in patients with mild to moderate dementia [4,5], cognitive impairment [6], after-stroke rehabilitation [7,8] and alcohol-induced toxic changes [9,10]. Also, phenotropil possesses nootropic drug-like activity in different experimental and clinical set-ups [1]. After chronic administration to Wistar rats, racemic phenotropil reduced the extent of neuralgic deficiency manifestations and retained the locomotor, research and memory functions in gravitational cerebral ischaemia. Piracetam was less effective compared with phenotropil [2]. Phenotropil also exhibited antiepileptic action after metrazol-induced seizures in mice [1] and significantly decreased the seizure and positive changes on the EEG in epileptic patients when used in combination with antiepileptic drugs [11,12].

Although phenotropil could be separated into R- and S-enantiomers, it is clinically used in the racemic form (fig. 1A,B). Information concerning the comparative pharmacological activity of R- and S-phenotropils is not available. Levetiracetam, an analogue of piracetam, is used in clinical practice as an optically pure substance, and the anticonvulsant action of levetiracetam is highly pronounced for the S-enantiomer [13]. In the case of oxiracetam, which could be separated into R- and S-enantiomers [14], a stereoselective pharmacological activity has not been described. In the present study, we explored the stereoselective pharmacological activity of racemic phenotropil and its enantiomers in different behavioural tests. We tested the effects of these compounds using general central nervous system (CNS) tests, as well as the locomotor, antidepressant and cognitive activity. In addition, the amount of compound in brain tissue extracts was determined after a single administration.

Materials and Methods

Chemicals. Racemic phenotropil and its optical isomers were prepared at the Latvian Institute of Organic Synthesis according to a previously published procedure [15]. Acetonitrile and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany), and 98% formic acid (LC/MS grade) was obtained from Fluka (Buchs, Switzerland).

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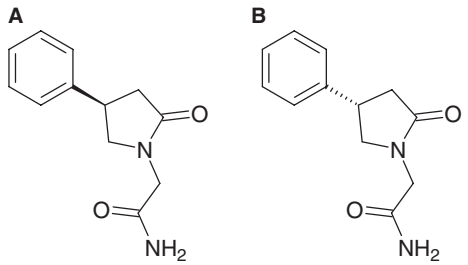

Fig. 1. Structures of R-phenotropil (A) and S-phenotropil (B).

Animals. Male ICR and CBA (antidepressant tests) mice (Laboratory Animal Breeding Facility, Riga Stradins University, Latvia) weighing 23–25 g were housed under standard conditions (21–23°C, 12-hr light/dark cycle) with unlimited access to standard food (Lactamin AB, Mjölby, Sweden) and water. All experimental procedures were carried out in accordance with guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Ethics Council of Animal Protection at the Veterinary and Food Service, Riga, Latvia.

Open-field test. The test apparatus was an octagonal arena (36 cm in diameter) with a black floor. The mice were gently placed in the centre of the field, and behavioural parameters were recorded using the EthoVision video tracking system (version 3.1.; Noldus, Wageningen, The Netherlands). Distance moved (cm) and velocity (cm/sec.) were recorded. Testing consisted of five successive 4-min. sessions that started 30, 60, 120, 180 and 240 min. after the intraperitoneal (i.p.) administration of test drugs at doses of 5, 10 and 50 mg/kg in the volume of 10 ml/kg body-weight. The control group received i.p. injection of saline.

Forced swim test. The forced swim test was performed as previously described by Porsolt *et al.* [16] with a slight modification. The mice were individually placed in a glass cylinder (26 cm high, 10 cm in diameter), containing 19 cm of water maintained at 22–25°C. The total duration of immobility was recorded during the last 4 min. of the 6-min. test period. The immobility time was recorded using the EthoVision video tracking system (version 3.1.; Noldus). A mouse was considered immobile whenever it floated passively in the water and only made movements necessary to keep its head above the water line. The animals received an i.p. injection of racemic R- and S-phenotropils at doses of 10, 50 and 100 mg/kg 30 min. prior to experiment in the volume of 10 ml/kg body-weight. The control group received i.p. injection of saline.

Passive avoidance response test. The passive avoidance response (PAR) test was used as described previously [17]. A step-through inhibitory avoidance apparatus (Ugo Basile, Comerio, Italy) consisted of two compartments, a light compartment (10 × 18 × 17 cm) and a dark compartment (10 × 18 × 17 cm). A sliding door opening (4 × 4 cm) was positioned on the floor in the centre of the partition between the two compartments. Stainless steel grids (2.5 mm in diameter) were placed at 8-mm intervals (the distance between the centres of the grids) on the floor of the dark compartment to produce a foot shock. Intermittent electric shocks were delivered to the grid floor of the dark compartment by an insulated stimulator.

On the acquisition day (training), each mouse was individually placed in the light compartment with no access to the dark compartment and allowed to explore for 60 sec. When 60 sec. expired, the sliding door was automatically opened and the mouse was allowed to cross over into the dark compartment. Upon entering the dark compartment, the mouse received a shock of 0.1 mA for 3 sec., the door was closed and the mouse was returned to its home cage after 20 sec. The latency to enter the dark compartment was recorded. Animals remaining in the light compartment for more than 100 sec. during the training session received no shock and were excluded from the retention test. The memory retention test was performed on the next day without any shock. The mice were again positioned in the illuminated compartment, and the time taken to enter the dark compartment was recorded as the retention latency. A maximum retention latency of 540 sec. was given to mice that did not enter the dark compartment. The apparatus was cleaned after each trial.

The animals received an i.p. injection of racemic R- and S-phenotropils at doses of 1 and 10 mg/kg 1 hr prior to acquisition trial in the volume of 10 ml/kg body-weight. The control group received i.p. injection of saline.

General central nervous system (CNS) tests. The effects of drugs were evaluated before and 30, 60, 120 and 180 min. after an i.p. administration at doses of 50, 100, 250 and 500 mg/kg in the volume of 10 ml/kg of body-weight. A rota-rod test [18] was used to measure motor coordination (Model 7600; Ugo Basile). One day before the experiment, the animals were trained on the apparatus, and the animals that failed to remain on the rotating rod for at least 90 sec. were excluded from further testing. On the experiment day, the animals were placed on a rota-rod (16 rpm), and the number of animals falling off the rota-rod within the 180-sec. session was recorded.

The effect of drugs on motor performance was tested also in the chimney test as described previously [19]. In this test, the mice had to climb backwards up a Pyrex glass tube (30 cm length, 3 cm inner diameter). Mice successfully reaching the 20 cm mark within 30 sec. were selected for further testing.

The effect of drugs on muscle strength was examined in the traction test. Hence, the forepaws of a mouse were placed on a horizontal firmly fixed stick. The untreated mice grasped the stick with both forepaws and, when allowed to hang free, placed at least one hind foot on the stick within 5 sec. Inability to perform that was scored as a failure of traction. The rectal temperature of animals was measured using a thermometer (Thermalert TH-5; Physitemp, Clifton, NJ, USA).

Determination of R- and S-phenotropils by UPLC/MS/MS. The mice received an i.p. injection of R- and S-phenotropils at a dose of 100 mg/kg 30 min. before decapitation. The brain tissue was gently removed and homogenized using a Cole Parmer 130-Watt ultrasonic processor set at 20 kHz twice for 10 sec. each in ice-cold Milli-Q water at w/v ratio 1:5. The obtained homogenate was centrifuged at 42,000 × *g* for 10 min. at 4°C. The supernatant was then decanted but the pellet was homogenized in the same volume of Milli-Q water as before. The obtained homogenate was centrifuged at 42,000 × *g* for 10 min. at 4°C. The supernatants were combined and stored frozen (−80°C) until analysed.

The concentration of R- and S-phenotropils in brain tissue extracts was determined using an ultra performance liquid chromatography–tandem mass spectrometry (UPLC/MS/MS) method in a positive ion electrospray mode. A sample preparation was performed by deproteinization with an acetonitrile/formic acid mixture. Brain tissue extract (100 µl) was mixed with 500 µl of 0.1% formic acid solution in acetonitrile (v/v), vortexed and centrifuged at 9279 × *g* for 20 min. One hundred microlitres of the supernatant was diluted with 500 µl of a mobile phase solution (methanol/0.1% formic acid, 70:30, v/v), transferred into UPLC vials and used for UPLC/MS/MS analysis. Calibration was made with external calibration standard solutions.

UPLC was carried out using the Waters Acquity UPLC system equipped with the Acquity BEH Shield RP18 column (2.1 × 100 mm, 1.7 µm). The injection volume was 5 µl. Chromatographic separation was performed in a gradient elution mode from 70% to 90% methanol in the mobile phase (methanol and aqueous 0.1% formic acid) at a flow rate of 0.15 ml/min. MS/MS analysis was performed on a Micromass Quattro Micro™ tandem mass spectrometer (Micromass, Manchester, UK) in positive ion mode using multiple reaction monitoring of the transition from *m/z* 219.0 to 173.9 (cone voltage 20 V, collision energy 16 eV) for phenotropil. MassLynx 4.1.

software with a QuanLynx 4.1. module (Waters Corporation, Milford, MA, USA) was used for data acquisition and processing.

Statistical analysis. All results are expressed as the mean ± S.E.M. The data were evaluated by a one-way, two-way or repeated-measures analysis of variance (ANOVA). *Post hoc* comparisons between individual groups were made with Newman–Keuls test. The effective dose 50 (ED_{50}) values were obtained by probit analysis [20]. *p*-values <0.05 were considered to be significant.

Results

Open-field test.

Locomotor activity was tested in an open-field test 30, 60, 120, 180 and 240 min. after a single i.p. administration of racemic phenotropil and R- and S-enantiomers at doses of 5, 10 and 50 mg/kg (fig. 2A–D). To compare the efficacy of racemic phenotropil and its optical isomers, the two-way ANOVA was calculated. The significance was found for the main effects of locomotor activity as represented by the moved distance [effects of drug ($F_{2,64}$ = 3.105; $p < 0.05$) and dose ($F_{2,64}$ = 3.243; $p < 0.05$), but no interaction between drug and dose ($F_{4,64}$ = 1.912; $p > 0.05$)] and velocity [effects of dose ($F_{2,64}$ = 4.382; $p < 0.05$), but no of drug ($F_{2,64}$ = 2.219; $p > 0.05$) and interaction between drug and dose ($F_{4,64}$ = 2.02; $p > 0.05$)] (fig. 2A,C). ANOVA with repeated measures showed that compounds induced a time-dependent increase in locomotor activity as represented by the moved distance [fig. 2B, main effect of time ($F_{4,124}$ = 60.32; $p < 0.001$) and drug ($F_{3,124}$ = 20.63; $p < 0.001$), but no interaction ($F_{12,124}$ = 0.994; $p > 0.05$)] and velocity (fig. 2D, main effect of time ($F_{4,124}$ = 65.43; $p < 0.001$) and drug ($F_{3,124}$ = 14.38; $p < 0.001$), but no interaction ($F_{12,124}$ = 1.419; $p > 0.05$)] in the open-field test.

R-phenotropil induced significant changes in locomotor activity as represented by the moved distance ($F_{3,31}$ = 10.37; $p < 0.0001$) and velocity ($F_{3,31}$ = 10.74; $p < 0.0001$) compared with saline-treated animals at all of the doses used (5, 10 and 50 mg/kg). Moreover, this action of R-phenotropil was maintained at a higher dose of 50 mg/kg for as long as 240 min. ($p < 0.0001$) (fig. 2A–D).

The administration of racemic phenotropil resulted in a somewhat lower activity than was observed for R-phenotropil, but the effect of R- and racemic phenotropils was not significantly different. Racemic phenotropil stimulated locomotor activity at doses of 10 and 50 mg/kg and was maintained for 180 min. after administration.

The S-enantiomer produced an increase in locomotor activity ($p < 0.05$) only at a dose of 50 mg/kg. Moreover, the action of S-phenotropil at a dose of 50 mg/kg was significantly lower than that of the R-enantiomer at the same dose in the open-field test ($F_{3,31}$ = 8.962; $p < 0.001$) (fig. 2A–D).

Forced swim test.

The antidepressant action of the compounds was tested in the forced swim test 30 min. after a single i.p. administration of racemic and R- and S-phenotropils at doses of 10, 50 and 100 mg/kg. The data presented in fig. 3 show that all of the tested compounds exerted an antidepressant-like action compared with the behaviour of saline-treated mice [two-way ANOVA, main effect of drug ($F_{2,81}$ = 21.94; $p < 0.0001$) and

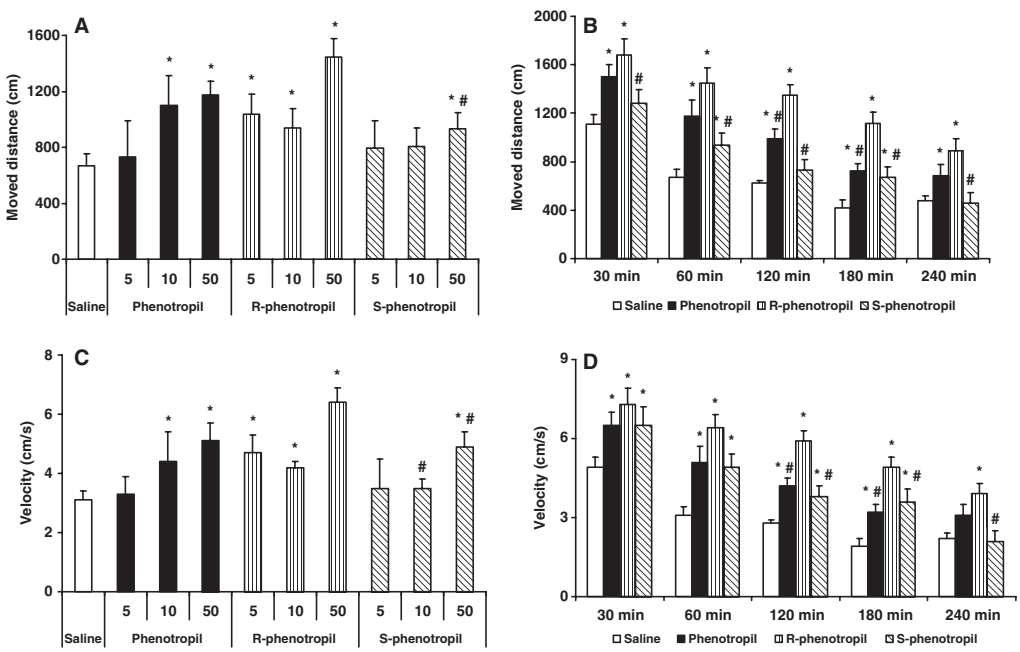

Fig. 2. Effects of racemic phenotropil, R- and S-phenotropils in the open-field test in mice. Compounds were administered at doses of 5, 10 and 50 mg/kg i.p. The mice were observed 30, 60, 120, 180 and 240 min. after injection. (A) Moved distance (cm/4 min.) 60 min. after injection; (B) moved distance (cm/4 min.) at all observed time-points at a dose of 50 mg/kg; (C) velocity (cm/sec.) 60 min. after injection; (D) velocity (cm/sec.) at all observed time-points at a dose of 50 mg/kg. Each column represents the mean ± S.E.M. of 8–10 animals. *$p > 0.05$ *versus* saline-treated group, #$p > 0.05$ *versus* the respective dose of R-phenotropil.

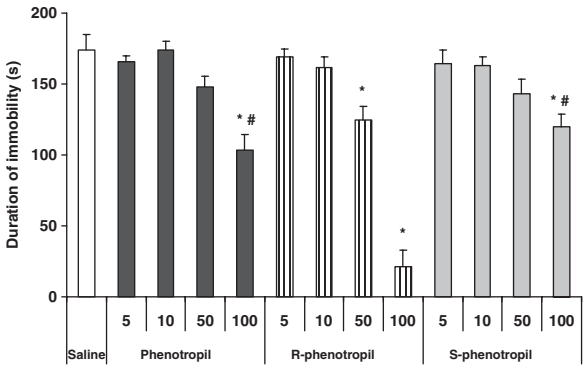

Fig. 3. Effects of racemic, R- and S-phenotropils in the forced swim test. The compounds were administered i.p. 30 min. prior to the experiment at doses of 10, 50 and 100 mg/kg. Each column represents the mean ± S.E.M. of 10 animals. $^{*}p > 0.05$ *versus* saline-treated group, $\#p > 0.05$ *versus* the respective dose of R-phenotropil.

dose ($F_{2,81} = 82{,}47$; $p < 0.0001$), significant interaction between drug and dose ($F_{4,81} = 10.19$; $p > 0.0001$)]. For animals treated with R-phenotropil, a decreased immobility time was observed in a dose-dependent manner compared with the control group at doses of 50 and 100 mg/kg ($F_{2,27} = 52.99$; $p < 0.0001$). At a dose of 100 mg/kg, R-phenotropil administration induced an 8-fold decrease in immobility time compared with the saline control group (21 ± 12 sec./4 min. and 174 ± 12 sec./4 min., respectively). The effect of R-phenotropil was significantly better compared with the effect of racemic and S-phenotropil at the same dose ($F_{2,27} = 39.13$; $p < 0.0001$), with the immobility times being five and six times longer (fig. 3), respectively.

Passive avoidance response test.

The influence of R-, S- and racemic phenotropils on retention latency was examined using the PAR test in mice (fig. 4). Fig. 4 shows the action of the R-enantiomer, where the retention latency was increased after R-phenotropil administration at doses of 1 and 10 mg/kg by 195% and 185%, respectively, compared with the control group

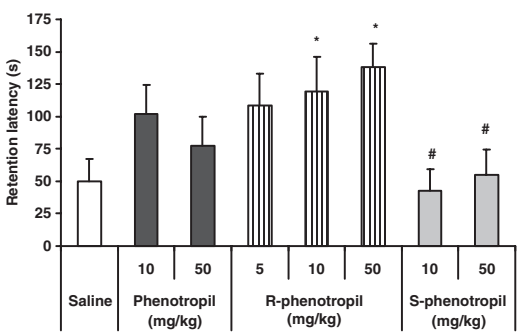

Fig. 4. Effects of racemic phenotropil, R- and S-phenotropils in the PAR test in mice. The animals received an i.p. injection of racemic, R- and S-phenotropils at doses of 1 and 10 mg/kg 1 hr prior to acquisition trial. Each column represents the mean ± S.E.M of nine animals. $^{*}p < 0.05$ *versus* saline-treated group. $^{\#}p < 0.05$ *versus* the respective dose of R-phenotropil.

Table 1.

Effects of racemic phenotropil and its optical isomers in the general CNS tests.

Tests	Racemic phenotropil ED_{50} (mg/kg)	R-phenotropil ED_{50} (mg/kg)	S-phenotropil ED_{50} (mg/kg)
Chimney	>500	258 ± 25	>500
Traction	>500	250 ± 40	>500
Rota-rod	>500	320 ± 65	>500
Rectal temperature	>500	>500	>500

Effects of racemic phenotropil and its optical isomers in the chimney, traction, rota-rod tests and rectal temperature. Compounds were administered i.p. at doses of 50, 100, 250 and 500 mg/kg. The effects were observed 30, 60, 120 and 180 min. after drug administration. ED_{50} value was calculated by probit analysis.

($F_{2,54} = 2.312$; $p < 0.05$); racemic phenotropil showed the same action (fig. 4). The administration of S-phenotropil did not influence the retention latency. Moreover, the retention latency in S-enantiomer-treated mice at a dose of 1 mg/kg differed significantly ($p < 0.05$) from the respective R-phenotropil-treated group (fig. 4).

There were no differences between the saline, racemic phenotropil and its optical isomer groups in entering the dark compartment during the acquisition trial (data not shown).

General CNS tests.

Only R-phenotropil exerted inhibitory activity on muscle strength and coordination in the chimney, traction and rota-rod tests. Pre-treatment with racemic or S-phenotropil did not influence muscle strength and coordination at doses up to 500 mg/kg (table 1). None of the compounds induced any changes in rectal temperature in the dose ranges used (table 1).

Determination of the brain tissue content of R- and S-phenotropils by UPLC/MS/MS.

R- and S-phenotropils were measured in brain tissue homogenates 30 min. after a single i.p. injection. The amount of

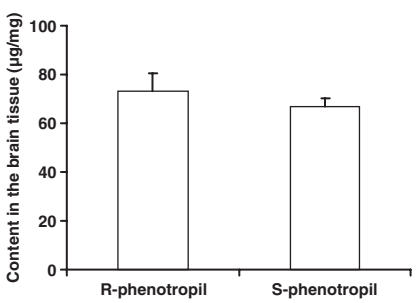

Fig. 5. The amount of R- and S-phenotropils (µg/g) in mice brain tissue. Mice received an i.p. injection of R- and S-phenotropils at a dose of 100 mg/kg 30 min. prior to tissue collection. The amount of compound in brain tissue extracts was measured using the *ultra* performance liquid chromatography–tandem mass spectrometry method in a positive ion electrospray mode. Each column represents the mean ± S.E.M. of seven mice.

both compounds was similar: 73 ± 7 μg/g for R-phenotropil and 67 ± 3 μg/g brain tissue for S-phenotropil. The concentration of compounds in brain tissue 30 min. after i.p. injection at a dose of 100 mg/kg was about 1% of administered amount (fig. 5).

Discussion

The results of the present study provide evidence that R-phenotropil is the most active enantiomer of phenotropil. After a single administration, it increased locomotor and antidepressant activity in the open-field and forced swimming tests, respectively, as well as enhanced memory in the PAR test. Racemic phenotropil and S-phenotropil also stimulated locomotor activity and induced some antidepressant effect after acute administration. However, S-phenotropil did not exert any activity in the PAR test, although the amount of the enantiomers in brain tissue was similar as shown by UPLC/MS/MS.

Because phenotropil is structurally related to piracetam, a known nootropic drug that increases physical capacity and cognition processes [3,4,6], we tested the pharmacological activity of racemic phenotropil and its enantiomers in experimental set-ups related to those activities. In the PAR test, a memory-improving activity was demonstrated by R- and racemic phenotropils. S-phenotropil did not exert any activity to improve cognition in the PAR test (fig. 4). Therefore, the presence of S-phenotropil in racemic phenotropil diluted or suppressed its pharmacological activity in the PAR test.

We also measured the influence of the enantiomers of phenotropil on locomotor activity in the open-field test and used the forced swimming test to determine the effect of the drug on depressive state. In all cases, R-phenotropil was the most active substance. At the same time, the racemic and S-phenotropils showed some activity after acute administration. In the forced swimming test, both racemic phenotropil and S-phenotropil showed antidepressant activity at a dose of 100 mg/kg, but a dose of 50 mg/kg did not influence immobility time compared with the control group (fig. 3). In the open-field test, racemic phenotropil increased locomotor activity at doses of 10 and 50 mg/kg, but S-phenotropil was active at a dose of only 50 mg/kg (fig. 2A–D). Considering that all compounds exerted significant effects in the open-field test at five times lower doses than in the forced swimming test, it could not be excluded that the decreased immobility time in the forced swimming test might result from the drug-induced increase in locomotor activity. In the general CNS tests, R-phenotropil affected motor coordination and muscle strength (ED_{50} 250–320 mg/kg) as shown by the rota-rod, chimney and traction tests. However, racemic and S-phenotropils did not influence motor function at doses up to 500 mg/kg (table 1). Moreover, the bioavailability of both compounds in brain tissue after acute administration was similar (fig. 5).

It can be suggested that the enantiomers of phenotropil might have different affinities for the possible drug target or that different targets could be involved in the pharmacological activity of R- and S-phenotropils. Previously, it was found that racemic phenotropil *in vitro* binds to nicotinic acetylcholine receptors with an IC_{50} of 5.86 μM/l [21]. In an *ex vivo* experiment in rats, a 7-day treatment with racemic phenotropil at a dose of 100 mg/kg significantly increased the B_{max} of *N*-methyl-D-aspartate and nicotinic acetylcholine receptors [21]. It has been shown in clinical settings and experimental models that nicotinic acetylcholine and *N*-methyl-D-aspartate receptors are involved in the modulation of cognition and memory processes [22,23]. This is in agreement with the results of the present study where a treatment of R-phenotropil improved the response time in the PAR test. The possible target receptors and mechanisms for the acute activity of phenotropil remain unclear. Racemic phenotropil does not bind to dopamine (D1, D2 and D3) or serotonin (HT_2) receptors [21]. In conclusion, using different behavioural tests in mice, we have shown that the antidepressant and increased locomotor activity of racemic phenotropil relies on both enantiomers and is more pronounced of R-phenotropil. Moreover, the memory-improving activity was only characteristic of R-phenotropil. This may be important for the clinical use of the optically pure isomers of phenotropil.

Acknowledgements

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