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Identification of inhibitory potential of acamprosate, roxindole and L-ascorbic acid against tryptophan 2, 3 dioxygenase using experimental and computational approaches

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Abstract: **Background**: Tryptophan 2,3-dioxygenase (TDO) is a haem-containing enzyme of the kynurenine pathway, which is imperative for tryptophan metabolism. Primarily found in the liver, TDO facilitates the breakdown of TRP into N-formyl kynurenine. TDO is regarded as a promising target for antidepressant therapies. As an enzyme responsible for tryptophan degradation, its inhibition may enhance serotonin availability, which plays a key role in mood regulation. **Objectives**: This study identifies TDO as a potential target for central nervous system drugs (acamprosate, roxindole, and L-ascorbic acid) that may alter brain activity and potentially impact mood and behavior. **Methods**: The study involved male Albino Wistar rats, each weighing between 150 and 200 grams. The rats were decapitated, the livers were promptly excised within 10 seconds, and perfused *in situ* with ice-cold saline. The perfused livers were then immediately frozen at -80 °C for subsequent analysis. *In vitro* TDO enzyme activity was assessed in frozen liver homogenates. Enzymatic activity was measured for both the holoenzyme and total enzyme spectrophotometrically. Molecular docking of the selected compounds with TDO was conducted using AutoDock Vina. The crystal structure of TDO was retrieved from the Protein Data Bank (PDB), while ligand structures were obtained from PubChem. **Results**: *In vitro* experiments revealed that these drugs inhibited apoenzyme activity by 68-85%, while total enzyme activity was reduced by 34%, 38% and 37% for acamprosate, roxindole, and L-ascorbic acid, respectively. Further validation through molecular docking analysis confirmed their strong binding affinity to the TDO active site, with l-ascorbic acid showing the highest binding energy (-7.2 kcal/mol), followed by acamprosate (-6.7 kcal/mol) and roxindole (-6.4 kcal/mol). **Conclusion**: These findings suggest that acamprosate, roxindole, and L-ascorbic acid act as competitive TDO inhibitors, potentially enhancing serotonin synthesis and mitigating depressive symptoms.

Keywords: Acamprosate; Tryptophan 2, 3 Dioxygenase (TDO); Inhibition; Roxindole; L-ascorbic acid

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INTRODUCTION

Tryptophan 2, 3-dioxygenase (TDO) is a haem-containing enzyme of the kynurenine pathway (KP), which is imperative for tryptophan (TRP) metabolism. The kynurenine pathway is the major route for approximately 95-99% of tryptophan degradation in the body. The residual amount is directed toward protein synthesis or converted into serotonin and melatonin (Badawy, 2022). Variations in plasma tryptophan levels, driven by TDO activity, can affect the amount of tryptophan that reaches the brain for serotonin synthesis. Activation of TDO in the liver can contribute to serotonin depletion by accelerating the breakdown of tryptophan, the amino acid required for serotonin production. This enzymatic activity is stimulated by glucocorticoids and catecholamine, which lower the amount of tryptophan available for serotonin synthesis in the brain. Increased TDO levels have been reported in individuals suffering from major depressive disorder (Liang *et al.,* 2024). This enzyme is reported to be involved either directly or indirectly in mental disorders, cancer and inflammatory disorders. It is an important factor affecting serotonin levels and significantly influences psychiatric conditions (Ye *et al.,* 2019). Liver TDO is known to control the supply of TRP for brain serotonin production. Recent studies have shown that mice lacking the TDO2 gene exhibited markedly elevated plasma tryptophan (TRP) levels, approximately 9.3 to 12.7 times higher than normal wild-type mice (Kanai *et al*., 2009; Too *et al*., 2016), which correlates with increased serotonin synthesis. Maintaining an equilibrium between the serotonin and kynurenine pathways is especially important in depression. This balance influences mood regulation and mental health outcomes. TDO enzymes are known to be activated during depression, underscoring the potential use of TDO as a therapeutic drug for treating depressive illness. It is essential to note that kynurenic acid functions as a neuroprotective compound, whereas qinolinic acid is neurotoxic. In depression, the equilibrium between these metabolites is disrupted. (Correria and Vale, 2022). Higher cortisol levels are associated with lower plasma TRP levels. Cortisol activates TDO, which in turn increases the production of kynurenine (kyn) (Oxenkrug, 2010). Chronic stress increases the conversion of TRP into kynurenine. This is primarily driven by higher levels of cortisol, which activate the TDO enzyme involved in this process (La Torre *et al.,* 2021). Moclobemide, sertraline (Bano *et al*., 2010) and citalopram (Ara and Bano, 2012) contribute to

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enhanced brain serotonin synthesis by elevating brain and plasma TRP concentrations, primarily through the inhibition of TDO activity. (Salter *et al.,* 1995). Venlafaxine and tianeptine have also been reported to decrease TDO activity and dock strongly to TDO (Dawood *et al.,* 2022). TDO has also been shown to play a role in modulating the immune system to promote resistance to tumors and enhance their proliferation (Pantouris *et al.,* 2016). There is evidence indicating that cancer cells that overexpress TDO and activate the aryl hydrocarbon receptor may evade detection by the immune system (Cheong and Sun, 2018). Several antidepressants have been shown to share the property of inhibiting TDO enzyme activity both *in vivo* and *in vitro* (Badawy, 2013). Antidepressants have been shown to effectively suppress TDO activity at doses as low as 0.5 mg/kg of body weight. Additionally, molecular docking, a computational approach, is a valuable tool for identifying possible inhibitors targeting specific proteins (Ferreira *et al.,* 2015). It can also be confirmed by the binding interactions of previously identified inhibitors. TDO is regarded as a promising target for antidepressant therapies. As an enzyme responsible for tryptophan degradation, its inhibition may enhance serotonin availability, which plays a key role in mood regulation. This study identifies TDO as a potential target for central nervous system drugs that may alter brain activity and potentially impact mood and behavior. In this study, we explored two distinct classes of drugs: roxindole, a serotonin reuptake inhibitor and 5HT1A agonist commonly used to treat alcohol abstinence, and acamprosate, a structural analogue of the neurotransmitter γ-aminobutyric acid (GABA), along with l-ascorbic acid, known for its neuroprotective and mood-enhancing properties. Our objective was to evaluate their potential antidepressant effects by inhibiting TDO enzyme activity. Furthermore, we confirmed their binding affinity to the receptor protein (TDO) using both *in vitro* and *in silico* methodologies.

MATERIALS AND METHODS

Acamprosate, L-ascorbic acid, L-tryptophan (≥98%), and Hematin hydrochloride were sourced from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.). Roxindole was purchased from Merck. Other reagents and chemicals were procured from BDH Chemicals Ltd. (Poole, Dorset, United Kingdom).

Animals and treatment

The experiment involved male albino Wistar rats, each weighing between 150 and 200 grams. The animals were kept under a natural light-dark cycle in a temperature-controlled room set at 25 ± 2.0°C, with unrestricted access to food and water. Between 1:00 PM and 2:00 PM, the rats were decapitated, the livers were promptly excised within 10 seconds and perfused *in situ* with ice-cold saline. The perfused livers were then immediately frozen at -80 °C for subsequent analysis.

Drug preparation

For *in-vitro* studies, L-ascorbic acid and acamprosate (calcium salt) were dissolved in deionized water, while roxindole was dissolved in DMSO and the pH of all solutions was adjusted to 7.0.

In-vitro TDO activity

TDO activity was assessed in frozen liver homogenates prepared using an Ultra-Turrax homogenizer. Enzymatic activity was measured for both the holoenzyme and total enzyme by supplementing with 2.0 µM haematin dissolved in 0.1 M NaOH (Badawy and Evans, 1976; Dawood *et al*., 2022).

Statistical analysis

Statistical comparisons between the two groups were performed using Student's t-test. A p-value < 0.05 was regarded as indicative of statistical significance.

In-silico analysis

Molecular docking of the selected compounds with TDO was conducted using AutoDock Vina (Trott and Olson, 2010). The crystal structure of TDO was retrieved from the Protein Data Bank (PDB), while ligand structures were obtained from PubChem. Ligand and receptor files, originally in SDF format, were converted to PDB format utilizing Marvin Sketch. Subsequently, AutoDock tools were used to generate PDBQT files for both the receptor and the ligands. Molecular docking was performed using the command prompt with a configuration file. The dimensions of grid box used were as follows: center_x = 40.269, center_y = -63.103, center_z = -30.244, size_x = 30, size_y = 30, size_z = 30. The results of molecular docking were analyzed using DS Visualizer.

RESULTS

TDO inhibition by Acamprosate, Roxindole and L-Ascorbic acid

In this study, the inhibitory effects of acamprosate, roxindole, and L-ascorbic acid on TDO enzyme activity were evaluated. TDO activity was assessed at drug concentrations of 10 µM, 0.1 mM, 0.5 mM and 1 mM. Roxindole significantly inhibited total enzyme activity at 0.5 mM and 1 mM by up to 38% ($p < 0.05$). Apoenzyme activity was reduced by 35% at 0.1 mM ($p < 0.05$) and by 38% at both 0.5 mM and 1 mM ($p < 0.01$). Acamprosate had no significant effect on holoenzyme or total enzyme activity. However, apoenzyme activity was inhibited at 10 µM by 47% ($p < 0.05$), at 0.1 mM and 0.5 mM by 59% ($p < 0.01$ and $p < 0.05$, respectively) and at 1 mM by 76% ($p < 0.01$). L-ascorbic acid did not significantly affect holoenzyme activity. Nevertheless, total and apoenzyme activities were inhibited at 10 µM, 0.1 mM and 0.5 mM by approximately 28–31% ($p < 0.05$) and at 1 mM by 68% ($p < 0.01$). Apoenzyme activity alone was significantly reduced at all concentrations—56% inhibition at 10 µM, 0.1 mM and 0.5 mM ($p < 0.001$ and $p < 0.05$) and 68% at 1 mM ($p < 0.05$). (Table 1&2)

Docking of TRP to TDO

In this study, TRP (reference ligand) was docked into the active site of the human TDO enzyme to confirm the validity of the molecular docking procedure. The findings, presented in tables 2 and 3, indicate that the TRP demonstrated the strongest binding affinity, with a binding energy of -9.0 kcal/mol, which is consistent with values reported in the literature (Fig. 1). The root mean square deviation (RMSD) between the native tryptophan ligand and the docked tryptophan ligand was also calculated and was found to be 1 A, which shows the reproducibility of the whole docking protocol. Key active site residues His76, Thr342, and Arg144 formed hydrogen bonds with TRP, while Hem401 (chain A) contributed to complex stability. The interaction profile also revealed hydrophobic contacts, including van der Waals and π-alkyl interactions. Residues such as Phe140, Leu336, Gly341, Gly343, Ser151, Gly152, Ser148, Tyr42 (B), and Tyr45 (B) engaged in van der Waals forces, while His76 and Phe72 formed π–π T-shaped interactions with TRP's aromatic ring. Additionally, Leu147 established a π-sigma bond. In total, sixteen active site residues were involved in ligand binding. A detailed interaction map is presented in Table 4.

Docking of L-ascorbic acid to TDO

Among the evaluated compounds, L-ascorbic acid showed the highest docking score, around -7.2 kcal/mol (Table 3). The active site residues of TDO involved in this interaction were carefully examined, as illustrated in fig. 2. Structurally, L-ascorbic acid has a six-carbon backbone with both an enol and a ketone group. The binding of the ligand to TDO was characterized by the formation of 4-H bonds with the main catalytic residues, His76, Arg144, and Thr342, as well as the heme group. The interactions were further reinforced through van der Waals forces contributed by additional active site residues (chain B). L-ascorbic acid formed a stable complex with twelve active site residues-nine from chain A, including the heme group and three from chain B. Significantly, the HEM moiety engaged in π–π stacking and π–alkyl interactions with the ligand. The binding mode of L-ascorbic acid in the TDO active site closely resembled that of TRP. Overall, sixteen amino acid residues participated in the interaction, highlighting L-ascorbic acid's potential as a promising TDO inhibitor.

Docking of Acamprosate to TDO

Acamprosate (3-acetamidopropane-1-sulfonic acid) exhibited a binding energy of approximately -6.7 kcal/mol, (Table 3) as determined by molecular docking analysis (Fig. 3). The compound exhibited strong affinity for the active site of the TDO enzyme, forming critical conventional hydrogen bonds within chain A. The catalytically crucial residue Arg144 formed a strong hydrogen bond with the -NH_2 group of acamprosate. Additionally, Thr342 contributed two hydrogen bonds: one with the oxygen atom of the acetyl group and another with a hydrogen atom of the -NH_2 group. The heme moiety further reinforced binding by forming a hydrogen bond with the amino group's hydrogen atom.

Interactions extended beyond hydrogen bonding: Tyr42 (B) formed a carbon-hydrogen bond, while Phe72 established a π-sulfur interaction with the sulfonic acid sulfur atom of the compound. Further van der Waals interactions were observed with multiple residues, including Ser151, Gly152, Leu147, Ala150, Phe140, Leu336, Gly341, Gly343, Leu40, Tyr45, and Leu46 of chain B. Collectively, these findings indicate that acamprosate can act as a potential inhibitor of the TDO enzyme, supported by its stable and multifaceted binding at the active site.

Docking Roxindole to TDO

Roxindole (5-hydroxy-3-[4-(4-phenyl-1, 2, 3, 6-tetrahydropyridyl (1) butyl (1) indole, mesylate) exhibited the lowest binding energy, approximately -6.4 kcal/mol, among the tested compounds. The 2D and 3D representations of its intermolecular interactions are illustrated in fig. 4. The ligand formed hydrogen bonds with Pro149 and Thr342; however, its interaction pattern differed significantly from the reference ligand, suggesting that Roxindole may not serve as a potent TDO inhibitor.

DISCUSSION

Emerging evidence highlights TDO as a therapeutic target for various brain disorders, and it has shown potential in cancer treatment and transplant medicine (Yu *et al*., 2016). Given its critical role in TRP degradation, TDO is also implicated in depression (Correia, 2022). This study investigated the effects of three compounds, acamprosate, roxindole, and L-ascorbic acid, on TDO activity. Among these, L-ascorbic acid demonstrated the most pronounced effects. Inhibitory activity was observed against both the apo form and the total enzyme, while the holo enzyme showed negligible inhibition. The selective inhibition of the apo and total forms is likely due to interference of these compounds with the binding of heme, the enzyme's essential cofactor (Badawy, 2013).

Experimental findings and molecular docking analyses confirmed that these compounds act as competitive inhibitors of TDO. Using AutoDock Vina, the compounds were docked into TDO's active site and binding energies were assessed. A more negative docking score indicates stronger binding affinity. Among the tested molecules, L-ascorbic acid had the strongest binding (-7.2 kcal/mol), acamprosate followed closely (-6.7 kcal/mol) and roxindole showed the weakest binding (-6.4 kcal/mol). L-ascorbic acid and acamprosate closely mimicked the interaction profile of the reference compound TRP, unlike roxindole. While roxindole's docking results were less impressive, it exhibited notable inhibition experimentally,

Table 1: *In-vitro* inhibitory activity of drugs

Drug	Concentration	Kynurenine formed (µmol/g wt/liver/h)		
		Holo enzyme	Total enzyme	Apo enzyme
Roxindole	0 µM	1.3 ± 0.12	2.6 ± 0.32	1.3 ± 0.20
	10 µM	1.4 ± 0.09	2.1 ± 0.20	0.7 ± 0.12
	0.1 mM	1.2 ± 0.03	1.7 ± 0.21	0.5 ± 0.18*
	0.5 mM	1.2 ± 0.11	1.6 ± 0.10*	0.3 ± 0.06**
	1 mM	1.4 ± 0.14	1.6 ± 0.13*	0.2 ± 0.10**
Acamprosate	0 µM	1.9 ± 0.23	3.5 ± 0.35	1.7 ± 0.22
	10 µM	1.9 ± 0.26	2.8 ± 0.35	0.9 ± 0.19*
	0.1 mM	1.9 ± 0.20	2.6 ± 0.29	0.7 ± 0.14**
	0.5 mM	1.9 ± 0.20	2.5 ± 0.39	0.7 ± 0.21*
	1 mM	1.9 ± 0.28	2.3 ± 0.27	0.4 ± 0.12**
L-ascorbic acid	0 µM	1.9 ± 0.05	3.5 ± 0.3	1.6 ± 0.20
	10 µM	1.7 ± 0.06	2.5 ± 0.26*	0.7 ± 0.17***
	0.1 mM	1.7 ± 0.17	2.4 ± 0.23*	0.7 ± 0.17***
	0.5 mM	1.8 ± 0.21	2.5 ± 0.25*	0.7 ± 0.18*
	1 mM	1.7 ± 0.17	2.2 ± 0.24**	0.5 ± 0.15***

TDO activity was assessed both without (holoenzyme activity) and with the addition of 2 µM hematin (total enzyme activity). Apoenzyme activity was calculated by subtracting holoenzyme from total activity. Data are presented as means ± SEM from a minimum of three independent experiments per drug. Statistical significance is denoted as follows: *P < 0.05; **P < 0.01; ***P < 0.001.

Table 2: Inhibition of the apo enzyme and total enzyme.

	% Inhibition of the apo enzyme				% Inhibition of total enzyme			
	10 µM	0.1 mM	0.5 mM	1 mM	10 µM	0.1 mM	0.5 mM	1 mM
Roxindole	46	61	77	85	19	35	38	38
Acamprosate	47	59	59	76	20	25	25	34
L-ascorbic acid	56	56	56	68	28	31	28	37

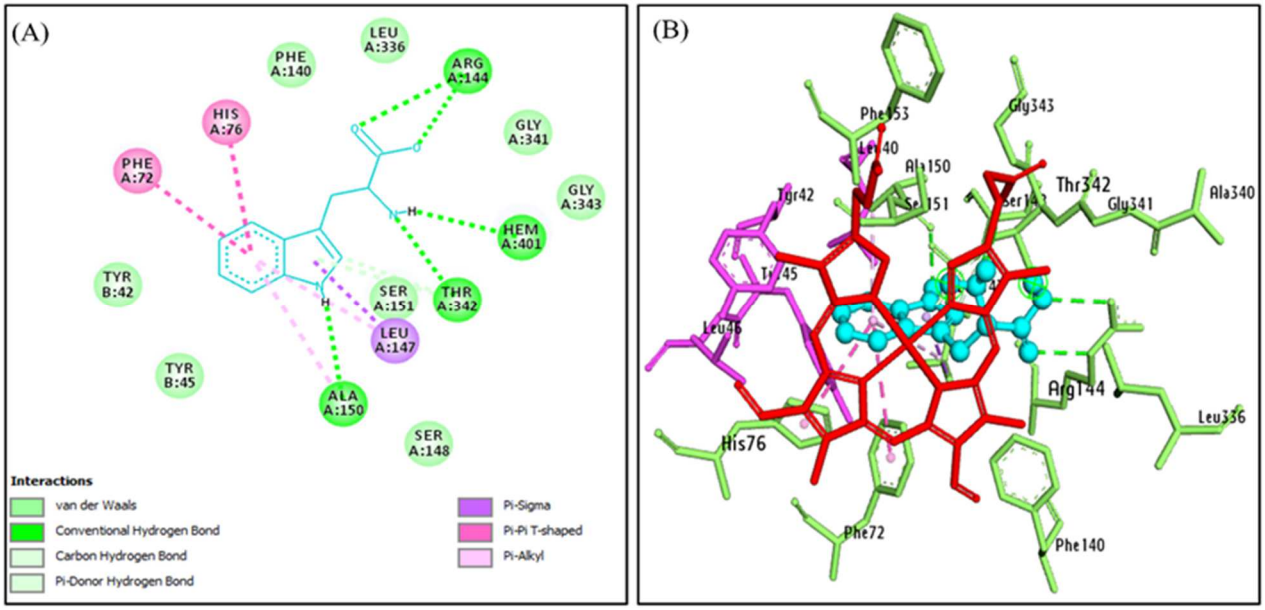

Fig. 1: Schematic representation of intermolecular interactions between tryptophan 2, 3 dioxygenase (TDO) and tryptophan.
(A) Two-dimensional representation generated by DS Visualizer depicting tryptophan interactions with active site residues of chain A and B. Different types of interactions are shown in different colors. (B) Three-dimensional mode showing tryptophan (cyan) in ball and stick representation interacting with heme (red) and active site residues of chain A (green) and B (pink) shown in stick representation.

Table 3: Drug structure, molecular formulae and docking scores of the drugs

S. No.	Drug	Structure	Molecular formula	Docking score (Kcal/mol)
1	TRP		$C_{11}H_{12}N_2O_2$	-9.0
2	L-ascorbic acid		$C_6H_8O_6$	-7.2
3	Acamprosate		$C_5H_{11}NO_4S$	-6.7
4	Roxindole		$C_{23}H_{26}N_2O$	-6.4

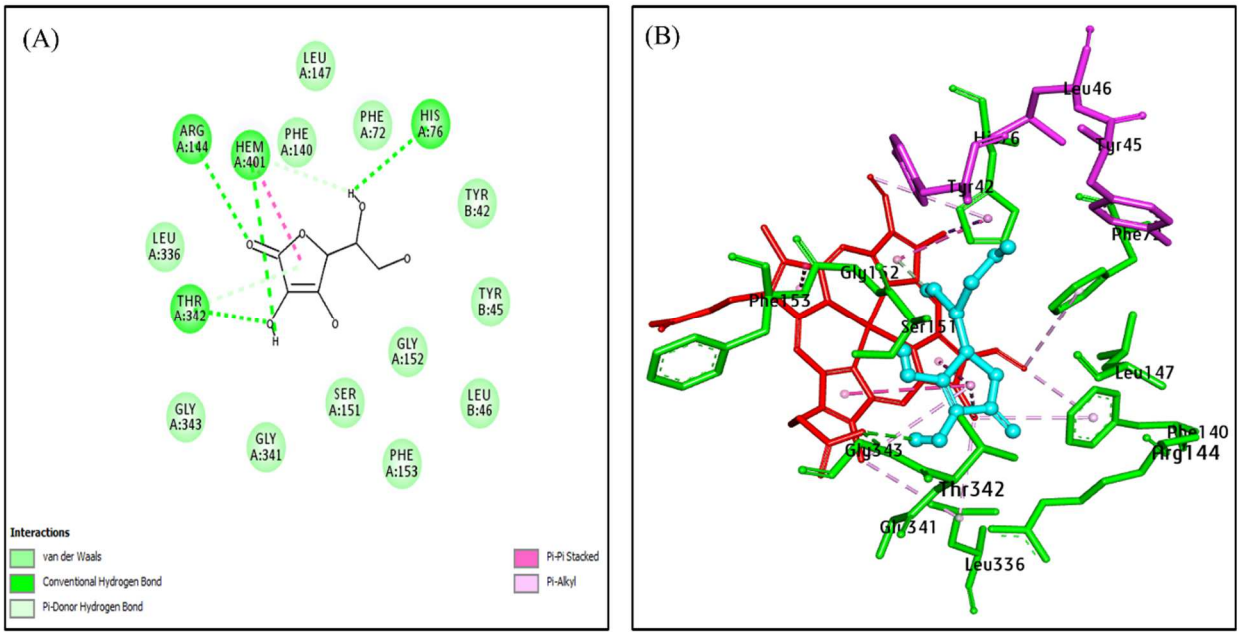

Fig. 2: Schematic representation of intermolecular interactions between TDO and L-ascorbic acid (A) two-dimensional and (B) three-dimensional modes.

Table 4: Nature of molecular interactions observed between the docked drugs and the amino acid residues at the TDO active.

S. No.	Drugs	Hydrogen bond	Vander Waal's forces	π donor hydrogen bond	π-π T-shaped	π-Alkyl	π-sulfur
1	TRP	ALA150 THR342 ARG144 HEM401	PHE140 GLY152 GLY343 GLY341 LEU336 SER151 SER148 B chain: TYR42 TYR45	--	A chain: PHE72 HIS76	A chain: LEU147 ALA150	--
2	Ascorbic Acid	HIS76 THR342 ARG144 HEM401	GLY152 PHE140 SER151 LEU147 PHE72 PHE153 GLY341 GLY343 LEU336 B chain: TYR45 LEU46	HEM401 ARG144	--	--	--
3	Acamprote	HEM401 THR342 ARG144	TYR42 GLY152 PHE140 ALA150 LEU147 GLY343 GLY341 LEU336 SER151 B chain: LEU40 LEU46	--	--	--	PHE72
4	Roxindole	PRO149	TYR45 SER148 SER151 GLY341 LYS339 GLY344 ARG144 B chain: ILE41			ALA340 LYS339 PRO149	

possibly due to π-alkyl interactions with residues Lys339 and Arg340, which may stabilize its binding within TDO's active site. Since π-alkyl interactions are vital for the stable binding of the ligand within the hydrophobic pocket of the protein. Some other factors, such as favorable pharmacokinetics, membrane permeability, may also be involved. L-ascorbic acid binds strongly (-7.2 kcal/mol) but likely does not block the active site or disrupt catalysis effectively, leading to weak inhibition (37%) at 1mM. In contrast, acamprosate, though binding more weakly, interferes functionally with enzyme activity, showing stronger inhibition (76%).

Earlier, Badawy and Evans (1981 & 1982), Badawy and Morgan (1991), Badawy *et al*. (1989; 1991) and Bano *et al*. (2010) also reported that antidepressants have TDO-inhibitory properties. Interestingly, tianeptine, despite its distinctive mechanism of enhancing serotonin uptake

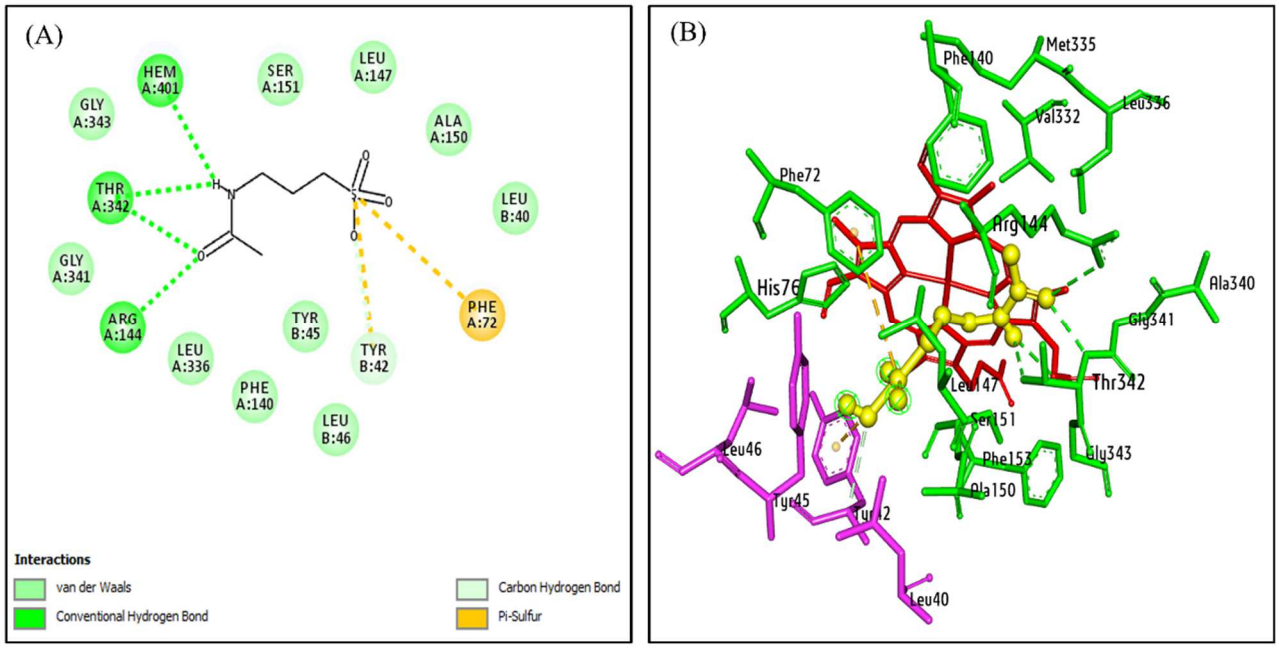

Fig. 3: Schematic representation of intermolecular interactions between TDO and acamprosate (A) two-dimensional and (B) three-dimensional modes.

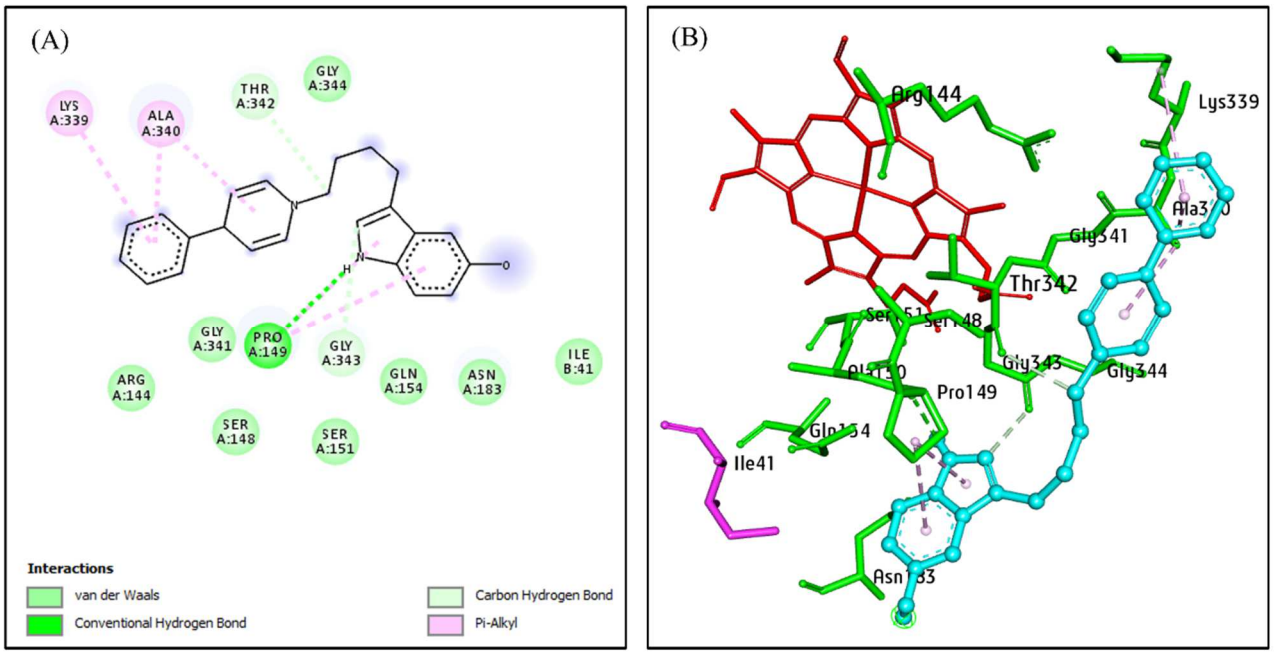

Fig. 4: Schematic representation of intermolecular interactions between TDO and roxindole (A) two-dimensional and (B) three-dimensional modes.

(Mennini *et al*., 1987), has also been recognized as a potent inhibitor of TDO (Bano *et al*., 2010).

While roxindole is categorized as an antidepressant, acamprosate has likewise demonstrated relevant pharmacological effects (Pałucha-Poniewiera and Pilc, 2012). L-ascorbic acid (Moretti *et al*., 2017) has shown antidepressant effects as well. These agents might enhance the effectiveness and onset time of traditional antidepressants. TDO influences serotonin biosynthesis by regulating the availability of free TRP in circulation. According to Badawy, TRP concentration rises as TDO activity decreases. Earlier, Badawy and Evans (1981) and later confirmed by Badawy and Morgan (1991) that TDO

inhibition increases brain TRP uptake and boosts serotonin (5-HT) synthesis. Unlike direct TRP supplementation, which may not significantly enhance serotonin levels, pharmacological inhibition of TDO presents a more effective approach (Modoux, 2021). This suggests that combination therapies targeting both TDO activity and serotonin pathways could represent a promising future direction for treating depression.

CONCLUSION

The study highlights that TDO, a key enzyme in the KP, is associated with depression and is significantly inhibited by acamprosate, roxindole and L-ascorbic acid. Increased TDO activity decreases serotonin levels by shifting tryptophan metabolism toward kynurenine compounds, so its inhibition could help restore serotonin balance. Acamprosate showed the strongest inhibition (up to 76%) of the enzyme's apo form, followed by L-ascorbic acid and roxindole. Molecular docking studies supported these findings, showing strong binding of these compounds to TDO's active site, particularly L-ascorbic acid. The results suggest these agents could serve as inhibitors of TDO, potentially boosting serotonin availability and alleviating depressive symptoms. The study opens doors to repurposing these drugs as adjunct treatments for depression.

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Authors' contributions
FJ: Statistical analysis, data collection, first draft
AQ: Manuscript editing, results interpretation
MH: Molecular docking simulation, data curation
YR: Docking analysis, results interpretation
SB: Concept design, reviewing, final draft

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None to declare

Data availability statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical approval
Ethical approval (IBC KU-282-A/2022) for the study was obtained from Institutional Bioethics Committee (IBC) University of Karachi, Pakistan.

Conflict of interest
Authors confirm that they have no conflict of interest.

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