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Benzodiazepine derivatives for the treatment of neuropharmacological disorders and pain management: Docking investigations and *in-vivo* studies

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ABSTRACT

Benzodiazepines are well-known for their medicinal properties. The current study aimed to evaluate and seek new therapeutic possibilities of some of these compounds for addressing anxiety and pain. Four benzodiazepine derivatives were synthesized, characterized, and subjected to computational assessment of the GABAergic response upon interaction with human GABA-A receptors (PDBID: 6×3x) relative to the drug diazepam (DZP) to achieve the objective. Apart from the docking analysis, ADME analysis of the compounds was also performed. Following the *in-silico* evaluations, two of the synthesized compounds (designated as P3 and P4) were identified for subsequent *in-vivo* investigations utilizing laboratory rats to explore their anxiolytic potential in comparison to diazepam, and their analgesic efficacy relative to diclofenac sodium. The combination of *in-silico* and *in-vivo* assessments uncovered the binding sites of P4 (a fluoro derivative) and its potential as a superior anxiolytic drug compared to P3 (a hydroxy derivative) for analgesic relief. The ADME pharmacokinetic evaluation of the synthesized compounds by Lipinski rule of 5 suggested the oral bioavailability of these drugs. These findings are important as they provide valuable insights into the potential development of new anxiolytic and analgesic agents from the benzodiazepine derivatives that can be easily synthesized and developed into affordable drugs.

1. Introduction

Benzodiazepines (BZDs) marked their importance in the 1960s as opium-free anxiolytics [1]. These compounds have various therapeutic potentials associated with them including hypnotic, anti-convulsant, and anti-depressant, and are used to treat insomnia, generalized anxiety disorder, panic disorder, and seizures and are also used to prevent alcohol withdrawals [2–4]. The benzodiazepines used for these purposes are sold as chlordiazepoxide and diazepam to treat anxiety [5], clonazepam as muscle relaxants [6], midazolam as general anesthetics [7], and olanzapine as an anti-psychotic drug as well as to treat bipolar disorders [8].

In addition to their use as pharmaceutical agents, benzodiazepines are a class of Schiff bases that have been incorporated in many fields owing to their structural diversity. The variational capacity of the benzodiazepines leads to the attachment of various donor-acceptor groups in conjugation with delocalized π-electron systems. These chromophores find application in a wide range of fields, including photo-optical devices, telecommunications, laser technology, medical imaging, optical sensors, material characterizations, quantum optics and computing, defense, and security systems. The vast variety of Schiff bases including benzodiazepines have also been used for their applications in material sciences [9].

Diversity arises from four major points within the structure. One is the relative positions of nitrogen atoms, the second is the point of saturation in the diazepine ring, third and fourth are either the derivatization of the benzene ring or the diazepine ring (Fig. 1) [10]. It is because of the relative positions of the nitrogen atoms inside the diazepine ring that the commonly known 1,5-benzodiazepine is 1*H*-benzo[*b*][1,4]diazepine while 1*H*-benzo[*e*][1,4]diazepine are commonly called as 1,4-benzodiazepines.

GABA-A is the chloride ion-regulated ligand-receptor site complex, which is structured by 5 glycoproteins 2α, 2β and 1γ (Fig. 2). Each complex has one site where BZD can get bound while two sites are specified for GABA units. The location of GABA-A receptors is at the post-synaptic membrane within the synapses. The BZD gets attached to the receptor in between the α and γ units [11].

The normal functioning of CNS has been maintained by an

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antagonistic pair of responses by the neurotransmitters; one being the excitatory glutamate receptors and the second being the inhibitory GABA (A, B and C) receptors [12]. Benzodiazepines work as positive allosteric modulators of GABA-A thus causing hyperpolarization of the membrane by influx of chloride ions at a greater frequency than usual. The resulting reduction in excitability of the neuronal activity leads to the calming effect thrusting towards the anxiolytic response of BZD [13].

Till now very few benzodiazepines have been marketed to treat neuropsychological disorders. Almost all the marketed benzodiazepines have nitrogen placements at positions 1 and 4 with respect to each other except for a few ones which have nitrogen placement at positions 1 and 5. The detailed literature survey provided evidence about the less toxicity as well as side-effects associated with clobazam (1,5-BZD) than diazepam (1,4-BZD) [14].

The main objective of the research was to synthesize 1,5-benzodiazepine derivatives and evaluate their *in-silico* and *in-vivo* neuropharmacological and analgesic activities. By systematically investigating the potential therapeutic properties of these compounds, the study aimed to contribute valuable insights to the field of neuropharmacology and pain management, paving the way for the development of innovative pharmacological agents.

2. Experimental part

2.1. Chemicals

1,2-Phenylenediamine (*o*-PDA) was purchased from Chemimpex (Illinois, USA), while the acetophenones (synthetic grades) and the solvents (analytical grade) were procured from Sigma-Aldrich (Steinheim, Germany) and diazepam and diclofenac sodium used for *in-vivo* studies were purchased in the form of marketed drugs (Lahore, Pakistan).

2.2. General method for the synthesis of BZD

The synthetic route was optimized by doing some modifications to an already reported method [15]. *o*-PDA and acetophenone were dissolved in methanol in a 1:2 mol. A few drops of GAA (Glacial acetic acid) were added to the reaction mixture to ensure the acidic pH. Stirring at room temperature was performed to ensure the complete dissolution of the mixture. The reaction was performed by providing heat with the help of a temperature-controlled hotplate. TLC (thin-layer chromatography) was performed periodically to monitor the progress of the reaction. The reaction mixture was cooled after the completion of the reaction, and precipitates were obtained that were washed with methanol. Recrystallization was performed using acetone. The reaction was repeated for all the products. The general scheme of reaction is given in Fig. 3.

2.3. Docking studies

Docking studies were performed on all four synthesized compounds using Auto dock tools 1.5.6 [16]. All the compounds prepared were drawn on ChemDraw (18.1) and the energy minimizations were performed on Avogadro using MMFF94 forcefield. The standard diazepam (ID#DB00829) was obtained from a drug bank and run through the same parameters. For docking studies, the GABA-A receptor (PDBID: 6×3X) was obtained from the RCSB [17]. The selection of protein was

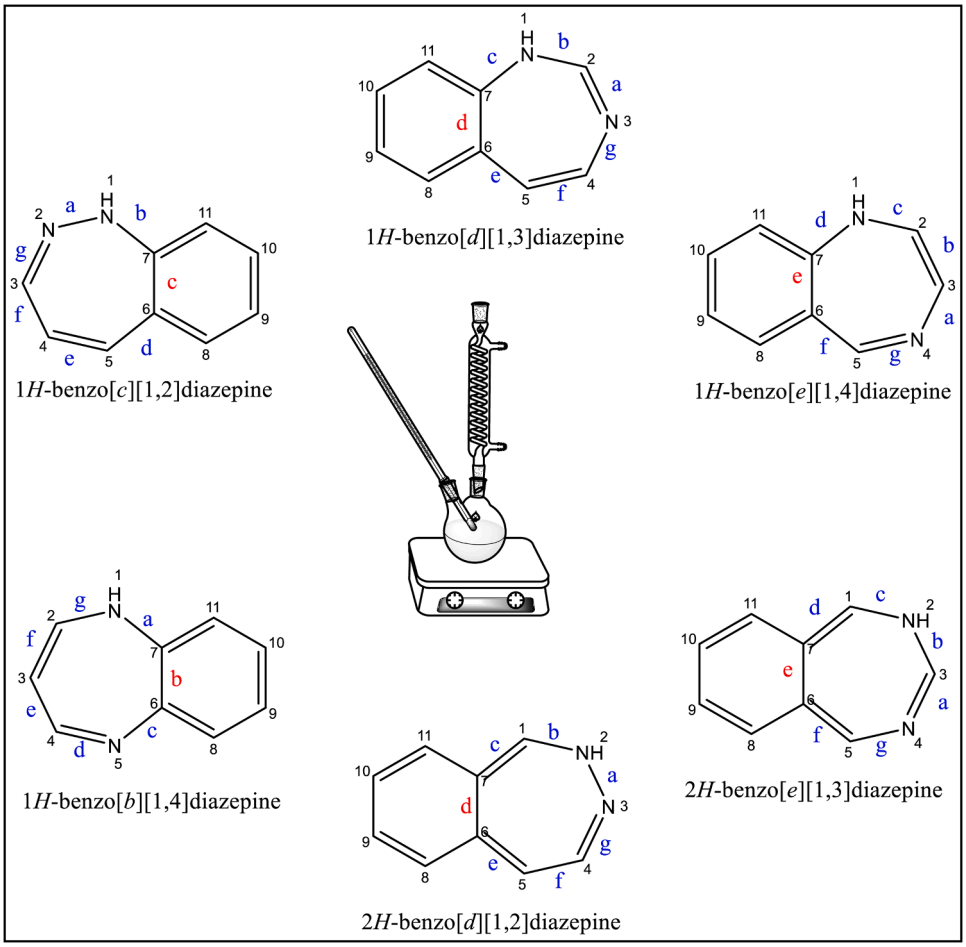

Fig. 1. Structural differences among various benzodiazepine classes based on the relative position of nitrogen atoms.

based on the fact that the 6×3X was obtained from *homo sapiens* with CryoEM resolution of 2.92 Å and has a co-crystallized ligand of interest (in our case that is diazepam). The protein obtained had a multimeric structure, therefore, it was prepared by removing extra chains using BIOVIA discovery studio 2021 client (Dassault Systèmes: San Diego, CA, USA) [18]. Validation of the docking protocol was performed by redocking the standard (DZP) into the binding pocket of the active site. For the docking common extracellular domain pocket was chosen which was exhibited by DZP 404 in the co-crystallized pdb6×3X structure [17].

2.4. Pharmacokinetics parameter evaluation

For the pharmacokinetics, drug likeliness and physicochemical studies SwissADME online tool was utilized to evaluate the ADME (absorption, distribution, metabolism, and excretion) of the four synthesized compounds. SwissADME utilizes physicochemical descriptors for the estimation of the gastrointestinal absorption and blood-brain-barrier penetration of the drug molecules; a technique known as BOILED-Egg (Brain Or Intestinal Permeation Method) estimation. The tool is advantageous in predicting the drug-likeliness of the compounds using Lipinski's rule of five. While the efficiency of the drug can be calculated by making use of such parameters; it is also seen that the metabolites of the drug may enhance or reduce the pharmacological potential of the drugs by enhancing or reducing the half-life of drug, oral clearance or bioavailability of the drug or its metabolites. ADME properties cover the structure of the drugs only not their metabolites [19,20].

2.5. In-vivo studies

2.5.1. Neuropharmacological activities

After the analysis of *in-silico* docking studies two compounds were selected for the evaluation of *in-vivo* neuropharmacological and analgesic activities against standard drugs diazepam and diclofenac sodium, respectively (Fig. 4). The activities were performed at the Faculty of Pharmacy and Allied Health Sciences, University of Balochistan, Quetta, Pakistan. The mice obtained were arranged from the animal house of CASVAB (Center for Advanced Studies in Vaccinology and Biotechnology, University of Balochistan). The weight of all the mice ranged from 25 to 28 g. All the animals were conscious and active 7 days prior administration of drugs. The animals were marked with identification numbers on their tails. All the mice were divided into three groups for the two synthesized drug trials. CTR P3 and STD P3 (represent the control and standard drug for the trial of P3) while CTR P4 and STD P4 (represent the control and standard drug for the trial of P4).

Group I: Vehicle Control group (0.5 mL normal saline)
Group II: Experimental administered drug (P3 or P4) 2 mg/Kg (treated group)
Group III: Positive control standard drug diazepam 2 mg/Kg (treated group).

Five different tests were performed to analyze the behavioral changes in the mice including forced swimming test, rearing test, open field test, cage crossing test, and traction test [21–25].

2.5.1.1. Forced swimming test (FST). The antidepressant behaviors of mice were observed by using the already reported method [21]. Mice were divided into three groups as mentioned in Section 2.5.1. The study was performed by first obtaining a control reading then the drug (P3 or P4) was administered in 5 mg while the standard drug(diazepam)was given in a dose of 2 mg. Mice were kept in an open cylinder apparatus, filled with water in such a manner that the forelimbs of mice did not touch the bottom surface of the cylinder. The time provided for swimming was 6 min. All the readings were analyzed for each of the 3 groups. The time of movement was calculated till the instance when the mice stopped moving. The floating time of mice by keeping their heads above water revealed immobility time. This time represented the antidepressant activity of mice.

2.5.1.2. Open field test (OFT). The locomotor activity was observed using this test. A plastic apparatus was designed to have an area marked

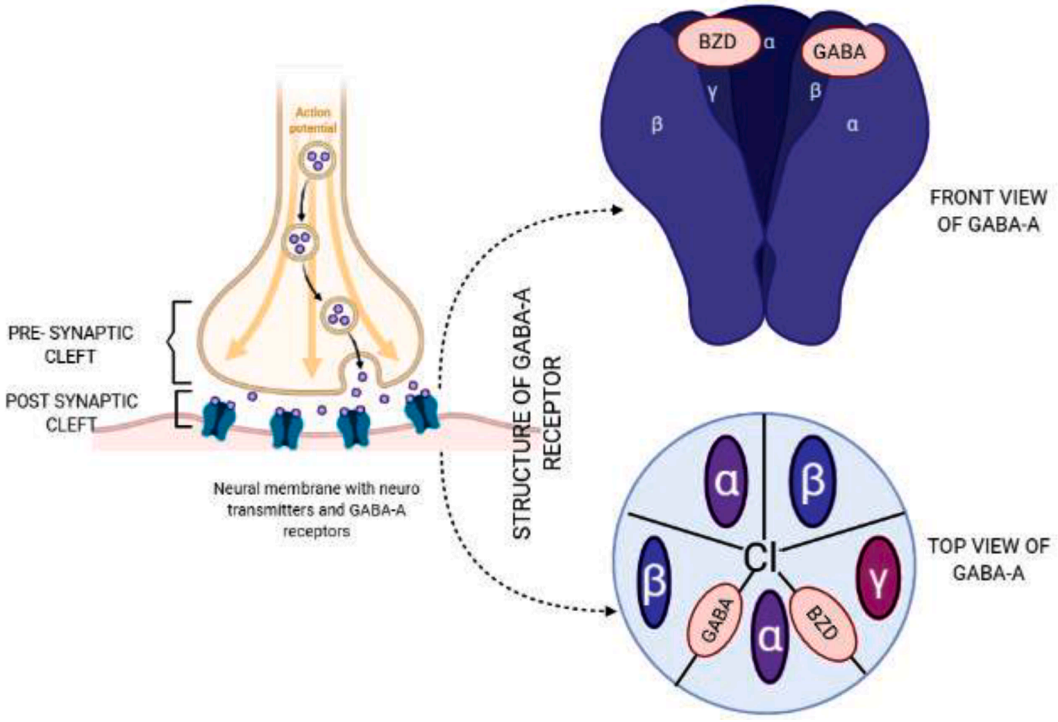

Fig. 2. Structure of A) neural membrane with the embedded GABA receptors and neurotransmitters B) front view of GABA-A and BZD sites on GABA-A receptors with 2α, 2β and 1γ site C) top view of the receptor.

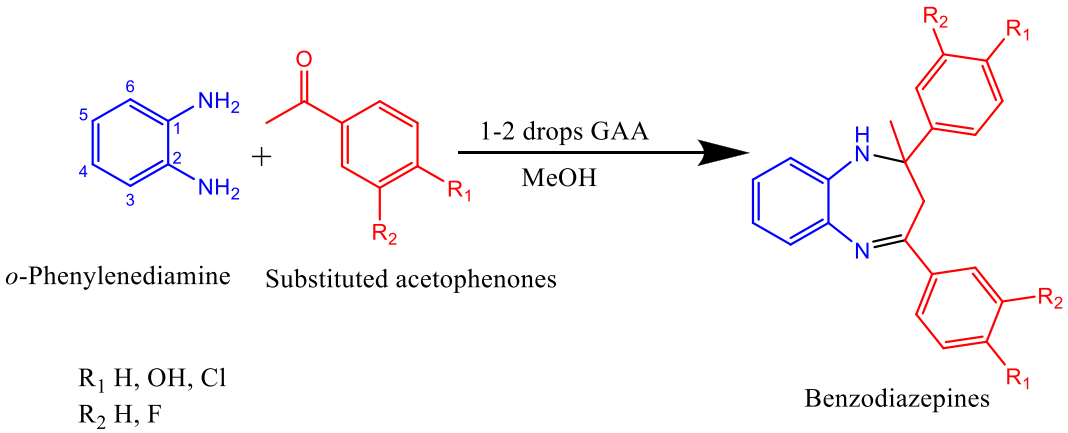

Fig. 3. General scheme for the synthesis of 1,5-benzodiazepines.

Fig. 4. Mice exhibiting different neuropharmacological activities. a). FST; b). RT; c). OFT; d). CCT.

by 25 squares. The effect of drugs on mice was checked after 30 min of the administration of vehicle control, experimental drug, and positive control. Each group is observed for 10 min and the effect is observed by the number of times the mice crossed a square [24].

2.5.1.3. Cage crossing test (CCT). In this test, a plastic cage is used, and the cage crossing activity was marked by the number of times the mice touched the sides of the cage after crossing it. The time provided for this test was 10 min [25].

2.5.1.4. Traction test (TT). An iron rod of length one meter was used for observing the traction activity. Before the activity, the training of animals to travel on the rod was done. The time enhancement/reduction for traveling showed the stimulant or sedative activity [25].

2.5.1.5. Rearing test (RT). This is a known test used for examining the central excitatory behavior of mice [22]. Mice were kept in a beaker and were observed for 10 min. The number of times that a mouse stands independent of forelimbs was counted.

2.5.2. Analgesic activities

A modified method proposed by Danbisya et al., 1999 was followed to conduct analgesic activity in mice. Four groups of mice having two experimental, one vehicle control, and one positive control were made. The pain was induced by injecting 2 % formalin using a 20 μL syringe in the dorsal region of the right hind paw. The activity was grouped into two phases. The 1st phase was initiated just after injecting the formalin dose and constituted 5 min, the number of licking and biting were observed in this phase. The second phase of licking and biting started

after 15 min of the initial dose of formalin and lasted for 15 min [26].

Group I: Vehicle control (0.5 mL normal saline)
Group II: Experimental administered drug (P3) 2 mg/Kg (treated group)
Group III: Experimental administered drug (P4) 2 mg/Kg (treated group)
Group IV: Positive control standard drug diclofenac sodium 2 mg/Kg (treated group).

2.6. Statistical analysis

Analysis of variance (ANOVA) with 95 % confidence level was applied for individual activities to test the variances among the means of different groups for neuropharmacological and analgesic activities. Tukey's HSD post-HOC (when there was homoscedasticity inside data) [27] and Dunnett T3 (where there was heteroscedasticity inside the data) [28] tests were implied to explore for possible group differences assuming that the omnibus test is significant.

3. Results

3.1. Synthesis and characterization of benzodiazepines

Synthesis of four benzodiazepines with slight modifications in structures was done according to the synthetic scheme. Among the four synthesized compounds P1, P2, and P3 are already reported [29,30] while P4 is a novel compound (not found in any of the previous research). All the compounds showed a characteristic peak of secondary N-H stretching (3200–3500 cm^{-1}), in addition to that P3 showed a broad band at 3183 cm^{-1} because of the phenolic hydroxyl.

Among the four synthesized compounds, hydroxy derivatives showed a maximum yield of 84.67 %. The physicochemical and spectral data of are presented in Sections 3.1.1, 3.1.2, 3.1.3, 3.1.4.

3.1.1. 2-Methyl-2,4-diphenyl-2,3-dihydro-1H-benzo[b][1,4]diazepine (P1)

Yield: 76.9%; brown flakes; m.p. 131 °C; ^{1}H NMR (DMSO d6, 60 MHz, δ with TMS = 0): 1.60 (s, 3H, CH_3), 2.92–3.39 (q, 2H, CH_2), 6.72 (q, 1 H, NH), 6.83–7.45 (m, 14 H, phenyl groups); EI-MS: $C_{22}H_{20}N_2$ calculated M^+: 312.16; observed [M^+]: 312.

3.1.2. 2,4-bis(4-Chlorophenyl)−2-methyl-2,3-dihydro-1H-benzo[b][1,4] diazepine (P2)

Yield: 78.2 %; yellow crystals; m.p. 156 °C; ^{1}H NMR (DMSO d6, 60 MHz, δ with TMS = 0): 1.60 (s, 3H, CH_3), 2.45- 3.27 (s, 2H, CH_2), 5.74 (s, 1 H, NH), 6.90 −7.75 (m, 12 H, phenyl groups); EI-MS: $C_{22}H_{18}Cl_2N_2$ calculated M^+: 380.087; observed [M^+]: 379.98.

3.1.3. 4,4′-(2-Methyl-2,3-dihydro-1H-benzo[b][1,4]diazepine-2,4-diyl) diphenol (P3)

Yield: 84.67 %; yellow flakes; m.p. 254 °C; ^{1}H NMR (DMSO d6, 60 MHz, δ with TMS = 0): 1.53 (s, 3H, CH_3), 2.45–3.26 (q, 2H, CH_2), 5.26 (s, 1 H, NH), 7.01–7.45 (m, 12 H, phenyl groups), 9.08–9.66 (d, 2H, OH); EI-MS: $C_{22}H_{22}N_2O_2$ calculated M^+: 344.15; observed [M^+]: 344.

3.1.4. 2,4-bis(3-Fluorophenyl)−2-methyl-2,3-dihydro-1H-benzo[b][1,4] diazepine (P4)

Yield: 79.5 %; brown crystals; m.p. 106 °C; ^{1}H NMR (DMSO d6, 60 MHz, δ with TMS = 0): 1.62 (s, 3H, CH_3), 2.45–3.51 (set, 2H, CH_2), 5.76 (q, 1 H, NH), 6.73 −7.45 (m, 12 H, phenyl groups); ESI-MS: $C_{22}H_{18}F_2N_2$ calculated M^+: 348.14; observed [M^+]: 348.

Fig. 5. 2d and 3d Molecular docking interactions of diazepam and synthesized BZDs with GABA-A (6×3X) receptor. The binding residues along with interaction types in colored indicators are shown. Color indicators: Green (conventional hydrogen bonding), Light green (Pi donor Hydrogen Bond) Purple (Pi-Alkyl), Pink (Pi-Pi stacked and Pi-Pi T shaped), Blue (Halogen).

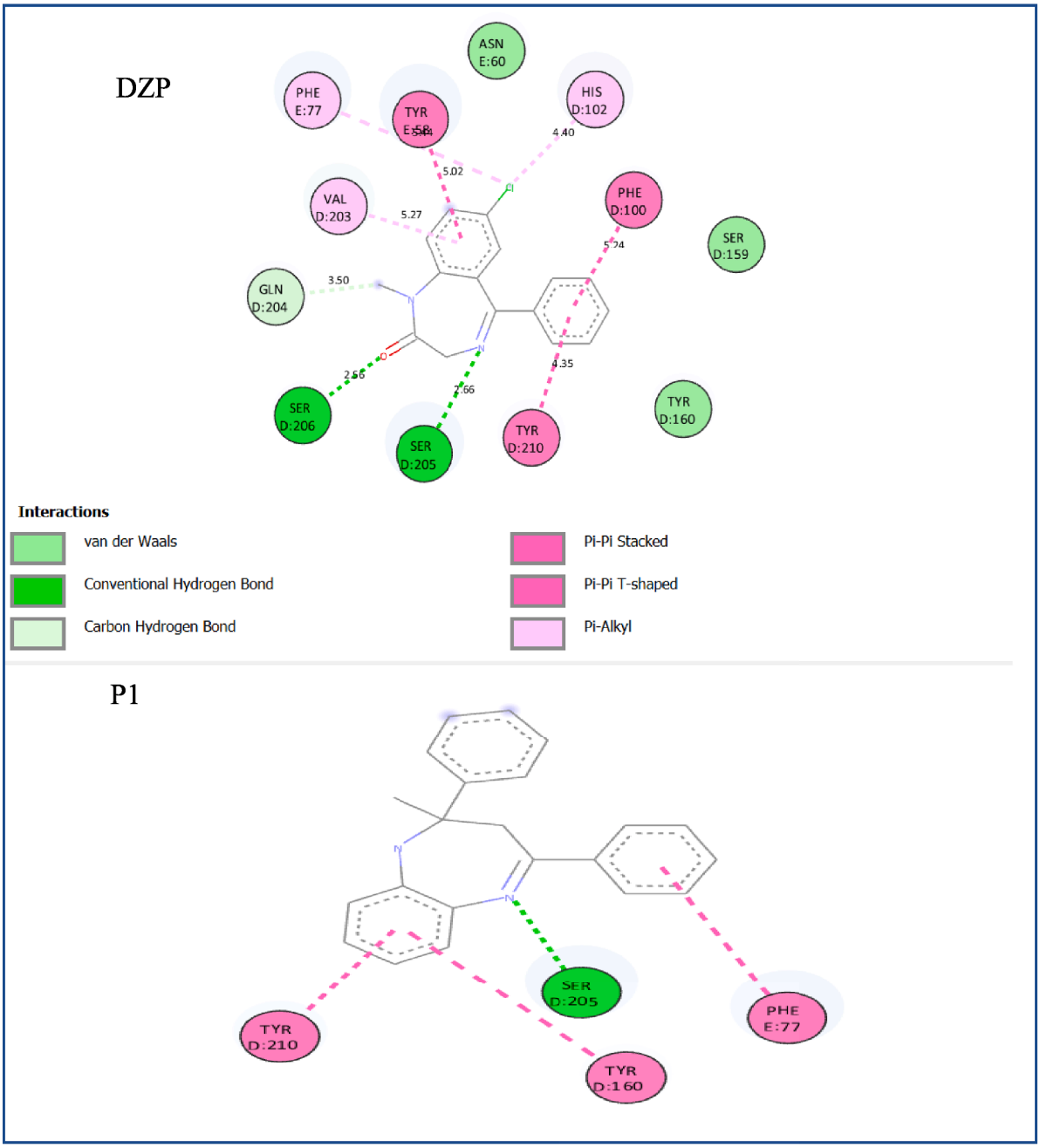

Fig. 5. (*continued*).

3.2. Docking results

The docking results are shown in Fig. 5.

3.3. Pharmacokinetic analysis

In-silico drug likeliness of the compounds is calculated for the target compounds as well as standard and marketed benzodiazepines (diazepam DZP, midazolam MDZ, brotizolam BRT) using SwissADME tool. Various molecular parameters were computed that are indicators of the CNS based activities. These include blood brain barrier, lipophilicity of the compounds in water-n-octanol system (Log P) as well as TPSA ~topological polar surface area. Drug- Likeliness of the compounds is studied by the help of Lipinski's rule of five [31]. The ADME properties are given in Table 1.

3.4. Neuropharmacological activities

The efficacy of drugs as potent anxiolytics was carried out on laboratory mice. For the evaluation of the anti-depressive/anxiolytic behavior the mice were categorized into three groups a vehicle, an experimental/ test group, and one positive control group. Two trials were carried out to analyze the activity of the test compounds with the control groups. All the groups were subjected to five different types of tests namely FST, OFT, CCT, TT, and RT, the data of which give the neuropharmacological activities (Fig. 6; Table 2).

3.4.1. Forced swimming test

The data from the Forced Swimming Test (MT) revealed distinct patterns across different conditions. In the drug trial study of P3, the control group exhibited a mean mobility time (MT) of 3.37 min, (SE= ±0.20 min). In comparison, the experimental group displayed a

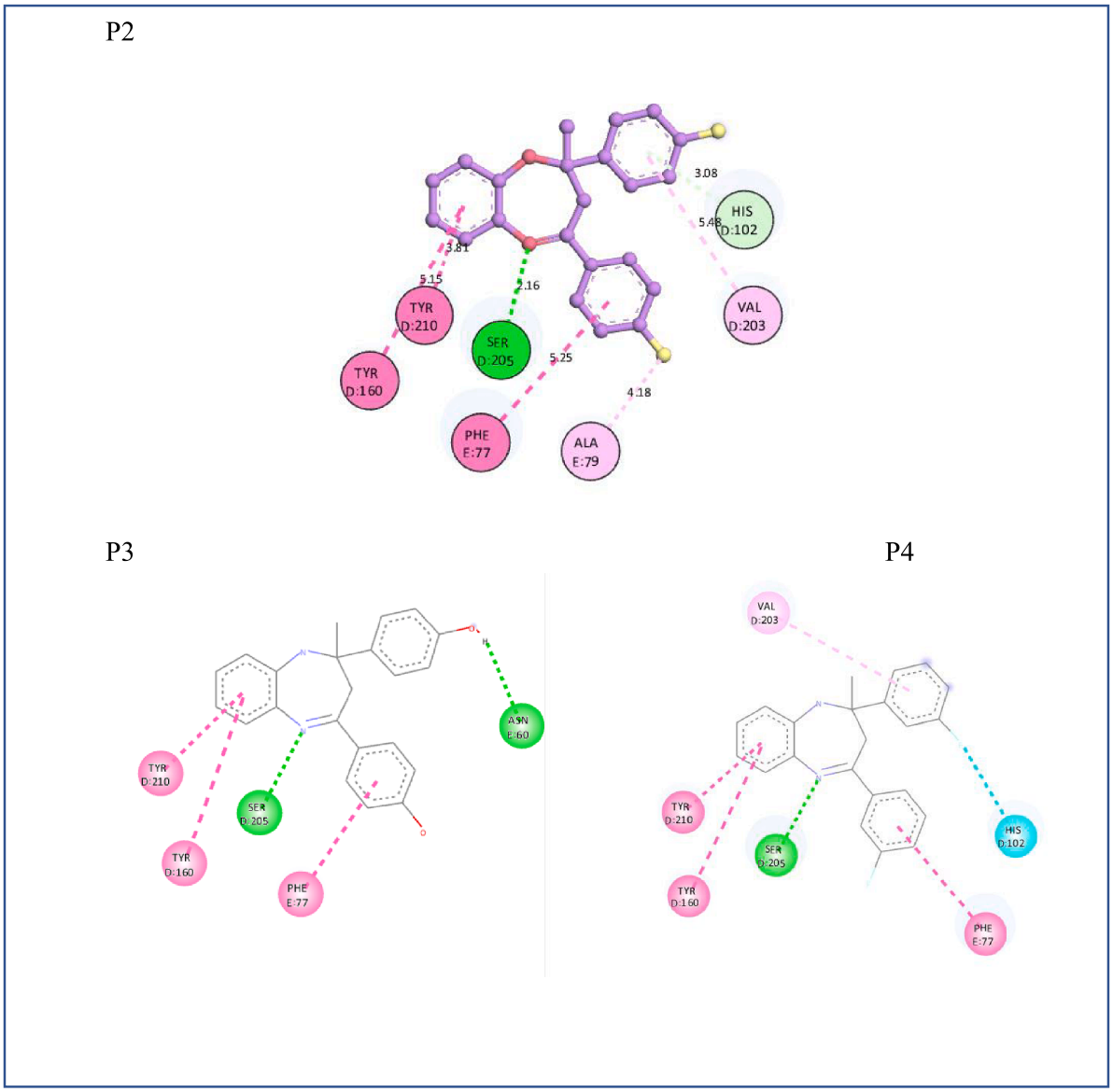

Fig. 5. (*continued*).

significantly lower mean MT of 2.04 min (SE = ± 0.15). Moreover, the standard group exhibited an even shorter mean MT of 1.10 min (SE = ± 0.08). The Tukey HSD test was conducted to compare the mean differences between different drug treatments in the Forced Swimming Test (MT) variable. The results indicate significant differences between certain pairs of drug treatments. The mean difference between CTR and P3 is 1.33 mins, with a standard error of 0.216, which was found to be statistically significant ($p = 0.001$). Similarly, the mean difference between STD P3 and P3 is 0.93 mins, indicating a significant difference ($p = 0.003$). Other significant differences include CTR vs. P3(mean difference = 2.26, $p < 0.001$).

Similar trends were observed for the control and experimental groups' drug trial study of P4. The control group demonstrated a mean MT of 3.37 min (SE =± 0.26 min). On the contrary, the experimental group had a significantly lower mean MT of 2.27 s (SE = ±0.36). The standard group in condition P4 had a mean MT of 1.74 (SE = ±0.12). The Tukey HSD test was conducted to compare the mean differences among various study groups in the Forced Swimming Test (MT) variable. The results indicate significant differences between certain pairs of drug treatments. The mean difference between CTR P4 and P4 is 1.10, with a standard error of 0.3791, which is statistically significant ($p =$ 0.034). Similarly, the mean difference between CTR and STD for drug trial P4 is 1.63, indicating a significant difference ($p = 0.003$). P4 and STD have a mean difference of 0.533 which is statistically non-significant. These results show that P4 has a comparable impact on the activity of mice as compared to the standard drug diazepam.

Analyzing the data from the Forced Swimming Test (IT) revealed notable differences among various conditions. The control group in condition P3 displayed a mean immobility time (IT) of 2.63 min (SE = ± 0.20), whereas the experimental group in the same condition exhibited a substantially higher mean IT of 3.96 (SE= ± 0.15). Additionally, the standard group demonstrated an even more prolonged mean IT of 4.90 s (SE = ± 0.08). In the Forced Swimming Test (IT) variable, the Tukey HSD test revealed significant differences between certain drug treatments. For instance, CTR P3 and P3 have a mean difference of −1.33 with a standard error of 0.216 ($p < 0.001$), indicating a significant distinction. Furthermore, there are substantial distinctions between STD and P3 (mean difference = - 0.93, $p = 0.003$), CTR and STD (mean difference = −2.26, $p <0.01$).

For P4, a similar trend was observed, with the control group having a mean IT of 2.63 min (SE = ±0.26) and the experimental group displaying a higher mean IT of 3.73 min (SE =± 0.36). The standard group

Table 1
ADME predictions of the synthesized compounds in comparison with the marketed drugs.

IDs	Physicochemical Properties							
	Heavy atoms	Aromatic Heavy atoms	Csp^3	Rot. bonds	HBA	HBD	MR	TPSA Å
P1	24	18	0.14	2	1	1	107.57	24.39
P2	26	18	0.14	2	1	1	117.59	24.39
P3	26	18	0.14	2	3	3	111.62	64.85
P4	26	18	0.14	2	3	1	107.49	24.39
DZP	20	12	0.12	1	2	0	87.95	32.67
MDZ	23	17	0.11	1	3	0	92.81	30.18
BRT	22	16	0.13	1	3	0	96.23	71.31
	Pharmacokinetics/Drug-Likeliness/Medicinal Alerts							
	Log $P_{o/w}$	GI-absorption	BBB penetration	P-gp Substrates	Inhibition	Non-inhibition	Lipinski; No of Violations	PAINS
P1	4.38	High	Yes	Yes	CYP1A2 CYP2C19 CYP2D6 CYP3A4	CYP2C9	Yes; O	0
P2	5.46	High	Yes	Yes	CYP1A2 CYP2C19 CYP2C9 CYP3A4	CYP2D6	Yes; 1 (MLogP>4.15)	0
P3	3.56	High	Yes	Yes	CYP1A2 CYP2C19 CYP2D6 CYP3A4	CYP2C9	Yes; 0	0
P4	5.01	High	Yes	Yes	CYP1A2 CYP2C19 CYP3A4	CYP2C9 CYP2D6	Yes; 1 (MLogP>4.15)	0
DZP	2.97	High	Yes	No	CYP1A2 CYP2C19 CYP2C9 CYP2D6 CYP3A4	–	Yes; 0	0
MDZ	3.61	High	Yes	Yes	CYP1A2 CYP2C19 CYP3A4	CYP2C9 CYP2D6	Yes; 0	0
BRT	3.66	High	Yes	Yes	CYP1A2 CYP2C19 CYP2C9	CYP2D6 CYP3A4	Yes; 0	0

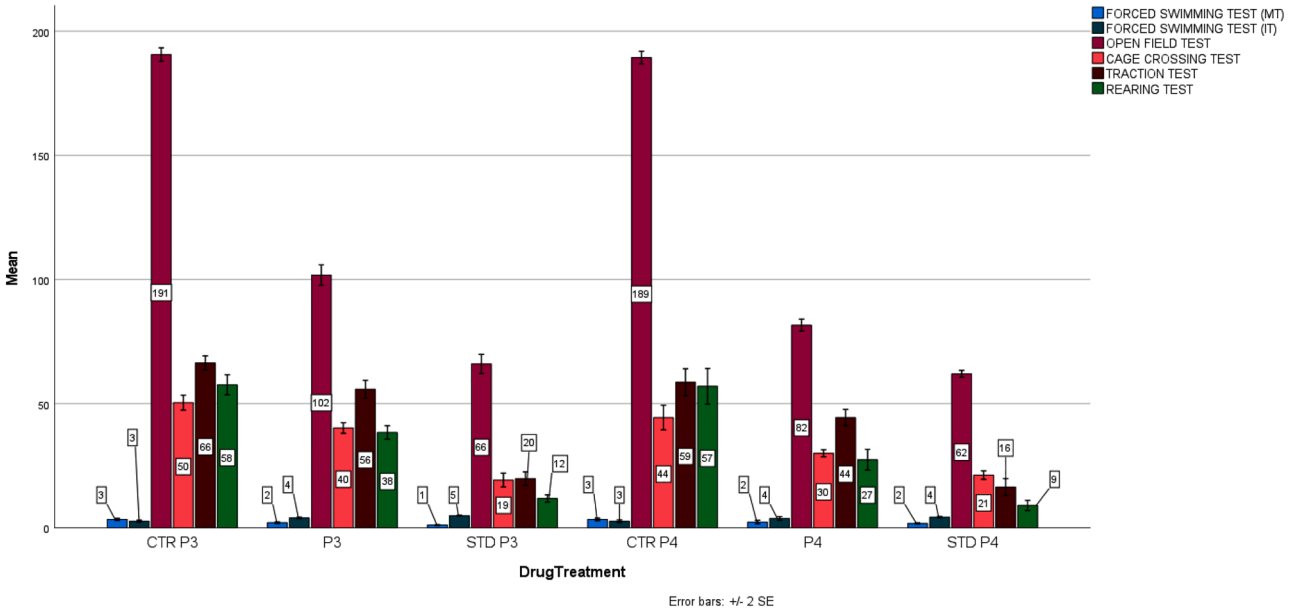

Fig. 6. The graph shows the treatment versus means ± Standard error of the mean at 95 % CI. FST is calculated as mobility timing in seconds, OFT is the number of squares mice crossed a square, CCT is the number of times mice crossed a cage, RT is the number of times a mouse stands independent of forelimbs, TT is time enhancement/reduction for traveling on the rod showing the stimulant or sedative activity.

in condition P4 exhibited a mean IT of 4.26 min (SE = 0.12). In the Forced Swimming Test (IT) variable, the Tukey HSD test revealed significant differences between certain drug treatments. For instance, CTR P4 and P4 have a mean difference of −1.10 ($p = 0.379$), indicating a significant distinction. Additionally, there are significant differences between CTR and STD (mean difference = −1.63, $p = 0.003$). On the contrary, STD P4 and P4 showed non-significant differences among the mean immobility time of mice (mean difference =−0.533, $p = 0.370$).

3.4.2. Open field test (OFT)

The open field test was evaluated by evaluating the number of times a mouse crossed the square in an open field. Examining the data from the Open Field Test revealed distinct performance patterns among the different conditions. For the test drug trial of P3, the experimental group had a lower mean score of 101.80 (SE = ±2.06) than the CTR group (mean score of 190.60, SE = ±1.36). Similarly, the standard group in trial testing of P3 exhibited a mean score of 66.00 (SE = ±1.92). In the Open Field Test variable, significant differences were observed between various drug treatments. Notably, there are significant differences between CTR P3 and P3 (mean difference = 88.800, SE=2.556, $p < 0.001$), CTR P3 and STD P3 (mean difference = 124.600, $p < 0.001$), P3 and STD P3 (mean difference = 35.800, $p < 0.001$.

Comparatively, the control group's mean score during the P4 trial testing was 189.40.(SE = ± 1.28), whereas the experimental group had

Table 2
Data indicating means ± standard error of the mean for the neuropharmacological activities.

	Drug Trial P3 (mean ± SE)			Drug Trial P4 (mean ± SE)		
	CTR P3	P3	STD P3	CTR P4	P4	STD P4
FST(MT)	3.37 ± 0.20	2.04 ± 0.15**, [a]	1.10 ± 0.01**, [a]	3.37 ± 0.26	2.27 ± 0.36*, [a]	1.74 ± 0.12*, [a]
FST (IT)	2.63 ± 0.20	3.96 ± 0.15**, [a]	4.90 ± 0.01**, [a]	2.63 ± 0.26	3.73 ± 0.36*, [a]	4.26 ± 0.12*, [a]
OFT	190.6 ± 1.36	101.8 ± 2.06**, [a]	66.0 ± 1.92**, [a]	189.4 ± 1.29	81.6 ± 1.21**, [a]	62.0 ± 0.71**, [a]
CCT	50.4 ± 1.50	40.2 ± 1.07**, [a]	19.2 ± 1.39**, [a]	44.4 ± 2.48	30.0 ± 0.71*, [b]	21.2 ± 0.86**, [b]
TT	66.40 ± 1.44	55.80 ± 1.80**, [a]	19.80 ± 1.36**, [a]	58.60 ± 2.73	44.40 ± 1.63**, [a]	16.40 ± 1.69**, [a]
RT	57.60 ± 2.01	38.40 ± 1.36**, [a]	11.80 ± 0.73**, [a]	57.00 ± 3.61	27.40 ± 2.09**,[b]	9.00 ± 1.00 **, [b]

[a] There was homoscedasticity in the data; Tukey's HSD as a post-HOC ANOVA was implied.
[b] There was heteroscedasticity in the data; Dunnet T3 as a post-HOC ANOVA test was used.
** $p \leq 0.001$.
* $p \leq 0.05$.

a lower mean score of 81.60 (SE = ± 1.21). The standard group in condition P4 had a mean score of 62.00 (SE =± 0.71). In the Open Field Test, significant differences were observed between various drug treatments in the trial study of P4. Notably, there are significant differences between CTR P4 and P4 (mean difference = 107.8, $p = 0.000$) with a standard error of 1.553, P4 and STD (mean difference = 19.6, $p = 0.000$), CTR and STD (mean difference = 127.400, $p = 0.000$). These results suggested that the three treatment groups were significantly different from each other.

3.4.3. Cage crossing test (CCT)

The number of times the mice crossed the cage gave the activity of the mouse as a behavioral pattern in the cage crossing test. Analysis of the data from the Cage Crossing Test revealed distinct behavioral patterns in different conditions. In the drug trial of P3, the CTR group displayed a mean score of 50.40 (SE = ±1.50). Contrarily, P3 administration displayed a slightly lower mean score of 40.20 (SE = ±1.07). Likewise, the standard group demonstrated a mean score of 19.20 (SE = ±1.39). The Cage Crossing Test also exhibited significant differences between drug treatments. Notable findings include significant differences between CTR P3 and P3 mean difference = 10.200, $p < 0.001$ with a standard error of 1.887, STD P3 and P3 (mean difference = 21.000, $p < 0.001$), CTR P3 and STD P3 (mean difference = 31.200, $p < 0.001$). In the trial test of P4, the control group had a mean score of 44.40 (SE = ±2.48), whereas the mice administered with P4 exhibited a lower mean score of 30.00 (SE = ±0.71). Additionally, the standard group in condition P4 demonstrated a mean score of 21.20 (SE = ±0.86). The cage crossing test exhibited significant differences between drug treatments when analyzed by using Dunnett T3. Notable findings include significant differences between CTR and P4 (mean difference = 14.4, $p = 0.008$, standard error =2.58), P4 and STD (mean difference = 8.80, $p = 0.000$, standard error = 1.11), CTR and STD (mean difference= 43.200, $p = 0.001$, standard error = 2.63).

3.4.4. Traction test (TT)

The traction test gave the number of seconds mice crossed the rod. Analyzing the data from the Traction Test revealed discernible performance variations across different conditions. In condition P3, a mean score of 66.40 s (SE = ±1.44) was displayed in the control group, while a lower mean score of 55.80 s (SE = ±1.80) was found in the P3 experimental group. The standard group in condition P3 exhibited a mean score of 19.80 s (SE = ±1.36). The Traction Test also exhibited significant differences between drug treatments. Notable findings include significant differences between CTR and P3 (mean difference = 10.6 s, $p = 0.001$), P3 and STD (mean difference = 36.00 s, $p = 0.000$), CTR and STD (mean difference= 46.60, $p = 0.000$).

Similarly, in the trial testing of drug P4, the control group exhibited a mean score of 58.60 s (SE = ± 2.73), whereas the experimental group had a lower mean score of 44.40 s (SE = ± 1.63). Additionally, the standard group in condition P4 displayed a mean score of 16.40 s (SE = ±1.69). The Post Hoc also exhibited significant differences between drug treatments. Notable findings include significant differences between CTR and P4 (mean difference = 14.2, $p = 0.001$), P4 and STD (mean difference = 28.00, $p = 0.000$), CTR and STD (mean difference= 42.20, $p = 0.000$).

3.4.5. Rearing test (RT)

Rearing activity was monitored on a mouse as the number of times the mouse stood independent of forelimbs. Evaluating the data from the Rearing Test revealed discernible patterns in the subjects' behavior across different conditions. The control group in condition P3 exhibited a mean score of 57.60 (SE = ±2.01), while the experimental group displayed a slightly lower mean score of 38.40 (SE = ±1.36). Additionally, the standard group in condition P3 demonstrated a mean score of 11.80 (SE = ±0.73). The rearing test exhibited significant differences between drug treatments when analyzed by using Tukey's HSD. Notable findings include significant differences between CTR and P3 (mean difference = 19.20, $p = 0.000$, standard error =2.075), P3 and STD (mean difference = 26.6, $p = 0.000$), CTR and STD (mean difference= 45.80, $p = 0.000$).

In the drug trial study for P4, the control group exhibited a mean score of 57.00 (SE = ±3.6), whereas the experimental group had a lower mean score of 27.40 (SE = ±2.09). Furthermore, the standard group in condition P4 demonstrated a mean score of 9.00 (SE = ±1).

The rearing test exhibited significant differences between drug treatments when analyzed by using Dunnett T3. Notable findings include significant differences between CTR and P4 (mean difference = 29.60, $p = 0.001$, standard error = 4.17), P4 and STD (mean difference = 18.4, $p = 0.001$, standard error = 2.32), CTR and STD (mean difference = 48.00, $p = 0.000$, standard error = 3.742).

3.5. Analgesic activities

The analgesic potential of drugs was determined by using the formalin-induced pain models (Fig. 7; Table 3). Formalin-induced analgesic activity is a biphasic response. The first phase consisted of 5 min while the second phase duration was from 15 to 30 min. The first phase is due to the nociceptor activation which is due to the release of tachykinins and bradykinins. The inhibition of first phase response is particular in opioid drugs. The inflammatory reaction is exhibited in the second phase. The release of inflammatory/allergic chemicals like prostaglandins, excitatory amino acids, serotonin, and histamine occurs in this period [32]. NSAIDs generally help reduce the inflammatory pain response. It was observed that both P3 and P4 along with diclofenac sodium (standard) showed anti-nociception response with a mean no of licking and biting of 29.4 ± 1.50, 23.6 ± 1.44, and 24.4 ± 2.38, respectively. However, in the late phase, there was a marked activity shown by P4 (30.2 ± 1.77) as compared to control (76.8±1.28) where the diclofenac sodium showed a reduced activity of 25.6 ± 2.69. It can

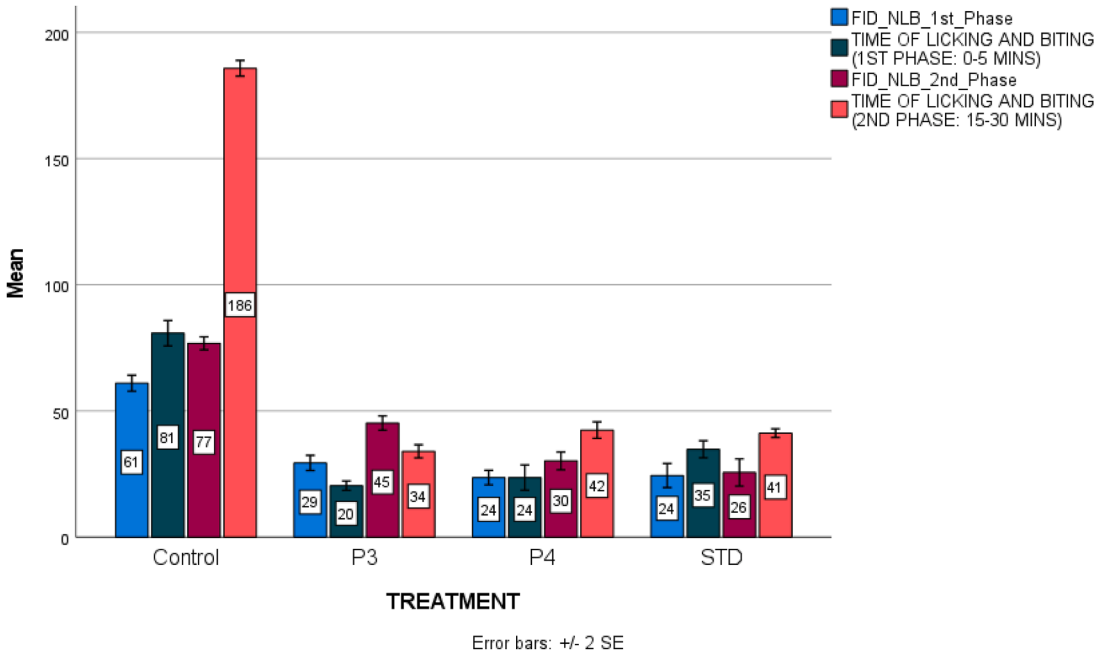

Fig. 7. Analgesic activities displaying anti-nociception (phase 1) and anti-inflammatory (phase 2) behavior of P3 and diclofenac sodium against control. The blue bars represent the number of licking and biting of phase 1 Formalin Induced Pain, the green bars represent the total time for licking and biting in phase 1, red bars represent the number of licking and biting of the forepaw in the second phase of formalin-induced pain response and the orange bar represents the total time spent for the response by mice during second phase.

Table 3
Data of formalin-induced inflammatory pain performed on mice divided into 4 groups (control, P3, P4, and standard-diclofenac sodium) of five mice each, expressed in the form of mean ± standard error of the mean. A biphasic response was observed and tabulated*. .

		CTR	P3	P4	STD
1st Phase (0–5 mins)	No of Licking and Biting	61.0 ± 1.58	29.4 ± 1.50**	23.6 ± 1.44**	24.4 ± 2.38**
	Time of Licking and Biting	80.8 ± 2.52	20.4 ± 0.93**	23.6 ± 2.50**	34.8 ± 1.68**
2nd Phase (15–30 mins)	No Licking and Biting	76.8 ± 1.28	45.2 ± 1.43**	30.2 ± 1.77**	25.6 ± 2.69**
	Time of Licking and Biting	185.8 ± 1.56	34.0 ± 1.30**	42.4 ± 1.63**	41.2 ± 0.86**

** $p \leq 0.001$.
* $p \leq 0.05$.

be inferred that P3 and P4 both showed anti-nociception as well as anti-inflammatory activity. On the other hand, P4 response in the first and second phases in terms of the number of licking and biting were highly significant for both phases suggesting both the drugs to be a potent broad-spectrum analgesic.

4. Discussion

4.1. Stability of products based on percentage yields

Comparison in the yield of the four synthesized compounds suggested that the hydroxy derivative (P3) was the most stable among all the synthesized compounds with a melting point of 254 °C. This may be due to the electron-donating effect of the hydroxy groups on the phenyl moieties [33,34].

Compounds were synthesized according to the method described in 2.2. All the synthesized compounds were evaluated by using spectroscopic analytical methods predominantly (IR and GCMS). The percentage yield of the compounds showed that **P3** was predominantly stable among all four derivatives, followed by **P4** while **P1** showed the lowest yields. This may be due to the resonance stability of the carbocation formed as an intermediate followed by imine, di-imine, and enamine functionalities (Fig. 8) [35].

4.2. In-silico investigations of test compounds

4.2.1. Docking investigations

The docking protocol was validated by getting the RMSD value of the redocked ligand from that of the co-crystallized (DZP404: 6×3X) by using an online dock RMSD calculator [36]. The RMSD value was 0.535 Å which was less than the permitted range (RMSD < 2 Å). From the *in-silico* screening, it was observed that the halogenated derivatives (**P2** and **P4)** of benzodiazepines give better scoring values than the non-derivatized phenyl rings (**P1)** and the hydroxy derivatized phenyl rings at position 4 (**P3**). According to the *in-silico* screenings, all of the docked ligands and co-crystallized ligands showed conventional hydrogen bonding characteristics with the SER205 amino acid, other common types of interactions included π-π stacking of ligands with TYR160, TYR210 (in the α-subunit presented as chain D) and with PHE77 (in the γ-subunit presented as chain E). Some unique interactions were also discovered in the docked structures of the synthesized compounds where P3 showed conventional hydrogen bonding with ASN60 (chain E), and P2 showed π-donor hydrogen bond interactions with HIS102 (chain D). P2 and P4 showed π-alkyl interactions with VAL203 (chain D) which was originally presented by co-crystallized ligand as alkyl interactions. The interactions and dock scores are presented in Fig. 9 and Table 4, respectively.

4.2.2. ADME properties

The compounds are analyzed for their drug potential by utilizing an online web tool. For a compound to be an effective CNS agent the TPSA value should not be greater than 90 $Å^2$ [37]. In the present study, TPSA shown by P1, P2, and P4 was 24.39 $Å^2$ while the P3 showed the TPSA value of 64.85 $Å^2$, this may be due to the presence of -OH group which

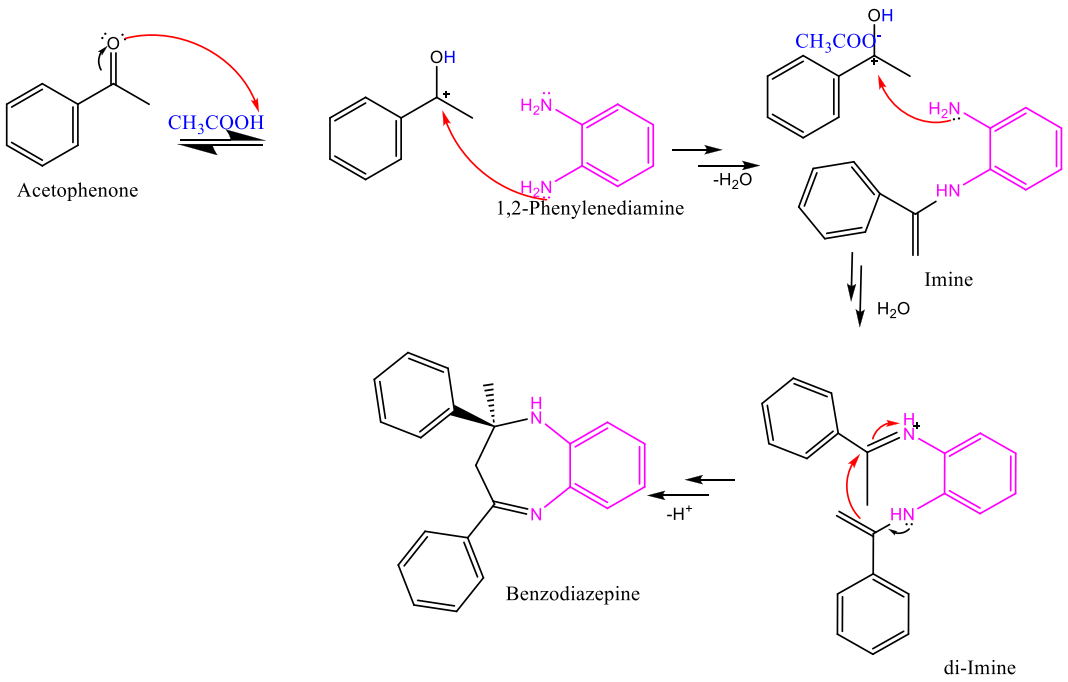

Fig. 8. General mechanism of the synthesis of benzodiazepines from acetophenone and *o*-PDA by using acetic acid as a catalyst.

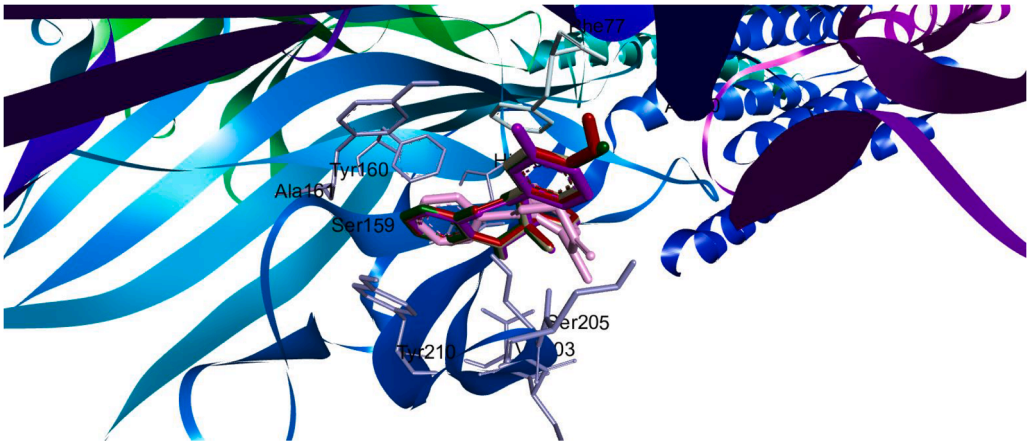

Fig. 9. Overlap of all docked compounds P1 (brown), P2 (green), P3 (red), P4 (light purple) and DZP (light pink).

Table 4
Table illustrating the dock RMSD of the redocked ligand validating the protocol of pocket identification for dockings and comparative Dock scores of synthesized compounds.

Compound Docked	Dock RMSD
DZP Redocked vs Co-crystallized DZP 404	0.535
Compound ID	**Autodock Vina Score**
DZP	−10.3
P1	−11.1
P2	−11.7
P3	−11.2
P4	−11.7

can form hydrogen bonds [38]. All of the synthesized compounds showed permeability across blood brain barrier as well as the absorption inside gastrointestinal tract was also high for those which illustrated the CNS activity of these. Surprisingly, these drugs were also P-gp substrates indicating the exsorption of the drugs back into the blood which may decrease their bioavailability, this type of pattern is also shown by midazolam and brotizolam). Most of the drugs are metabolized in the liver by two metabolic processes. Oxidative reactions comprise phase I of the metabolism where the liver CYP450 enzymes are involved, the others comprise phase II in the metabolic stage are conjugative reactions in which UDP-glucuronosyltransferases ~UGTs are involved. The inhibition of some of the CYP450 isomorphs was also shown by the four synthesized derivatives which may be useful in case of multiple drug resistant cancers, further study in this respect may be required. The compounds showed zero alerts in PAINS (PAN interference compounds) and Brenk assays, yielding no toxicity, chemical reactivity, metabolic unstability or poor pharmacokinetic effects. The drug likeliness of the synthesized moieties was confirmed by Lipinski's rule of five, in which the drug-like compounds must not violate two parameters out of five where the drugs must not have molecular weight greater than 500 Da, there should not be more than five hydrogen bond donors and 10 hydrogen bond acceptors, permissible range of molar refractivity is 40 −130, and MLogP not more than 4.15. P1 and P3 showed all the parameters within the range of Lipinski's five criteria while P2 and P4 showed one violation of the rule where MLogP > 4.15 [39].

Molecular docking results showed the compounds to have better binding affinity than the standard drug diazepam. In the ADME studies it was shown that the synthesized compounds are also P-gp substrates which may indicate their low therapeutic potential in comparison to the DZP [40].

4.3. *In-vivo neuropharmacological potentials*

From the data, it was observed that P4 exhibited better neuropharmacological activities (except for the forced swimming test) than P3. The ANOVA table for all the compounds and standard against control gave $p<0.01$ for all the tests except for P4 which gave $p<0.05$ for the forced swimming test, which was highly significant, providing the statistical measure that both drugs were potent as anxiolytics. The immobility timing is the reverse of the mobility timing and tells the measure of performing activities therefore the percentage activity is only analyzed for the mobility timings. To get the activity potential of drugs the percentage activity before and after treatment is recorded in Table 5. It is seen from all the neuropharmacological activities that the compound containing halogen is potent as compared to non-halogenated compound, this result is in accordance with the neuropharmacological studies performed by a group of researchers in 2014 [41]. The synthesized compounds were half as potent as compared to diazepam, which is also reported for 1,5-benzodiazepines in a study conducted in 1978 by Gerhard [42]. From the results, it can be inferred that the diazepam induces greater sedation as compared to the 1,5-benzodiazepines. In the past trials have been made between diazepam and a commonly known 1, 5-benzodiazepine (clobazam), which depicted similar patterns of sedation [43,44]. Moreover, diazepam is considered to be more neurotoxic as compared to the 1,5-benzodiazepine (clobazam) which can give further insight into the current study that the synthesized compounds are safer than diazepam.

4.4. *In-vivo analgesic potentials*

The analgesic potential mimicked the same trend as that of the neuropharmacological potentials where P4 showed better response as compared to P3 (Table 6). Moreover, the ANOVA table suggested that the anti-inflammatory response of P4 was not significantly different from the standard which means that P4 is as potent as that of the standard.

5. Conclusions

As the literature shows, many studies have been done to synthesize benzodiazepines in the laboratory. In the present research work, four benzodiazepines (P1, P2, P3, and P4) were synthesized by using glacial acetic acid (GAA) as a catalyst. The structures of the compounds were determined based on spectroscopic data. The maximum yield was reported for the compound P3 which was found to be 84.67 %. The *in-silico* studies proposed that these compounds can give comparable binding affinities as anxiolytic as compared to the standard diazepam. ADME analysis of the synthesized compounds gave more insight into the bioavailability of the drug. The lesser binding affinity of diazepam with the GABA suggested that the DZP is less active than the synthesized compounds which may be augmented by the fact that diazepam is a poor substrate of P-gp while all the synthesized compounds were good substrates. Among the two compounds selected for *in-vivo* evaluations of neuropharmacological and analgesic activities, it was found that P4 proved to be a more potent anxiolytic as well as anti-inflammatory drug than P3.

Table 5
Neuropharmacological activities of P3 and P4 as compared to standard diazepam.

FORCED SWIMMING TEST				
Sr. No	**Treatment**	**Without Treatment**	**With Treatment**	**Percentage**
1	P3	3.37	2.04	39.46
2	P4	3.37	2.27	32.64
3	Diazepam	3.37	1.74	48.37
OPEN FIELD LOCOMOTOR ACTIVITY				
Sr. No	**Treatment**	**Without Treatment**	**After Treatment**	**Percentage**
1	P3	190.6	101.8	46.59
2	P4	189.4	81.6	56.92
3	Diazepam	189.4	62	67.27
CAGE CROSSING TEST				
Sr. No	**Treatment**	**Without Treatment**	**After Treatment**	**Percentage**
1	P3	50.4	40.2	20.24
2	P4	44.4	30	32.43
3	Diazepam	44.4	21.2	52.25
TRACTION TEST				
Sr. No	**Treatment**	**Without Treatment**	**After Treatment**	**Percentage**
1	P3	66.4	55.8	15.96
2	P4	58.6	44.4	24.23
3	Diazepam	58.6	16.4	72.01
REARING TEST				
Sr. No	**Treatment**	**Without Treatment**	**After Treatment**	**Percentage**
1	P3	57.6	38.4	33.33
2	P4	57	27.4	51.92
3	Diazepam	57	9	84.21

Table 6
Analgesic activities of P3 and P4 as compared to standard diclofenac sodium.

1st Phase (No of Licking and Biting)				
Sr. No	**Treatment**	**Without Treatment**	**With Treatment**	**Percentage**
1	P3	61	29.4	51.8
2	P4	61	23.6	61.31
3	Diclofenac Sodium	61	24.4	64.92
2nd Phase (No of Licking and Biting)				
Sr. No	**Treatment**	**Without Treatment**	**After Treatment**	**Percentage**
1	P3	76.8	45.2	41.14
2	P4	76.8	30.2	60.68
3	Diazepam	76.8	25.6	66.67

CRediT authorship contribution statement

Masooma Hyder Khan: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Dildar Ahmed:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Taufiq Ahmad:** Methodology, Investigation, Formal analysis, Data curation. **Haroon Iftikhar:** Validation, Software, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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