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Effect of benzothiazole/piperazine derivatives on intracerebroventricular streptozotocin-induced cognitive deficits

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Abstract:
Background: In this study, benzothiazole-piperazine compounds were synthesized by condensing the functional groups of donepezil (DNP), FK-960, and sabeluzole, which are known to have therapeutic potential against Alzheimer's disease, with the aim of obtaining new and potent anti-Alzheimer agents.
Methods: Initially, acetylcholinesterase/butyrylcholinesterase enzyme inhibition activities of the synthesized test compounds were investigated by Ellman's method. Effects of the compounds on a streptozotocin (STZ) model of Alzheimer's disease (SMAD) were investigated in rats. SMAD was established by intracerebroventricular (*icv*) injection of STZ (3 mg/kg), bilaterally. The elevated plus maze, Morris water maze, and active avoidance tests were used to examine the effects of test compounds (1, 5, and 10 mg/kg) on learning and memory parameters of *icv* STZ-injected rats. Effects of the test compounds on spontaneous locomotor activities of rats were examined with the activity cage test.
Results: The compounds **B2–B5** and DNP exhibited significant selective inhibitory potencies against acetylcholinesterase. Compounds **B2** and **B3** at 10 mg/kg doses and compounds **B4** and **B5** at 5 and 10 mg/kg doses, as well as the reference drug DNP (1 and 3 mg/kg), significantly improved the learning and memory parameters of animals in all cognition tests. None of the test compounds changed spontaneous locomotor activities.
Conclusion: Results of the present study revealed that compounds **B2–B5** repaired the parameters related to the learning and memory deficits of *icv* STZ-injected rats. Potencies of these test compounds were comparable to the activity of DNP.

Key words:
Alzheimer's disease, streptozotocin, donepezil, acetylcholinesterase, elevated plus maze test, Morris water maze test, active avoidance test, benzothiazole/piperazine

Abbreviations: AChE – acetylcholinesterase, AD – Alzheimer's disease, BChE – butyrylcholinesterase, DNP – donepezil, DTNB – 5,5-dithiobis(2-nitrobenzoic acid), IAL – initial acquisition latency, ITL – initial transfer latency, *icv* – intracerebroventricular, 1st RL – first retention latency, 2nd RL – second retention latency, 1st RTL – first retention transfer latency, 2nd RTL – second retention transfer latency, SEM – standard error of the mean, SMAD – streptozotocin model of Alzheimer's disease, STZ – streptozotocin

Introduction

Alzheimer's disease (AD) is the most common type of dementia, comprising 60–70% of all cases [8]. It is a chronic and irreversible neurodegenerative disorder characterized by a progressive deterioration of intellectual functions, including memory, language, visiospatial skills, problem-solving ability, and abstract

reasoning, as well as behavioral disturbances [12, 62]. AD is a major health problem and the third largest cause of death in the world after cardiovascular diseases and cancer [53]. Global prevalence of AD was reported as 26.6 million in 2006, and it is estimated that the worldwide prevalence of this disease will reach 106.2 million by 2050. Its impact on health care costs was predicted at $156 billion per year [51].

Until now, several therapeutic approaches have been offered, but only non-competitive *N*-methyl-D-aspartate receptor antagonist, memantine, and acetylcholinesterase (AChE) inhibitors, tacrine, donepezil (DNP), rivastigmine, and galantamine have been approved by the Food and Drug Administration and have been applied in the treatment of AD [3, 21, 29]. However, the effectiveness of these drugs on AD is limited, and they only provide a palliative therapy for patients [29, 61]. Besides, treatment with these drugs has several limitations such as short half-life or side-effects like hepatotoxicity, nausea, diarrhoea, vomiting, anorexia, weight loss, muscle cramps, dizziness, hallucinations, confusion, and headache [21, 29]. Therefore, there is still great interest in the development of more potent and safer agents against AD.

Benzothiazole, a heterocyclic ring, is often subjected to various drug discovery studies [17]. Some clinically available drugs carrying this ring system are used in the management of several central nervous system (CNS) diseases. For instance, riluzole, a benzothiazole derivative, is effective in retarding the progression of amyotrophic lateral sclerosis [15]. Another benzothiazole derivative, ethoxzolamide, has been reported to suppress epileptic seizures [46]. This pharmacophoric group is also important for drug discovery studies in the AD area with several benzothiazole-bearing compounds demonstrating potential therapeutic effects against AD [6, 11, 30, 38, 41]. Furthermore, another benzothiazole-based agent, sabeluzole, has been shown to delay the clinical progression of AD [13].

Piperidine is another important ring for the development of new chemical agents, demonstrating therapeutic effects against CNS disorders in particular. Numerous piperidine-carrying compounds have been synthesized and investigated for their anti-Alzheimer potential [1, 4, 14, 24, 27, 40, 42, 49]. The chemical structure of DNP, an important and widely used drug for the treatment of Alzheimer patients in neurology clinics, is also based on a piperidine ring [9, 52, 56, 60]. Like piperidine, its bioisostere, piperazine has also been subjected to the development of new agents. FK-960, a new anti-Alzheimer drug candidate, and some other piperazine derivative compounds have been demonstrated to possess activities against AD [5, 7, 18, 25, 26, 31–36, 53, 58].

In light of the aforementioned literature and the limitations of drugs currently used for the treatment of AD, our research group sought to design compounds carrying benzothiazole and piperazine moieties on the same chemical skeleton, with the intention of investigating their potential to treat cognitive defects. Condensation studies were carried out according to the molecular hybridization strategy, which is an approach

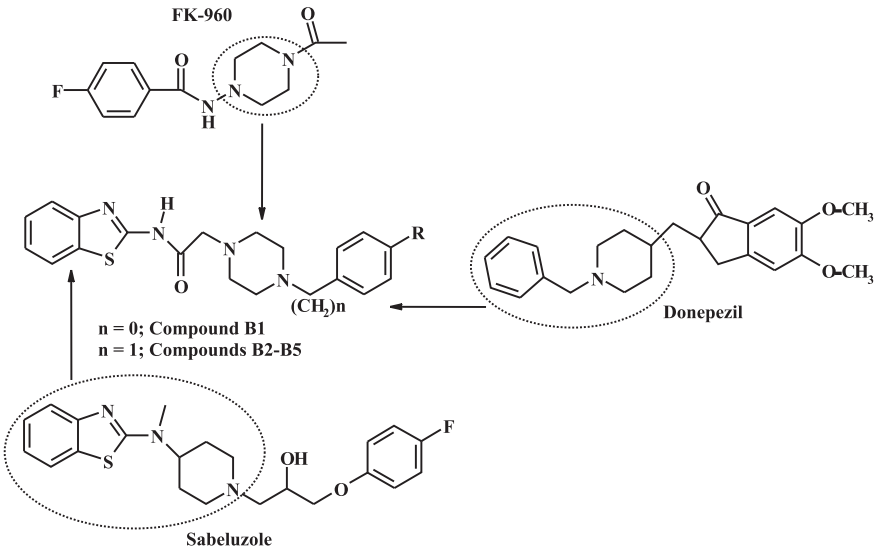


Fig. 1. Designing of the compounds **B1**–**B5** based on chemical structures of anti-Alzheimer agents

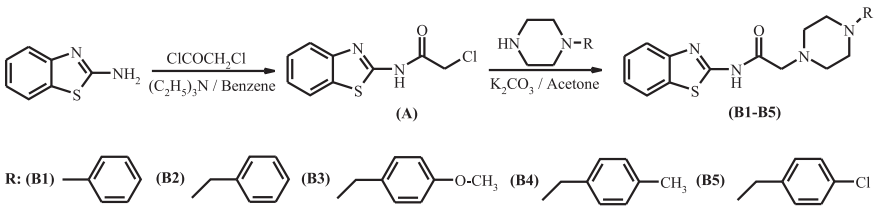


Fig. 2. Synthesis of the compounds **B1**–**B5**

for the logical design of new compounds based on the recognition of pharmacophoric sub-unities in 2 or more bioactive agents that form new hybrid structures whilst preserving pre-selected characteristics of the original templates [59]. For this purpose, DNP, FK-960, and sabeluzole, which contain piperidine, piperazine, and benzothiazole groups, respectively, were selected as model agents (Fig. 1). After a careful literature survey, we observed that no previous CNS-related study included the designed compounds.

Materials and Methods

Drugs and chemicals

Purified AChE (cholinesterase, acetyl C 2888 Type V-S: from electric eel; 1000–2000 U/mg), purified butyrylcholinesterase (BChE) (cholinesterase, butyryl C 4290 from horse serum, highly purified minimum 500 U/mg), streptozotocin (STZ), 1-(4-methylbenzyl) piperazine, 1-(4-methoxybenzyl)piperazine, and 1-(4-chlorobenzyl)piperazine were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO, USA. 5,5-Dithiobis(2-nitrobenzoic acid) (DTNB), potassium dihydrogen phosphate, potassium hydroxide, sodium hydrogen carbonate, acetylthiocholine iodide, and butyrylthiocholine iodide, used as substrates, were obtained from Fluka, Buchs, Switzerland. All other chemicals were purchased from Merck, Darmstadt, Germany. DNP (Doenza®, Sanovel, İstanbul, Turkey), ketamine (Alfamine®, Woerden, Holland), and xylazine (Alfazyne®, Woerden, Holland) were of commercial grade.

Synthesis of compounds

Target compounds were synthesized over 2 steps. Initially, 2-aminobenzothiazole was acetylated with chloroacetyl chloride in benzene to give 2-(2-chloroacetamido)benzothiazole (**A**), which was then treated with appropriate piperazine derivatives to obtain final products (**B1**–**B5**). The synthetic method is given in Figure 2.

2-[2-(4-Phenylpiperazin-1-yl)acetylamino]benzothiazole (B1)

M.p. 184–186°C. Yield 75%. IR (KBr, cm^{-1}): 3350 (N-H), 1676 (C=O), 1599–1395 (C=C and C=N). ^{1}H-NMR (500 MHz, DMSO-d_6, δ, ppm): 3.47–3.64 (8H, m, piperazine), 3.92 (2H, s, $COCH_2$), 7.34–7.58 (7H, m, Ar-H), 7.84 (H, d, J = 7.96 Hz, benzothiazole), 8.02 (H, d, J = 7.73 Hz, benzothiazole), 10.96 (H, s, NH-CO). MS (Es) m/z: 353 [M + 1].

2-[2-(4-Benzylpiperazin-1-yl)acetylamino]benzothiazole (B2)

M.p. 118–119°C. Yield 76%. IR (KBr, cm^{-1}): 3350 (N-H), 1676 (C=O), 1599–1395 (C=C and C=N). ^{1}H-NMR (500 MHz, DMSO-d_6, δ, ppm): 2.14 (2H, s, $C_6H_5CH_2$), 2.37–2.66 (8H, m, piperazine), 3.62 (2H, s, $COCH_2$), 7.24–7.48 (7H, m, Ar-H), 7.83 (H, d, J = 7.94 Hz, benzothiazole), 8.00 (H, d, J = 7.78 Hz, benzothiazole), 11.56 (H, s, NH-CO). MS (Es) m/z: 367 [M + 1].

2-[2-[4-(4-Methoxybenzyl)piperazin-1-yl]acetylamino]benzothiazole (B3)

M.p. 114–115°C. Yield 77%. IR (KBr, cm^{-1}): 3349 (N-H), 1676 (C=O), 1599–1397 (C=C and C=N). ^{1}H-NMR (500 MHz, DMSO-d_6, δ, ppm): 2.34 (2H, s, $C_6H_5CH_2$), 3.26–3.44 (8H, m, piperazine), 3.82 (2H, s, $COCH_2$), 3.98 (3H, s, OCH_3), 6.84–7.38 (6H, m, Ar-H), 7.71 (H, d, J = 7.98 Hz, benzothiazole), 7.92 (H, d, J = 7.74 Hz, benzothiazole), 11.54 (H, s, NH-CO). MS (ES) m/z: 397 [M + 1].

2-[2-[4-(4-Methylbenzyl)piperazin-1-yl]acetylamino]benzothiazole (B4)

M.p. 140–142°C. Yield 74%. IR (KBr, cm^{-1}): 3348 (N-H), 1676 (C=O), 1599–1395 (C=C and C=N). ^{1}H-NMR (500 MHz, DMSO-d_6, δ, ppm): 1.18 (3H, s, CH_3), 2.25 (2H, s, $C_6H_5CH_2$), 2.41–2.64 (8H, m, piperazine), 3.56 (2H, s, $COCH_2$), 7.04–7.46 (6H, m, Ar-H), 7.78 (H, d, J = 7.93 Hz, benzothiazole), 7.98 (H, d, J = 7.71 Hz, benzothiazole), 12.04 (H, s, NH-CO). MS (ES) m/z: 381 [M + 1].

2-[2-[4-(4-Chlorobenzyl)piperazin-1-yl]acetylamino]benzothiazole (B5)

M.p. 131–133°C. Yield 71%. IR (KBr, cm^{-1}): 3237 (N-H), 1694 (C=O), 1605–1398 (C=C and C=N). ^{1}H-NMR (500 MHz, DMSO-d_6, δ, ppm): 2.11 (2H, s, $C_6H_5CH_2$), 2.36–2.68 (8H, m, piperazine), 3.65 (2H, s, $COCH_2$), 7.25–7.44 (6H, m, Ar-H), 7.84 (H, d, J = 7.93 Hz, benzothiazole), 7.98 (H, d, J = 7.77 Hz, benzothiazole), 12.10 (H, s, NH-CO). MS (ES) m/z: 401 [M + 1].

Conversion of DNP HCl to DNP form

Ninety Doenza® tablets, including 900 mg DNP HCl, were granulated and dissolved in 250 ml of distilled water. The obtained suspension was stirred at room temperature for 30 min and filtered. The filtrate was treated with 10% NaOH (10 ml) and sequentially extracted with ethyl acetate (3 × 10 ml). Ethyl acetate extracts were pooled and dried over anhydrous Na_2SO4; the solvent was removed under reduced pressure to give 780 mg DNP.

AChE/BChE activity assay

The *in vitro* AChE/BChE activities of test compounds were evaluated prior to animal studies. Enzyme activity was investigated using a slightly modified colorimetric method of Ellman et al. [10]. The evaluation of enzyme activity was performed using a specific chromogenic reagent, DTNB. DTNB solution (100 ml) was prepared in water containing DTNB (0.396 g) and sodium hydrogen carbonate (0.15 g). Solutions of acetylthiocholine iodide and butyrylthiocholine iodide were prepared in 10 ml of water by adjusting concentrations to 0.075 M. AChE or BChE (500 units) was dissolved in gelatin solution (1 ml, 1%) and put into a 100-ml volumetric flask. The volume was made up with water to 5 units/ml and further diluted to 2.5 units/ml. All solutions were freshly prepared before enzymatic assays and used rapidly. Stock solutions of the synthesized compounds were prepared in 2% DMSO. Enzyme activities were determined in the presence of 6 different concentrations of test compound. Each concentration was assayed in triplicate. The samples were investigated immediately after preparation. Enzyme solution (100 µl) and test compound solution (100 µl) were added to a cuvette containing the phosphate buffer (3.0 ml, 0.1 M; pH = 8.0). After 5-min incubation, the required aliquots of DTNB solution (100 µl) and acetylthiocholine iodide (20 µl) or butyrylthiocholine iodide (20 µl) were added. After rapid and immediate mixing, the absorption was measured at 412 nm. As a reference, an identical solution of the enzyme without the inhibitor was processed following the same protocol. DNP was used as a positive control [20].

Animals

All experiments were carried out using adult Wistar rats weighing 200–250 g. The animals were housed in a temperature controlled (24 ± 1°C) room under a 12-h light/12-h dark cycle and were acclimatized to the laboratory environment at least 48 h before the experimental session. Rats were maintained in groups no greater than 4 per cage prior to and 1 per cage after intracerebroventricular (*icv*) STZ injections. During experiments, food and tap water were allowed *ad libitum*. The experimental protocols were approved by the Local Ethical Committee on Animal Experimentation of the Eskişehir Osmangazi University, Turkey.

Icv administration of STZ

Prior to surgery, rats were anesthetized immediately with a combination of ketamine (100 mg/kg, *ip*) and xylazine (5 mg/kg, *ip*). The animal's head was fixed in the stereotaxic frame (Harvard Apparatus, 51650, Massachusetts, USA). The scalp was cleaned with iodine solution, and a sagittal incision was made to the midline of the scalp. Two holes were drilled (Harvard Apparatus, BS4 72-4951, Massachusetts, USA) in the skull bilaterally over the lateral ventricles. According to Paxinos and Watson's atlas [45], the following coordinates were used for *icv* injection: 0.8 mm poste-

rior to the bregma, 1.5 mm lateral to the sagittal suture, and 3.6 mm ventral from the surface of the brain.

STZ was dissolved in citrate buffer (pH 4.4) shortly before application. Bilateral *icv* injection of STZ (3 mg/kg, 10 μl/injection site) was applied gradually in 2 divided doses, on days 1 and 3. The skin was sutured after injections. The same surgical procedures were also performed in the control group, although citrate buffer (10 μl/injection site) was injected instead of STZ, on the first and third day. After surgery, rats were housed individually, and special care was undertaken until spontaneous feeding resumed [2, 37, 44, 57].

Administration of drugs and test compounds

The animals were randomly divided into the following groups: the control group, which received *icv* citrate buffer; the STZ model of AD (SMAD) group, which received *icv* STZ; the DNP-injected SMAD groups; and the test compound-injected SMAD groups.

Two weeks after injection of STZ, DNP (1 and 3 mg/kg) was applied to the 'DNP-injected SMAD groups', and 5 test compounds (1, 5, and 10 mg/kg) were applied to the respective 'test compound-injected SMAD groups' *via* intraperitoneal injection over 1 week. Control groups were treated with sunflower oil, which is the solvent of the reference drug DNP and test compounds.

Behavioral tests

Activity cage, elevated plus maze, and Morris water maze tests were performed in the same experimental groups. In order to avoid potential interactions between the tests, different experimental groups were used for active avoidance measurements.

1. Activity cage measurements

The horizontal and vertical locomotor activities of rats were recorded by the activity cage apparatus, which contains 2 pairs of 16 photocells positioned 3 cm and 12 cm above the floor (UgoBasile, 7420, Varese, Italy). Interruptions of light beams to the photocells during horizontal and vertical movements of the animals were automatically recorded for 10 min. The apparatus was placed in a sound-attenuated and well-ventilated room [44, 48, 57].

Activity cage tests were performed just before (on day 14) and after 'DNP' and 'test compounds' treatments (on day 21) to check the presence of possible alterations in spontaneous locomotor activities of rats caused by these compounds [44, 48, 57].

2. Elevated plus maze measurements

The acquisition and retention of memory processes were evaluated using the elevated plus maze. The apparatus was made of wood and comprised 2 opposite open arms (50 × 10 cm), crossed with 2 closed arms (50 × 10 cm) with 40 cm high walls. The arms were connected by a central square (10 × 10 cm), and the maze was elevated 50 cm above the floor. The rats were placed individually at one end of an open arm, facing away from the central square. The latency time to enter into the closed arms was recorded as initial transfer latency (ITL) and then, rats were allowed to explore the maze for 30 s. The cut-off time was fixed at 300 s and rats that could not find the closed arms within this period were eliminated from the experiments.

Elevated plus maze tests were performed on days 13, 14, and 21 after *icv* STZ injection. Acquisition of memory was assessed on day 13. On days 14 and 21, latency times to enter the closed arms were measured again and recorded as first retention transfer latency (1^{st} RTL) and second retention transfer latency (2^{nd} RTL), respectively [22, 57].

3. Morris water maze measurements

Morris water maze tests were performed to evaluate spatial learning and memory of animals [39]. The water maze apparatus consisted of a circular water tank (150 cm diameter × 60 cm high) which was filled up to 40 cm with water at 25 ± 1°C. The pool was divided virtually into 4 equal quadrants. A round platform was placed 2 cm below the surface of water and remained in the same quadrant for the entire experiment [16, 28, 54].

Before the training session, rats were allowed to swim freely in the pool for 60 s without a platform. On day 13, animals were subjected to 4 trials. The rats were left in the water, facing the wall of the maze from the starting points, which were in the middle of each quadrant. The latency time to find the hidden platform was recorded as initial acquisition latency (IAL) [22, 23]. Trials were given to rats for a maximum time of 60

s (cut-off time) to find the hidden platform, and rats were allowed to stay on it for 30 s [16, 19].

On days 14 and 21, latency times to attain the hidden platform were recorded as first retention latency (1st RL) and second retention latency (2nd RL), respectively [22, 23].

4. Active avoidance measurements

Learning-related behaviors of rats were assessed by two-way active avoidance apparatus (UgoBasile, 7530, Varese, Italy). An auditory (70 dB, 670 Hz) and light stimulus (10 W), administered for a maximum of 11 s, served as the conditioned stimulus. One second later, a 0.4 mA electric shock was administered for a maximum time of 10 s, which served as the unconditioned stimulus. The total time for each trial was 22 s; the time between 2 consecutive trials was 4 s. Fifty trials were given every 3 consecutive days. The first session was carried out 10 min after the accommodation period. The total number of avoidances (number of crossings during unconditioned stimulus) and latency periods (as time before the first crossing) of animals were recorded on days 11, 14, and 21 [55].

Statistical analyses

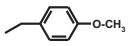
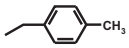
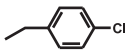
The data used in statistical analyses were obtained from 7 animals for each group. Experimental data of all tests were analyzed by repeated measures ANOVA followed by Tukey's HSD multiple comparison test using GraphPad Prism 4.03 (GraphPad Software, San Diego, CA, USA). The results were expressed as the mean ± standard error of the mean (SEM). Differences between data sets were considered as significant when p value was less than 0.05.

Results

Cholinesterase activity

The inhibitory potencies of the test compounds against AChE and BChE enzyme activities were evaluated by Ellman's test. The IC_{50} values for the inhibition of cholinesterase activity are shown in Table 1. Phenyl-substituted compound **B1** exhibited a poor inhibitory activity on both AChE and BChE. However, compounds **B4** and **B5** exhibited notable activity, which was comparable with the reference drug DNP. Moderate inhibitory activity was observed with compounds **B2** and **B3** (Tab. 1).

Tab. 1. AChE IC_{50} (μM) and BChE IC_{50} (μM) of synthesized compounds. Results are presented as the mean ± SEM

Comp.	R	AChE IC_{50} (μM)	BChE IC_{50} (μM)	Selectivity*
B1		22.6 ± 3.07	104.8 ± 9.15	4.64
B2		0.52 ± 0.072	28.9 ± 4.63	55.6
B3		0.13 ± 0.019	16.6 ± 1.27	127.7
B4		0.061 ± 0.0044	8.74 ± 0.36	143.3
B5		0.086 ± 0.0029	9.27 ± 0.41	107.8
DNP		0.023 ± 0.0015	4.18 ± 0.34	181.7

* BChE IC_{50} (μM)/AChE IC_{50} (μM)

Confirmation of the cognitive deficits with behavioral tests

Before administration of DNP and test compounds, elevated plus maze, Morris water maze, and active avoidance tests were performed on control and *icv* STZ-injected animals in order to confirm the establishment of an AD model in *icv* STZ-injected rats.

In the elevated plus maze test, there was a significant difference between the ITL (on day 13) and 1st RTL (on day 14) values of the *icv* citrate buffer-injected control group, reflecting the unimpaired cognitive ability and memory behavior of control animals. However, the difference between these parameters was not significant in the *icv* STZ-injected groups, indicating the establishment of SMAD in these groups, as expected (Fig. 3). Similar results were observed for the IAL (on day 13) and 1st RL (on day 14) parameters in Morris water maze (Fig. 4), and for latency times (Fig. 5) and number of avoidance (Fig. 6) parameters in active avoidance tests. Spontaneous locomotor activity did not differ significantly between

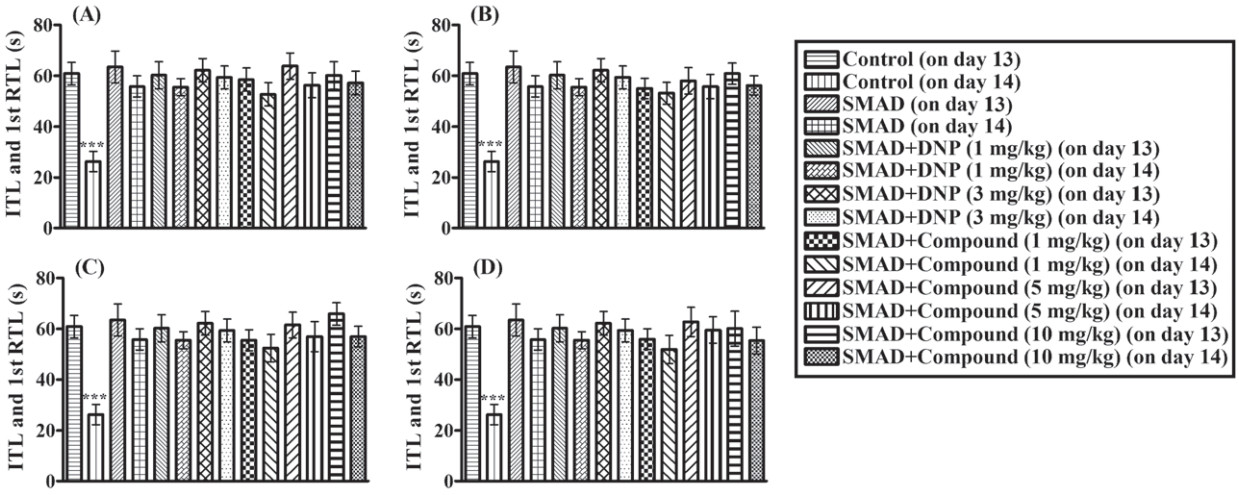


Fig. 3. Elevated plus maze response latencies of the animals in the experimental groups before the DNP and test compounds administrations. (**A**) SMAD groups which would be treated with DNP and compound **B2**. (**B**) SMAD groups which would be treated with DNP and compound **B3**. (**C**) SMAD groups which would be treated with DNP and compound **B4**. (**D**) SMAD groups which would be treated with DNP and compound **B5**. Significance against control values, *** $p < 0.001$. Values are given as the mean ± SEM

the control and *icv* STZ-injected groups on day 14 (data not shown).

DNP and test compounds (**B2**, **B3**, **B4**, and **B5**) were administrated to animals just after impairments in learning and memory parameters of *icv* STZ-injected animals, which were confirmed by the above-mentioned behavioral tests. Due to the poor potency against AChE, compound **B1** was not assessed in the *in vivo* tests.

Behavioral tests

STZ-injected animals (*icv*) were administrated 1, 5, and 10 mg/kg doses of test compounds **B2**, **B3**, **B4**, and **B5** and 1 and 3 mg/kg doses of DNP over 1 week. After this application period, on day 21, behavioral experiments were repeated to observe the effect of treatments on animals with AD.

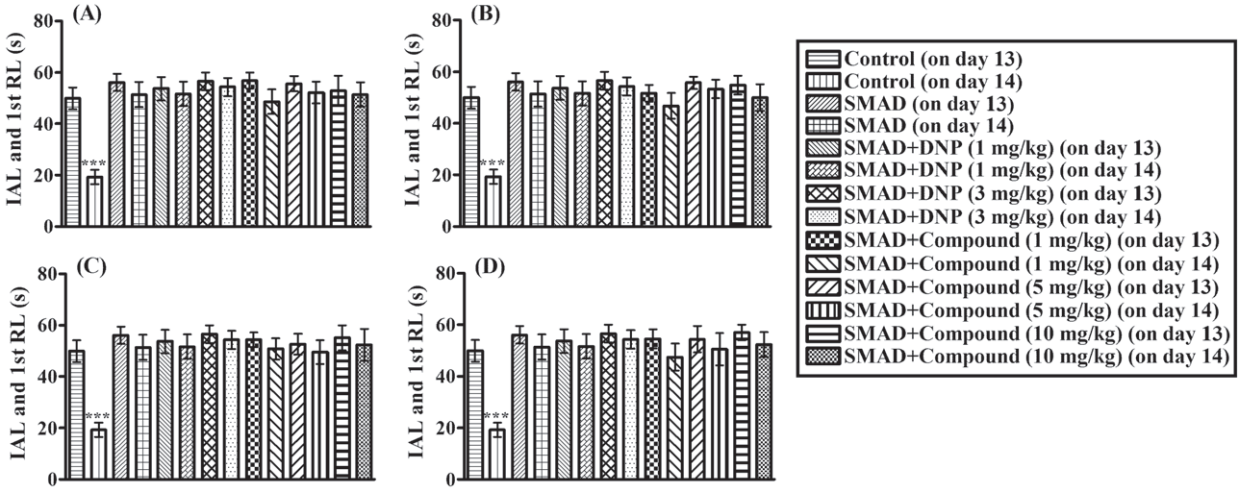


Fig. 4. IAL and 1st RL values of experimental groups in Morris water maze test before the DNP and test compounds administrations. (**A**) SMAD groups which would be treated with DNP and compound **B2**. (**B**) SMAD groups which would be treated with DNP and compound **B3**. (**C**) SMAD groups which would be treated with DNP and compound **B4**. (**D**) SMAD groups which would be treated with DNP and compound **B5**. Significance against control values, *** $p < 0.001$. Values are given as the mean ± SEM

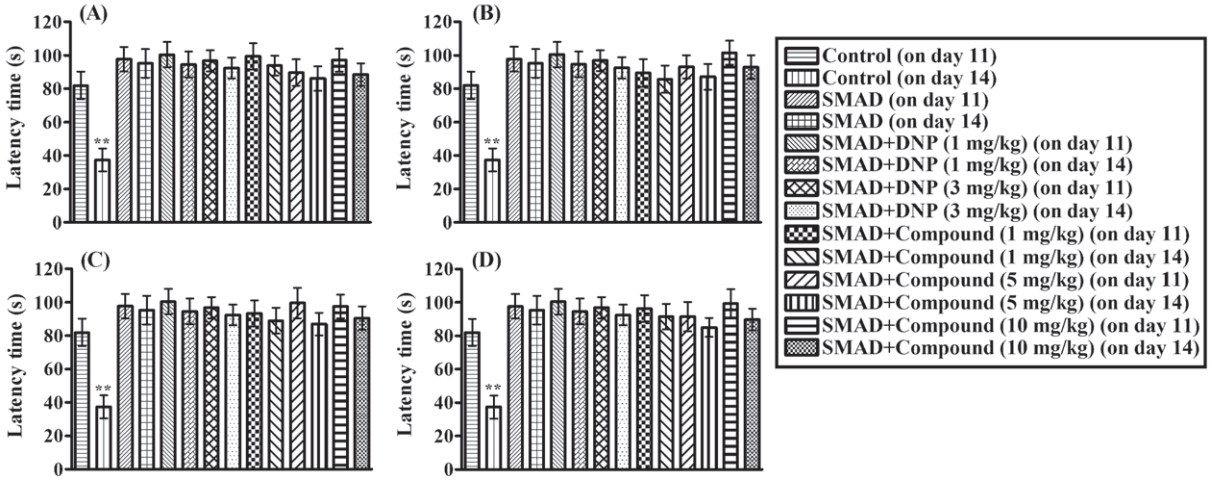


Fig. 5. Latency times of experimental groups in active avoidance test before the DNP and test compounds administrations. (**A**) SMAD groups which would be treated with DNP and compound **B2**. SMAD groups which would be treated with DNP and compound **B3**. (**C**) SMAD groups which would be treated with DNP and compound **B4**. (**D**) SMAD groups which would be treated with DNP and compound **B5**. Significance against control values, ** $p < 0.01$. Values are given as the mean ± SEM

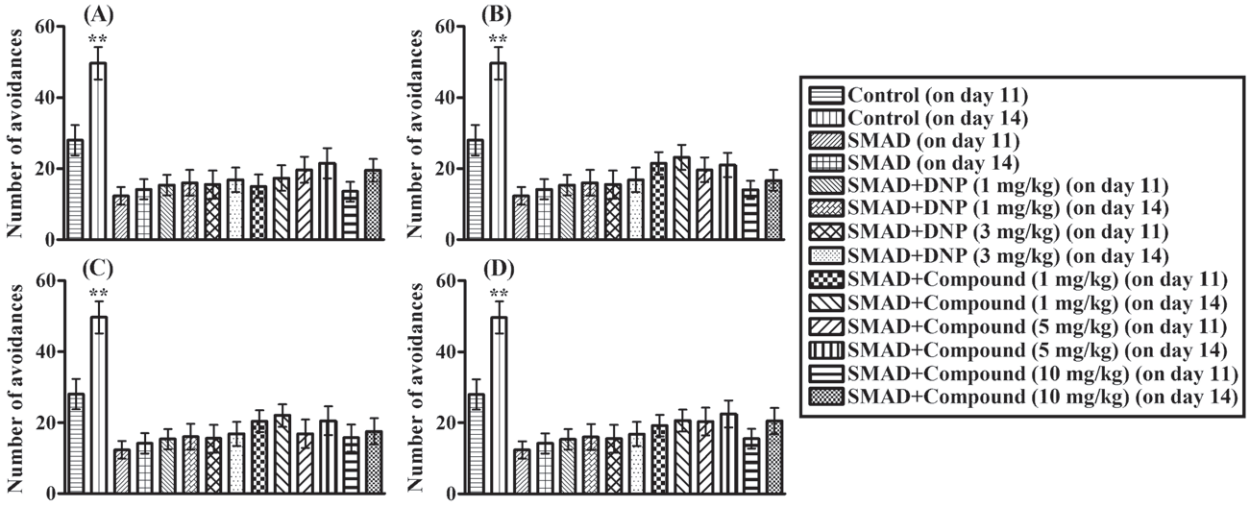


Fig. 6. Number of avoidances of experimental groups in active avoidance test before the DNP and test compounds administrations. (**A**) SMAD groups which would be treated with DNP and compound **B2**. (**B**) SMAD groups which would be treated with DNP and compound **B3**. (**C**) SMAD groups which would be treated with DNP and compound **B4**. (**D**) SMAD groups which would be treated with DNP and compound **B5**. Significance against control values, **$p < 0.01$. Values are given as the mean ± SEM

In elevated plus maze tests, compounds **B4** and **B5** at 5 and 10 mg/kg doses and compounds **B2** and **B3** at 10 mg/kg doses significantly decreased the 2nd RTL times of animals. **B4** and **B5** were significantly more effective than **B2** and **B3** (at 10 mg/kg doses) in terms of the 2nd RTL time-shortening effects (Fig. 7). At 1 and 3 mg/kg doses, the reference drug DNP decreased the 2nd RTL times, as expected. The effects of **B2**, **B3**, **B4**, and **B5** were comparable to that of DNP, as shown in Figure 7.

In Morris water maze tests, similarly to the results of elevated plus maze tests, **B4** and **B5** at 5 and 10 mg/kg and **B2** and **B3** at 10 mg/kg significantly reduced the 2nd RL times of animals. At 10 mg/kg

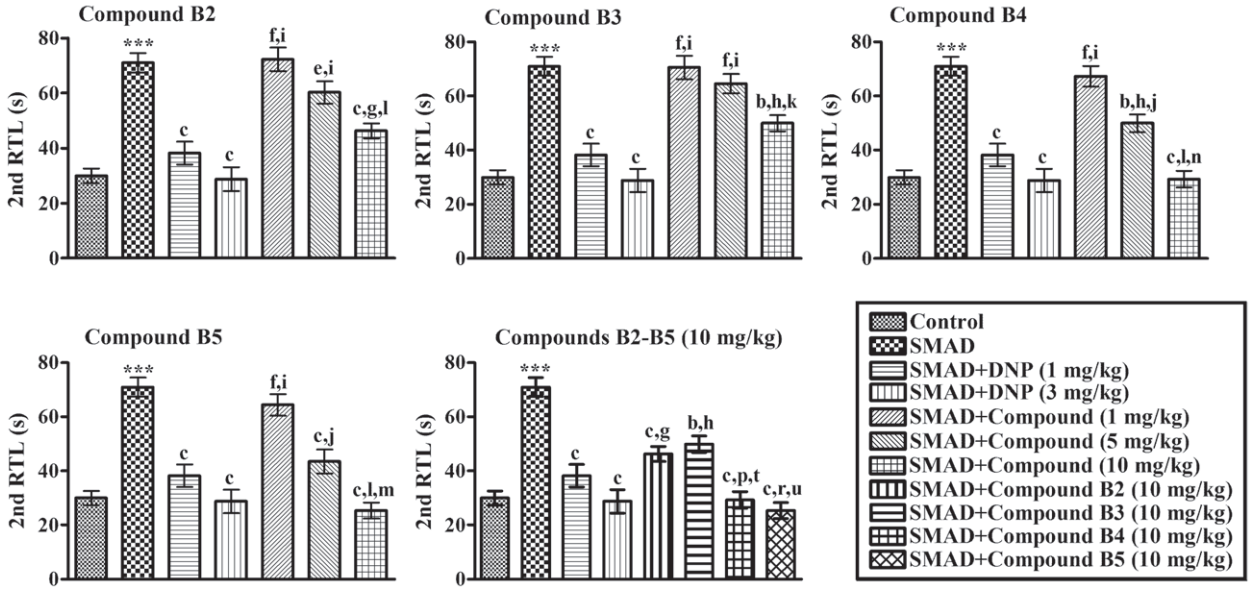


Fig. 7. 2nd RTL values of control, SMAD, DNP-treated (1 and 3 mg/kg) SMAD and compound-treated (1, 5 and 10 mg/kg **B2**–**B5**) SMAD groups in elevated plus maze test on day 21. Significance against control values, *** $p < 0.001$; significance against SMAD group values, [b] $p < 0.01$, [c] $p < 0.001$; significance against 1 mg/kg DNP-treated group values, [e] $p < 0.01$, [f] $p < 0.001$; significance against 3 mg/kg DNP-treated group values, [g] $p < 0.05$, [h] $p < 0.01$, [i] $p < 0.001$; significance against 1 mg/kg compound-treated group values, [j] $p < 0.05$, [k] $p < 0.01$, [l] $p < 0.001$; significance against 5 mg/kg compound-treated group values, [m] $p < 0.05$, [n] $p < 0.01$; significance against 10 mg/kg compound **B2**-treated group values, [p] $p < 0.05$, [r] $p < 0.01$; significance against 10 mg/kg compound **B3**-treated group values, [t] $p < 0.01$, [u] $p < 0.001$. Values are given as the mean ± SEM

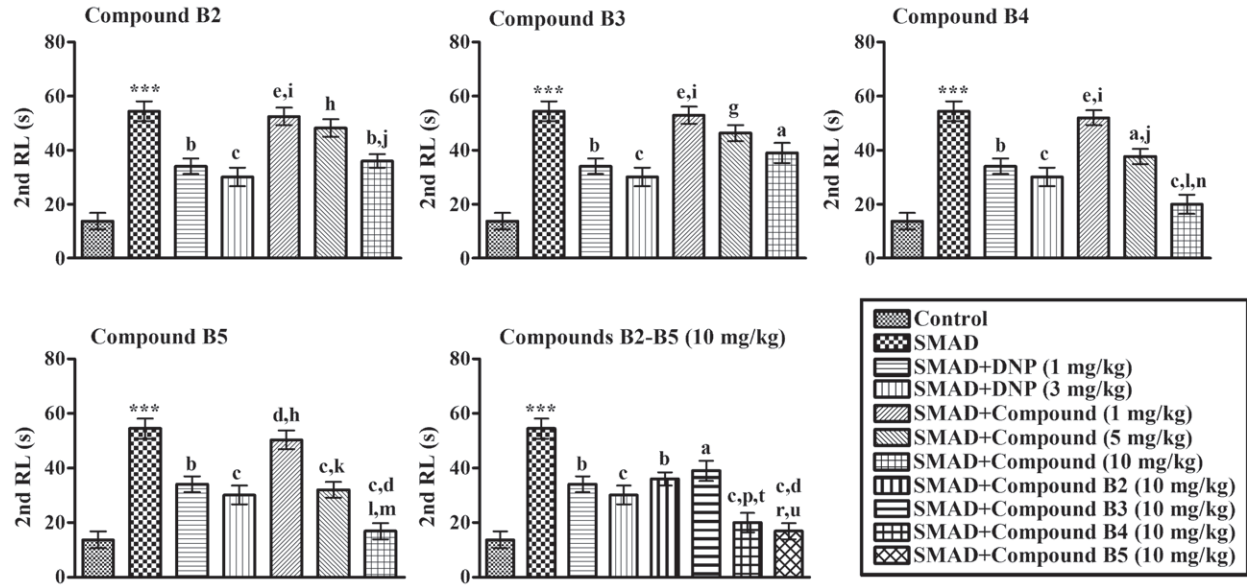


Fig. 8. 2nd RL values of control, SMAD, DNP-treated (1 and 3 mg/kg) SMAD and compound-treated (1, 5 and 10 mg/kg **B2**–**B5**) SMAD groups in Morris water maze test on day 21. Significance against control values, ***$p < 0.001$; significance against SMAD group values, [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$; significance against 1 mg/kg DNP-treated group values, [d] $p < 0.05$, [e] $p < 0.01$; significance against 3 mg/kg DNP-treated group values, [g] $p < 0.05$, [h] $p < 0.01$, [i] $p < 0.001$; significance against 1 mg/kg compound-treated group values, [j] $p < 0.05$, [k] $p < 0.01$, [l] $p < 0.001$; significance against 5 mg/kg compound-treated group values, [m] $p < 0.05$, [n] $p < 0.01$; significance against 10 mg/kg compound **B2**-treated group values, [p] $p < 0.05$, [r] $p < 0.01$, significance against 10 mg/kg compound **B3**-treated group values, [t] $p < 0.01$, [u] $p < 0.001$. Values are given as the mean ± SEM

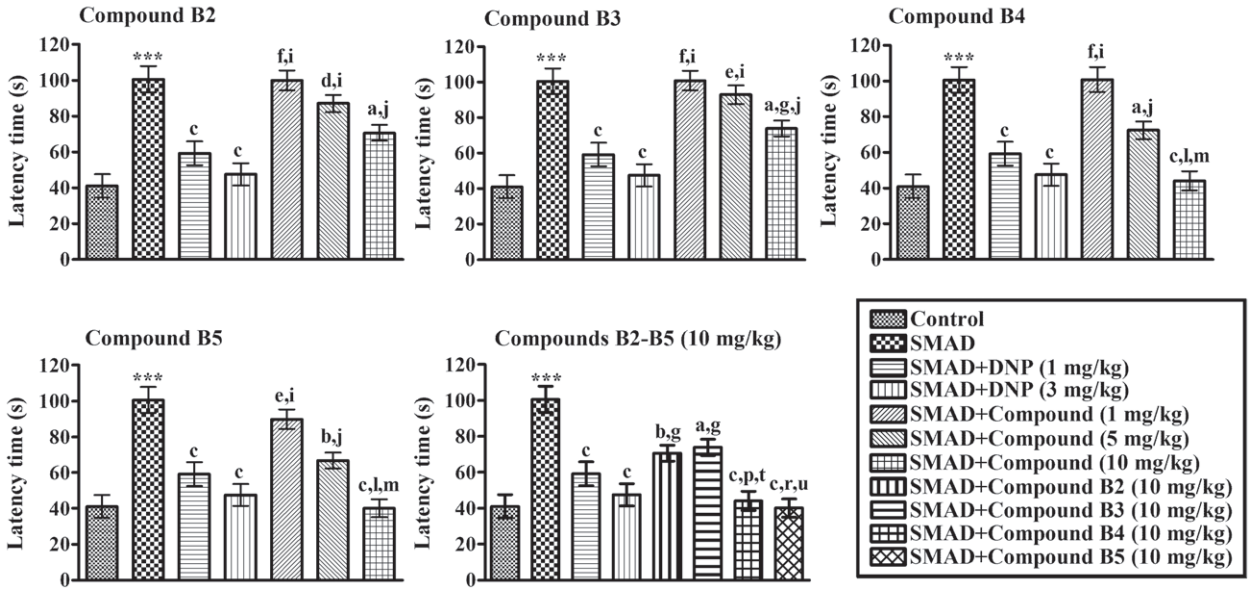


Fig. 9. Latency time values of control, SMAD, DNP-treated (1 and 3 mg/kg) SMAD and compound-treated (1, 5 and 10 mg/kg **B2**–**B5**) SMAD groups in avoidance test on day 21. Significance against control values, *** $p < 0.001$; significance against SMAD group values, [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$; significance against 1 mg/kg DNP-treated group values, [d] $p < 0.05$, [e] $p < 0.01$, [f] $p < 0.001$; significance against 3 mg/kg DNP-treated group values, [g] $p < 0.05$, [i] $p < 0.001$; significance against 1 mg/kg compound-treated group values, [j] $p < 0.05$, [l] $p < 0.001$; significance against 5 mg/kg compound-treated group values, [m] $p < 0.05$; significance against 10 mg/kg compound **B2**-treated group values, [p] $p < 0.05$, [r] $p < 0.01$; significance against 10 mg/kg compound **B3**-treated group values, [t] $p < 0.01$, [u] $p < 0.001$. Values are given as the mean ± SEM

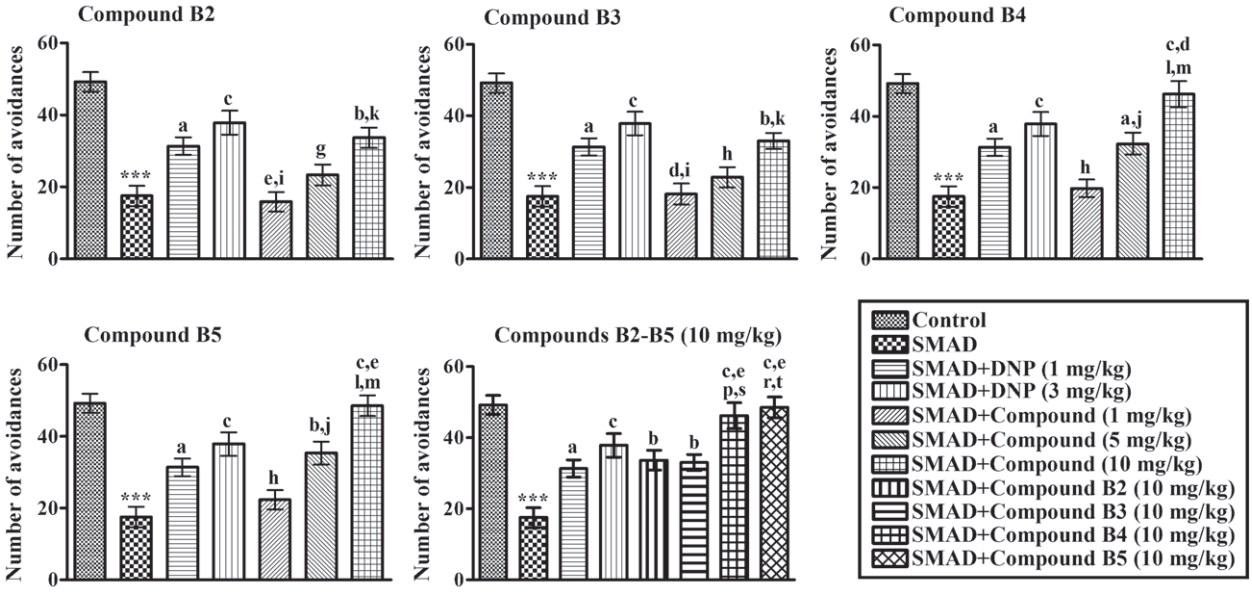


Fig. 10. Number of avoidances of control, SMAD, DNP-treated (1 and 3 mg/kg) SMAD and compound-treated (1, 5 and 10 mg/kg **B2**–**B5**) SMAD groups in avoidance test on day 21. Significance against control values, *** $p < 0.001$; significance against SMAD group values, [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$; significance against 1 mg/kg DNP-treated group values, [d] $p < 0.05$, [e] $p < 0.01$; significance against 3 mg/ kg DNP-treated group values, [g] $p < 0.05$, [h] $p < 0.01$, [i] $p < 0.001$; significance against 1 mg/kg compound-treated group values, [j] $p < 0.05$, [k] $p < 0.01$, [l] $p < 0.001$; significance against 5 mg/kg compound-treated group values, [m] $p < 0.05$; significance against 10 mg/kg compound **B2**-treated group values, [p] $p < 0.05$, [r] $p < 0.01$; significance against 10 mg/kg compound **B3**-treated group values, [s] $p < 0.05$, [t] $p < 0.01$. Values are given as the mean ± SEM

doses, **B4** and **B5** were significantly more effective than **B2** and **B3** (Fig. 8). DNP was effective in decreasing 2nd RL times, as expected (Fig. 8).

In active avoidance tests, latency times were significantly decreased (Fig. 9), and avoidance numbers were significantly increased (Fig. 10) by **B4** and **B5** (5 and 10 mg/kg) and **B2** and **B3** (10 mg/kg) treatment. **B4** and **B5** were significantly more effective than **B2** and **B3**, at 10 mg/kg doses (Fig. 9 and 10). Once again, DNP improved the measured learning and memory parameters in this test (Fig. 9 and 10).

Differences in the numbers of horizontal and vertical movements between the groups were not statistically significant on day 21 (data not shown).

Discussion

In the present study, benzothiazole-piperazine compounds were synthesized by condensing the functional groups of DNP, FK-960, and sabeluzole, which are known to have therapeutic potential against AD, with the aim of obtaining new and potent anti-Alzheimer agents.

The first step of this study was to evaluate the possible inhibitory activity of synthesized compounds on AChE and BChE enzymes by Ellman's method. Following this *in vitro* method, based on the relationship between inhibition of cholinesterase activities and treatment of AD, we planned to continue with *in vivo* tests to assess the learning and memory parameters of the experimental animals.

Results from Ellman's method revealed that some of the tested benzothiazole-piperazine derivatives exhibited a remarkable AChE inhibitory potential. Compounds **B2**, **B3**, **B4**, and **B5** possessed significant inhibitory potencies against AChE, whereas BChE activity was not affected by the test compounds. DNP also selectively inhibited AChE.

It is well established that acetylcholine, a neurotransmitter degraded by AChE in nerve synapses, is required for the proper function of cholinergic transmission in the regulation of learning and memory processes [47]. The enhancement of cholinergic activity by the inhibition of AChE is the mainstay of symptomatic treatment of AD [47]. For example, the centrally acting cholinesterase inhibitor DNP is used for the symptomatic treatment of AD. It has been reported that DNP preferentially inhibits AChE rather than BChE [43]. This finding was in accordance with the results of our enzymatic study. The results from *in vitro* AChE enzyme inhibition assays encouraged us to investigate the potential anti-Alzheimer effects of compounds **B2, B3, B4,** and **B5** *in vivo*, whereas compound **B1** was not evaluated *in vivo* due to its poor potency against AChE.

Icv injection of STZ to rats at 3 mg/kg has been accepted as an appropriate animal model for sporadic AD. The glucosamine-nitrosourea compound STZ when applied into the brain bilaterally induces significant cognitive impairment in animals without affecting peripheral blood glucose levels. SMAD is characterized by progressive deterioration of memory, cerebral glucose, and energy metabolism. Desensitization of neuronal insulin receptors has been suggested as the cause of impaired cerebral glucose and energy metabolism in this animal model. *Icv* injection of STZ causes a cholinergic deficiency by inhibiting the synthesis of adenosine triphosphate and acetyl-CoA. Cholinergic deficiency was accompanied by reduced choline acetyltransferase activity in the hippocampus and increased AChE activity in rat brains [37, 50]. Bilateral injection of STZ causes more widespread damage and marked impairment in memory compared to unilateral damage [50, 57].

The occurrence of SMAD in rats was confirmed with behavioral tests. The elevated plus maze, Morris water maze, and active avoidance tests were applied to the *icv* citrate buffer-injected control groups and indicated that learning and memory functions of these control groups were intact (Figs. 3–6). In contrast, learning and memory parameters of *icv* STZ-injected animals were significantly impaired when compared to control groups (Figs. 3–6), as expected [2, 37, 44, 57].

Just after these behavioral tests, DNP and test compounds **B2**, **B3**, **B4**, and **B5** were administrated to '*icv* STZ-injected animals' for 1 week. The rats were taken through elevated plus maze, Morris water maze, and active avoidance tests after all administrations were completed. In all these cognitive tests, compounds **B2**, **B3**, **B4**, and **B5** improved the impaired learning and memory parameters of *icv* STZ-injected animals. **B4** and **B5** were found to be more effective than **B2** and **B3**, in all performed tests. STZ-injected rats treated with reference drug DNP demonstrated significant improvement in the measured learning parameters, as expected (Figs. 7–10).

The locomotor activity values of reference and test compound-treated groups were similar to those of the control animals. Therefore, it may be suggested that these compounds did not change the spontaneous locomotor activities of animals or affect the results of cognitive tests.

Chemical structure-pharmacological activity relationships revealed that substituents on the fourth position of the piperazine ring have a substantial effect on pharmacological activity. Compounds **B2**–**B5**, which bear 4-substituted benzyl piperazines, indicated significant pharmacological activity *in vitro*, compared with the phenyl-substituted piperazine compound **B1**. This result suggests that the benzyl moiety on the piperazine ring substantially increases the AChE inhibitory activity. This may be explained by chemical differences between compounds **B1** and **B2**–**B5**. In compound **B1**, *N*-phenyl substitution decreases the electron density of the piperazine ring. On the other hand, a methylene group between aromatic and piperazine rings enhances the electron density of piperazine in compounds **B2**–**B5**. It is well known that electron density plays an important role in biomolecular interactions, which is a pre-condition of pharmacological activity. Thus, it may be suggested that varying electron density caused differences in the activities of **B1** and **B2**–**B5** *in vitro*. Lipophilicity is another important structural feature affecting pharmacological activity. Due to the different substitution patterns of compounds **B2**–**B5**, their lipophilicity varies and can be ordered as **B5** > **B4** > **B3** > **B2**. They are similarly ordered in terms of their efficacies. The compounds **B4** and **B5** possess greater pharmacological activity than compounds **B2** and **B3** both in *in vitro* and *in vivo* tests. This interesting finding suggests that compounds **B4** and **B5** may pass through the CNS more readily than **B2** and **B3** due to their more lipophilic nature, which accounts for their improved pharmacological activity.

Conclusion

Compounds **B2**–**B5**, which were synthesized by condensing the functional groups of the DNP, FK-960, and sabeluzole, restored the impaired learning and memory parameters of *icv* STZ-injected rats, comparable to the activity of DNP. The mechanisms for the proposed anti-Alzheimer activities were related, at least partially, to the AChE enzyme inhibitory activities of the test compounds. However, probable additional mechanisms need to be investigated in further, detailed studies.

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