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Original Paper

Biochemical and Biophysical in Vitro Studies and Systematic Literature Review on the Antioxidant and Antiglycation Activities of Trazodone

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Key Words
Trazodone • Antioxidant activity • Protein glycation • Carbonyl stress • Oxidative stress

Abstract
Background/Aims: Trazodone is a selective serotonin reuptake inhibitor; however, other mechanisms of the drug's anti-depressive properties have also been postulated. Hence, the aim of the study was to perform a systematic review and assess antiglycoxidative properties of trazodone in *in vitro* models. ***Methods:*** Trazodone's scavenging and chelating properties were measured with spectrophotometric method. The impact of the drug on carbonyl/oxidative stress was marked in the bovine serum albumin (BSA) model where sugars (glucose, fructose, galactose, ribose) and aldehydes (glyoxal and methylglyoxal) were used as glycation agents. Aminoguanidine and N-acetylcysteine (NAC) were applied as reference glycation/free radical inhibitors. Glycation biomarkers (kynurenine, N-formylkynurenine, dityrosine as well as advanced glycation end products contents) were assessed spectrofluorometrically. Concentrations of oxidation parameters (total thiols (TTs), protein carbonyls (PCs) and also advanced oxidation protein products (AOPPs) levels) were determined spectrophotometrically. ***Results:*** We demonstrated that trazodone poorly scavenged radicals (hydroxyl radical, nitric oxide, hydrogen peroxide and 2,2-diphenyl-1-picrylhydrazyl radical) and showed low ferrous ion chelating, unlike aminoguanidine and NAC. Sugars/aldehydes caused enhancement of glycation parameters, as well as a decrease of TTs and an increase of PCs and AOPPs levels compared to BSA incubated alone. Trazodone did not reduce oxidation parameters to the baseline (BSA) and significantly exacerbated glycation markers in comparison with both BSA and BSA+glycators. The content of glycation products was markedly lower in aminoguanidine

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and NAC than in trazodone. The molecular docking of trazodone to BSA revealed its very low affinity, which may indicate non-specific binding of trazodone, facilitating the attachment of glycation factors. ***Conclusion:*** According to our findings, it may be concluded that trazodone poorly counteracts oxidation and intensifies glycation *in vitro*. A possible mechanism for antiglycoxidative effect of trazodone *in vivo* may be the enhancement of the body's adaptive response, as indicated by the results of our systematic review.

Introduction

According to the report of the World Health Organization (WHO) of 2015, about 4.4% of the world's population, that is 322 million people, suffer from depression [1]. This is an increase by 18.4% since 2005 [2]. The prevalence of depression is the lowest among men of the Western Pacific Region (2.6%) and the highest among African and American women (5.9%) [1]. Since depression worsens patients' daily functioning, it often prevents them from working. According to the WHO's predictions, depression will be the leading cause of disability worldwide by 2030 [1, 2].

Depression is a multifactorial disorder. The main biological factor in depression is disrupted interplay between neurotransmitters in the central nervous system – serotonin, norepinephrine, or dopamine [3]. Moreover, imbalance between neuroprotective (e.g., brain-derived neurotrophic factor [BDNF], progranulin, cystatin C) and neuroprogressive (e.g., nuclear factor kappa-light-chain-enhancer of activated B cells [NF-κB] and nitric oxide [NO]) factors in favor of the latter bring negative effects [3, 4]. Another known cause of depression is telomerase shortening and inflammation, which increases oxidative damage at both the central (brain) and systemic (blood) level [5, 6]. Polymorphisms of various genes, including those encoding enzymes of the tryptophan catabolite (TRYCAT) pathway, are also related to overproduction of reactive oxygen species (ROS) [6]. Indeed, oxidative stress and the associated protein glycoxidation play a key role in the pathogenesis of depression [6–8]. Although proteins, lipids and nucleic acids undergo glycoxidative modifications, proteins are the primary target of an ROS attack [6]. Oxidative and carbonyl stress products aggregate and accumulate in the nerve cells, thus disrupting neurotransmitter synthesis/secretion and inducing neuronal apoptosis [9]. Therefore, it is not surprising that the content of glycoxidant protein products correlates with the severity of depression or suicidal tendencies [10]. Compounds that could inhibit oxidation/glycation of brain biomolecules are being intensively searched for as a new therapeutical target in depression [11–13].

Trazodone ($C_{19}H_{22}ClN_5O$; 2-{3-[4-(3-chlorophenyl)piperazin-1-yl]propyl}[1, 2,4] triazolo [4, 3-a]pyridin-3(2H)-one; Fig. 1) is a substance used for depression of various etiologies, including depression accompanied by anxiety [14, 15]. Trazodone is a serotonin reuptake inhibitor – it blocks serotonin transport from the synaptic space and directs it back into the nerve cell. Trazodone, exhibiting strong affinity for 5-HT_{2A} receptors, antagonizes their action by increasing the binding of serotonin to the 5-HT_{1A} receptor. On the other hand, trazodone blocks the inhibitory effect of 5-HT_{2A} on the 5-HT_{1A} receptor [16, 17]. However, not all mechanisms of trazodone action are well known. The drug administered orally is absorbed well and the maximum serum concentration is reached after four hours.

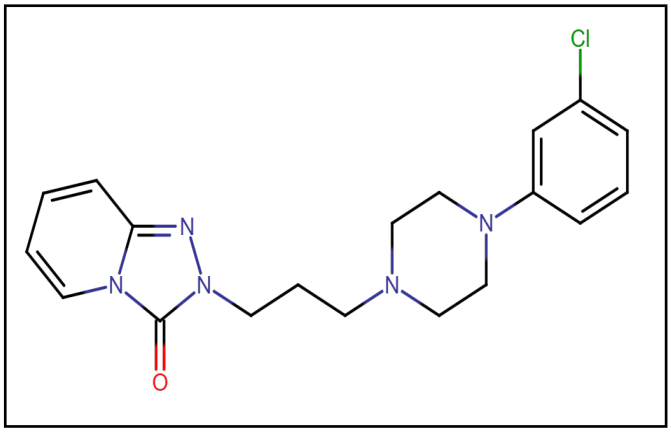

Fig. 1. Trazodone chemical formula.

Trazodone is metabolized in the liver, excreted in the urine, and the biological half-life of the drug is about 12 h [15, 18].

Literature data on the effects of trazodone on oxidative stress are scarce, the results remain inconclusive, and only one paper addresses the issue of the effect of trazodone on protein glycation. The authors demonstrated the inhibitory effect of trazodone on the ROS-mediated generation of tau fibrils in a laboratory assay. Moreover, this drug prevents the formation of tau oligomers in a cell culture, thus reducing the mortality of cells [19]. Since carbonyl stress plays a crucial role in developing psychiatric disorders [20–22], trazodone may also exhibit effective antidepressant results by preventing glycoxidation. Therefore, we decided to investigate this mechanism holistically.

Materials and Methods

The scheme of the study is shown in the schematic workflow (Fig. 2).

Systematic Review

The literature review was performed from 1995 to September 2022 on Medline (PubMed) database. The available references were trawled through based on the following keywords: [trazodone and antioxidant], [trazodone and oxidative stress], [trazodone and ROS], [trazodone and glycation], [trazodone and glycemia], [trazodone and hyperglycemia], as well as [trazodone and amyloid]. Inclusion and exclusion criteria are presented in Table 1.

Two researchers explored the provisional data by independently assessing the titles of articles and abstracts. Then, all the previously selected publications were reviewed by two other investigators. Next, manuscripts meeting the set criteria were selected for the final analysis. The Cohen's kappa coefficient (κ) of the researchers' reliability level was 0.92. The methodology of all papers was evaluated. The following variables were assessed: authors, publication year, design of research, experiment population size, inclusion and exclusion criteria as well as experiment duration and results.

Chemicals and Equipment

The analytical grade reagents used were acquired from Sigma-Aldrich Co. (St. Louis, MO, USA). In order to sterilize the chemical solutions, they were filtrated with 0.2-mm membrane filters immediately prior to utilization. Tecan Infinite 200 Pro Microplate reader was employed to calculate absorbance and fluorescence.

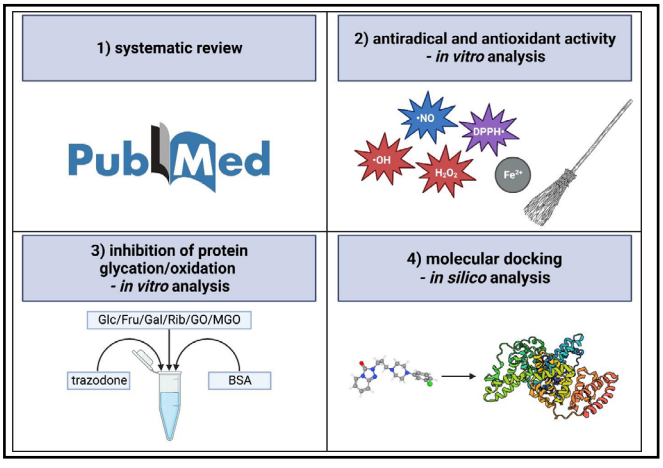

Fig. 2. Schematic workflow of the study.

Table 1. Inclusion and exclusion criteria of the analysed publications

Inclusion criteria	Exclusion criteria
publications only in English	publications in other languages
articles describing the antiglycoxidative activity of amantadine	articles not describing the antiglycoxidative activity of amantadine
manuscripts relevant to human, as well as in vivo and in vitro experiments	review manuscripts, surveys, and case descriptions

Antiradical and Antioxidant Activity

Scavenging of Hydroxyl Radical (OH). OH scavenging activity of trazodone was determined based on the interaction of sodium salicylate with the residual radicals. OH was produced by the $FeSO_4$-hydrogen peroxide (H_2O_2) system in the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^-$). Initially, the reaction mixture containing 50 µL of $FeSO_4$ (8 mM), 80 µL of H_2O_2 (6 mM), 50 µL of distilled water, 100 µL of the tested solutions (terminal strength of 1 mM) as well as 20 µL of sodium salicylate (20 mM) was prepared and then incubated at 37°C for one hour. Mixture absorbance was assessed spectrophotometrically at a wavelength of 562 nm. The inhibition rate of OH percentage value was determined using the following formula: $[1 - \{(A_1 - A_2)/A_0\}] \times 100\%$ (A_0 – control absorbance [without additives], A_1 – absorbance after the drugs are mixed, A_2 – absorbance without sodium salicylate) [23].

Scavenging of Nitric Oxide (NO). To 50 µL of samples, 100 µL of phosphate buffered saline, including 5 mM sodium nitroprusside was added. Then, 150-minute incubation of this mixture was conducted at 25°C. After that, 150 µL of Griess reagent (1% sulfanilamide, 2% H_3PO_4 as well as 0.1% N-(1-naphthyl) ethylenediamine) was added to the reaction mixture. As a consequence of nitrite diazotization by sulfanilamide and its conjugation with N-(1-naphthyl)ethylenediamine, chromophore was released. The absorbance of this product was marked by means of a spectrophotometer at 546 nm. The scavenging activity was calculated according to the formula: $[1 - (A_1/A_2)] \times 100\%$ (A_0 – absorbance of the control [without drugs], A_1 – sample absorbance after reaction) [24].

Scavenging of Hydrogen Peroxide (H_2O_2)

At first, in order to produce ferrous ion oxidation-xylenol orange (FOX), 87.3 mg of butylated hydroxytoluene (BHT), 10 µL of H_2SO_4, 7.6 mg of xylenol orange and 10 mg of ferrous ammonium sulphate were mixed in 100 mL of 90% methanol-water solution. Next, 30-minute incubation of a mixture (1:1, v/v) of H_2O_2 (50 mM) with the samples (at terminal strength of 1 mM) was performed at room temperature. After that, 10 µL of high-performance liquid chromatography (HPLC)-grade methanol was introduced to 90 µL of the above-mentioned solution. Then, 0.9 mL of the FOX was added to this mixture, whereupon it was vortexed and incubated at room temperature for 30 minutes. The absorbance of ferric-xylenol orange complex generated in the reaction was measured at 560 nm wavelength via the spectrophotometer method. The percentage of H_2O_2 inhibition rate was calculated based on the following formula: $[1 - \{(A_1 - A_2)/A_0\}] \times 100\%$ (A_0 – control absorbance [without drugs], A_1 – absorbance after the introduction of additives, A_2 – absorbance without the FOX reagent) [23].

Scavenging of 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical. The assessment of DPPH radical (DPPH) scavenging activity was estimated based on decolorization of this radical. The amount of 180 µL of 0.13 mg/mL DPPH was mixed with 30 µL of diluted samples, after which the solution was supplemented with methanol to the volume of 210 µL. The DPPH solution was served as a control. Next, 20-minute incubation was performed at room temperature. After that, the absorbance was evaluated at 517 nm wavelength by a spectrophotometer. The following formula was applied to estimate DPPH• Elimination: $[(A_{blank} - A_{sample})/A_{blank}] \times 100\%$, where: A_{blank} – the solution of blank DPPH absorbance, A_{sample} – DPPH absorbance in the sample mixture [23].

Ferrous Ion Chelating (FIC)

FIC was determined by examining the decrease in the generation of Fe^{2+}-ferrozine complex. To 90 µL of samples (at the terminal strength of 0.5 mg/mL) or BHT (used as a control), 18 µL of 0.6 mM $FeCl_2$ solution and 162 µL of methanol were added. Then, the reaction mixture was incubated for 10 minutes at room temperature. After that, 18 µL of 5 mM solution of ferrozine was added. Next, the mixture was incubated for 5 more minutes at room temperature. The absorbance was assayed spectrophotometrically at a wavelength of 562 nm. FIC was calculated as a percentage of absorbance decrease of the control [23].

Bovine Serum Albumin (BSA) Model

The glycation of BSA was performed based on the previously applied methods [25–28]. Promptly, the solution of 96% BSA was added to 0.1 M sodium phosphate buffer of pH 7.4, where 0.02% sodium azide served as a preservative. The terminal concentration of BSA in incubated mixtures was 0.09 mM. Sugars (glucose [Glc], fructose [Fru], galactose [Gal], and ribose [Rib]), as well as aldehydes (glyoxal [GO]

and methylglyoxal [MGO]) were used as glycation agents. BSA incubation was conducted in the presence of 1 mM trazodone with 0.5 M Glc, Fru, Gal, and Rib for 6 days, or with 2.5 mM GO and also MGO for 12 h, in order to evaluate the impact of the drugs on protein glycation [25–28]. GO as well as MGO were utilized within a period of a month after delivery. The solutions of these aldehydes were prepared shortly before the experiment [26].

According to earlier kinetic studies, glycation agent concentrations and the most optimal incubation conditions were measured and verified in order to examine the modulation of the glycoxidation rate by additives [25, 26]. The levels of glycation agents were significantly enhanced in comparison with their concentrations found in the human body. However, they were nonetheless beneficial for simulating the physiological processes in a relatively short time, which usually lasts for weeks or even months [25, 26, 29]. The antiglycation characteristics of novel compounds are frequently evaluated under experimental conditions [25–28, 30].

To compare the results obtained for trazodone, we used aminoguanidine – protein glycation inhibitor, as well as N-acetylcysteine (NAC) – free radical scavenger [25, 26]. All additives were employed at a concentration of 1 mM which was calculated proportionally to the high levels of glycation agents based on the previous *in vitro* experiments [25–28]. The study was conducted in three series, and each of them was repeated twice.

Products of Protein Glycation

The levels of kynurenine (KN), N-formylkynurenine (NFK) and dityrosine (DT) were measured spectrofluorimetrically at 365/480, 325/434 and 330/415 nm wavelengths of emission and excitation, respectively. Before the reading, 0.1 M H_2SO_4 (1:5, v/v) was used to dilute the samples. Results were standardized according to the fluorescence of 0.1 mg/mL quinine sulfate solution in 0.1 M H_2SO_4 [31, 32].

Advanced Glycation End Products (AGEs)

The content of AGEs was evaluated by means of a spectrofluorometer. The fluorescence of AGEs was calculated at 440/370 nm wavelength. Before the assessment, the assayed solutions were diluted with PBS (1:5, v/v). Evaluation of AGEs was also performed via the commercial method of enzyme-linked immunosorbent assay (ELISA) (USCN, Life Science, Wuhan, China), in compliance with the manufacturer's instructions [33].

Products of Protein Oxidation

Total Thiols (TTs)

The concentration of TTs was measured with a spectrophotometer at 412 nm using Ellman's reagent. The level of TTs was determined based on the reduced glutathione (GSH) standard curve [34].

Protein Carbonyls (PCs) level

The level of PCs was assayed based on the reaction of 2, 4-dinitrophenylhydrazine (DNPH) and carbonyls in oxidation-damaged proteins. The absorbance of the color reaction products was evaluated spectrophotometrically at 355 nm wavelength. The 2, 4-DNPH absorption coefficient of 22, 000 M^{-1} cm^{-1} was used as a standard [35].

Advanced Oxidation Protein Products (AOPPs)

The concentration of AOPPs was determined via the spectrophotometric method. 200 µL of the tested solutions were diluted by PBS at a ratio of 1:5 (v/v). Thus prepared samples as well as standard solutions at concentrations of 0 to 100 µM and 200 µL of blank solution of PBS were placed on a 96-well microplate. After that, 10 µL of KI (1.16 M) and 20 µL of CH_3COOH were added. The absorbance of the samples was assayed promptly at 340 nm wavelength against the blank solution of: 200 µL PBS, 10 µL KI and 20 µL CH_3COOH [36].

Molecular Docking

Molecular docking is recognized in the *in silico* technique which aims to anticipate the best suitable mode of ligand binding with a macromolecule (usually protein) [37]. Our study investigated the interaction between trazodone hydrochloride and BSA particles. The 3D crystal structure of BSA (ID: 4F5S) sourced from the Protein Data Bank (PDB) was obtained in a form of a .pdb file. The protein structure was determined using the method of X-ray diffraction (resolution value: 2.47 Å). The 3D trazodone hydrochloride (ID: 62935) was downloaded from the National Library of Medicine website in an .sdf format. Firstly, all water molecules were removed using AutoDock MGL Tools, and polar hydrogens and Kollman's partial charges

were introduced. After that, processed protein particle was saved as a .pdbqt file. The docking was studied in a grid box of 40 × 40 × 40 with 0.375 Å spacing located at 34.885, 23.976 as well as 98.792, respectively. It was determined that the value of exhaustiveness was 8. Simulation of molecular docking was obtained with the use of AutoDock Vina software, and the docking was visualized using PyMOL 2.5 [27, 28].

Statistical Analysis

The statistical analysis was obtained using GraphPad Prism 9.0.0 (GraphPad Software, San Diego, California, USA). The results were presented as a percentage of relative values of the controls (BSA with glycation agents). A one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons were applied to assess differences between the groups. The value of $p < 0.05$ was found to be statistically significant. A multiplicity adjusted p-value was marked as well.

Results

Systematic Review

The systematic bibliography review led to the selection of 81 articles in the Medline (PubMed) database, 53 of which were rejected due to the title. A total of 28 abstracts were read, and 19 of them met the inclusion and exclusion criteria. Out of the remaining works, 7 were not associated with the topic of our study. Ultimately, 12 manuscripts were included in the research (Fig. 3). Table 2 below presents the results of our systematic review.

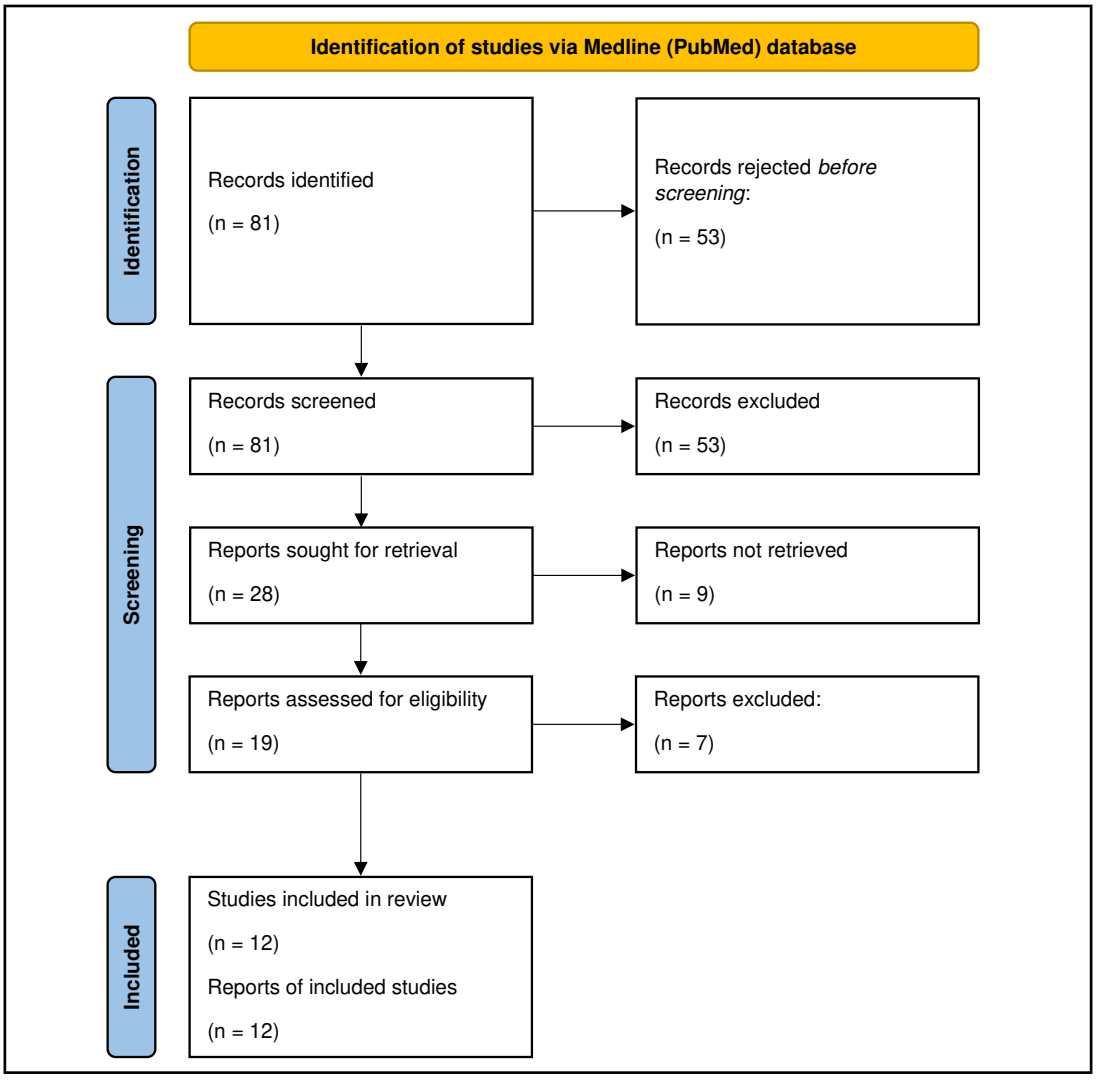

Fig. 3. Systematic review methodology flow diagram (Prisma).

Table 2. Antiglycoxidation effects of trazodone in experimental and clinical studies. 3-NP: 3-nitropropionic acid; CAT: catalase; GSH: reduced glutathione; GSH/GSSG: glutathione redox ratio; GSSG: oxidized glutathione; GST: glutathione-S-transferase; iNOS: inducible nitric oxide synthase; LPS: lipopolysaccharide; MDA: malondialdehyde; •NO: nitric oxide; ROS: reactive oxygen species; SOD: superoxide dismutase; SOD2: superoxide dismutase 2; TTs: total thiols

Study design	Endpoints	References
in vitro studies		
rat hepatic BRL3A superoxide dismutase 2 (SOD2) –knockdown cells exposed to a trazodone solution (75 μM)	the decline of SOD2 activity potentiated trazodone-induced cytotoxicity, which indicates that the hepatotoxicity of this drug is mediated by oxidative stress	(Yoshikawa et al., 2009)
rat hepatocytes treated with a solution of trazodone (300 μM)	the study drug enhanced reactive oxygen species (ROS) fluorescence, malondialdehyde (MDA), and oxidized glutathione (GSSG) concentration, as well as decreased reduced glutathione (GSH) level and mitochondrial membrane potential	(Taziki et al., 2013)
rat hepatocytes exposed to a trazodone solution (450 μM)	trazodone decreased GSH concentration and increased MDA level	(Najibi et al., 2016)
in vitro thioflavin T-based fluorimetric assay of trazodone solutions (10, 30, and 50 μM); SHSY5Y cells induced to tau aggregation treated with a trazodone solution (50 μM), evaluated via flow cytometry	trazodone at 50 μM inhibited tau fibers generation by almost 40%; the drug nearly completely reduced intracellular oligomerization of tau	(Akbari et al., 2020)
in vivo studies		
lipopolysaccharide (LPS)-treated rats exposed to trazodone intravenous administration (1 mg/kg)	trazodone reduced cerebral lipid peroxidation and MDA levels, but did not scavenge peroxyl radicals or prevent hyperglycemia	(Shen et al., 2005)
rats with LPS-induced endotoxin shock pretreated intravenously with trazodone (1 mg/kg)	the drug lowered the concentration of nitric oxide (•NO) in plasma as well as inducible nitric oxide synthase (iNOS) in lymphocytes, but not in the aorta and the liver	(Shen et al., 2007)
male albino mice exposed to trazodone (5 mg/kg and 10 mg/kg) 30 minutes before a forced swim test	trazodone dose-dependently diminished concentrations of MDA and nitrite in the brain, and elevated cerebral levels of GSH and catalase (CAT) activity	(Kumar et al., 2008)
ischemia/reperfusion damage in the brain of Laca mice after trazodone administration (5 and 10 mg/kg)	the study drug reduced MDA and nitrite levels, boosted the activity of CAT, superoxide dismutase (SOD) and glutathione-S-transferase (GST), as well as augmented glutathione redox ratio (GSH/GSSG)	(A. Kumar et al., 2010)
rats treated with trazodone (5, 10, and 20 mg/kg) and then systemically administrated 3-nitropropionic acid (3-NP)	trazodone prevented GSH/GSSG decrease as well as mitochondrial complex enzyme impairment in the hippocampus caused by 3-NP	(Kumar et al., 2011; P. Kumar et al., 2010)
male Wistar rats administered trazodone orally to induce testicular injury	the drug potentiated MDA level and suppressed the content of total thiols (TTs) as well as CAT activity	(Khedr and Werida, 2022)
male albino rats administrated trazodone orally (200 mg/kg/day)	trazodone increased MDA concentration and decreased TTs fluorescence as well as CAT activity	(Khedr et al., 2022)

Antiradical and Antioxidant Activity

Excess ROS and reactive nitrogen species (RNS) lead to oxidative stress. Oxidative stress is exacerbated by accumulation of redox-active metals (such as iron). Thus, antioxidant properties of a substance depend not only on the scavenging capacity of free radicals (e.g., OH and DPPH) and other ROS/RNS (NO or H_2O_2) but also on its ability to chelate metals (like FIC) [6, 38, 39].

Scavenging of Hydroxyl Radical (OH)

OH inhibition rate of trazodone was 41%. Aminoguanidine and NAC scavenged OH more efficiently compared to trazodone (+34% and +53%, respectively) (Fig. 4A).

Scavenging of Nitric Oxide (NO)

Trazodone scavenged NO at the level of 1%. Both aminoguanidine (+13%) and NAC (+40%) showed substantially higher inhibition rates than trazodone (Fig. 4B).

Scavenging of Hydrogen Peroxide (H_2O_2)

H_2O_2 scavenging capacity of aminoguanidine (+61%) as well as NAC (+79%) was significantly elevated compared to trazodone (6%) (Fig. 4C).

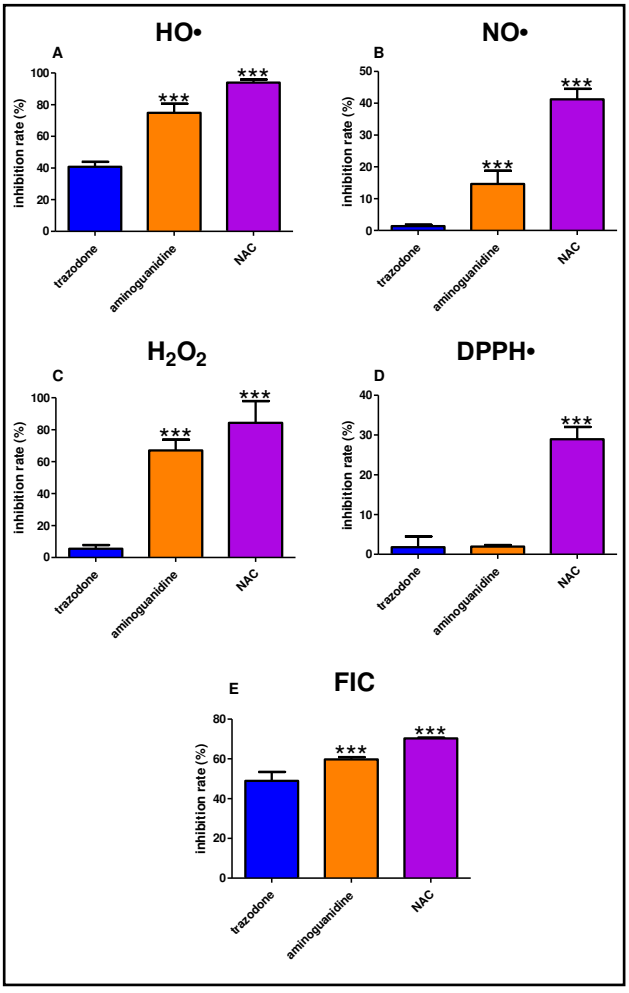

Fig. 4. The effect of trazodone, protein glycation (aminoguanidine) and free radical (N-acetylcysteine, NAC) inhibitors on hydroxyl radical (OH, Fig. 4A), nitric oxide (NO, Fig. 4B), hydrogen peroxide (H2O2, Fig. 4C) and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH , Fig. 4D) scavenging as well as ferrous iron chelating (FIC, Fig. 4E). ***p<0.001 vs. control (trazodone)

Scavenging of 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical

Trazodone presented a DPPH• Inhibition rate of 2%. Only DPPH Scavenging of NAC was markedly increased in comparison with the study drug (+27%) (Fig. 4D).

Ferrous Ion Chelating (FIC)

The FIC of trazodone was 49%. This parameter was effectively increased in aminoguanidine and NAC compared to trazodone (+11% and +21%, respectively) (Fig. 4E).

Products of Protein Glycation

Protein glycation leads to modifications of amino acids, including tyrosine (Tyr) which is particularly susceptible to the glycation process generating DT. Moreover, in the Maillard reaction, glycoxidation factors react with amino acids of proteins to form Schiff bases. These, in turn, are transformed into Amadori products (APs) from which AGEs are ultimately formed [40, 41].

Kynurenine (KN), N-formylkynurenine (NFK) and dityrosine (DT)

The content of KN was significantly potentiated in Glc+trazodone (+244%) compared to Glc. The marker was relevantly decreased in Glc+aminoguanidine as well as Glc+NAC versus Glc alone (−88% and −82%, respectively). The fluorescence of KN was considerably elevated in Glc (+1371%), Glc+trazodone (+4963%), Glc+aminoguanidine (+84%) and Glc+NAC (+166%) in comparison with BSA (Fig. 5A). This parameter was substantially higher in Fru+trazodone (+185%) but markedly lower in Fru+aminoguanidine and Fru+NAC (−87% and −86%, respectively) than in Fru. KN fluorescence was significantly augmented in Fru (+858%), Fru+trazodone (+2632%) as well as Fru+NAC (+34%) versus BSA (Fig. 5B). The biomarker was significantly enhanced in Gal+trazodone (+279%) compared to Gal alone. The content of KN was pointedly attenuated in both Gal+aminoguanidine and Gal+NAC versus Gal (−81% and −85%, respectively). The parameter in question was meaningfully increased in Gal (+879%), Gal+trazodone (+3609%), Gal+aminoguanidine (+82%) and Gal+NAC (+49%) compared to BSA (Fig. 5C). The level of KN was relevantly potentiated in Rib+trazodone (+84%); however, the marker was markedly reduced in Rib+aminoguanidine (−39%) as well as Rib+NAC (−43%) versus Rib alone. The fluorescence of KN was substantially increased in Rib, Rib+trazodone, Rib+aminoguanidine and Rib+NAC versus BSA (+328%, +687%, +162% and +146%, respectively) (Fig. 5D). This parameter was considerably elevated in GO+trazodone (+277%) in comparison with GO. Nevertheless, KN fluorescence was effectively decreased in GO+aminoguanidine (−31%) as well as GO+NAC (−59%) over against GO alone. The biomarker was significantly elevated in GO (+693%), GO+trazodone (+2890%), GO+aminoguanidine (+450%) and GO+NAC (+226%) versus BSA (Fig. 5E). The content of KN was meaningfully higher in MGO+trazodone (+37%) but relevantly lower in MGO+NAC (−29%) than in MGO. This parameter was considerably improved in MGO (+1192%), MGO+trazodone (+1670%), MGO+aminoguanidine (+1007%) as well as MGO+NAC (+822%) in comparison with BSA (Fig. 5F).

The fluorescence of NFK was significantly higher in Glc+trazodone (+669%) but markedly lower in Glc+aminoguanidine (−87%) and Glc+NAC (−81%) than in Glc alone. The content of NFK was considerably augmented in Glc, Glc+trazodone, Glc+aminoguanidine as well as Glc+NAC compared to BSA (+1356%, +11089%, +93%, and +171%, respectively) (Fig. 5G). The biomarker was elevated in Fru+trazodone (+579%) versus Fru. However, NFK fluorescence in Fru+aminoguanidine as well as Fru+NAC was substantially diminished versus Fru alone (−86% in both). This parameter was significantly enhanced in Fru (+909%), Fru+trazodone (+6748%), Fru+aminoguanidine (+38%) and Fru+NAC (+38%) in comparison with BSA (Fig. 5H). The content of NFK was relevantly higher in Gal+trazodone (+780%), but significantly lower in Gal+aminoguanidine as well as Gal+NAC than in Gal (−83% and −84%, respectively). The content of the marker was increased in Gal, Gal+trazodone, Gal+aminoguanidine and Gal+NAC versus BSA (+898%, +8679%, +71% and +60%, respectively) (Fig. 5I). The fluorescence of NFK was markedly improved in Rib+trazodone (+418%) and Rib+aminoguanidine (+44%) compared to Rib alone. This parameter was substantially lowered in Rib+NAC (−12%) over against Rib. The concentration of NFK was markedly potentiated in Rib (+137%), Rib+trazodone (+1125%), Rib+aminoguanidine (+242%) as well as Rib+NAC (+108%) versus BSA (Fig. 5J). The biomarker was substantially higher in GO+trazodone (+1352%) but significantly lower in GO+aminoguanidine and GO+NAC than in GO alone (−37% and −57%, respectively). The fluorescence of NFK was largely enhanced in GO (+586%), GO+trazodone (+9859%), GO+aminoguanidine (+331%), GO+NAC (+192%) versus BSA (Fig. 5K). This parameter was significantly increased in MGO+trazodone (+583%), MGO+aminoguanidine (+33%) and MGO+NAC (+35%) than in MGO. The content of NFK parameter was substantially elevated in MGO, MGO+trazodone, MGO+aminoguanidine and MGO+NAC compared to BSA (+753%, +5726%, +1035% and +1048%, respectively) (Fig. 5L).

The fluorescence of DT was relevantly improved in Glc+trazodone (+136%) compared to Glc alone. The biomarker was considerably decreased in Glc+aminoguanidine as well as

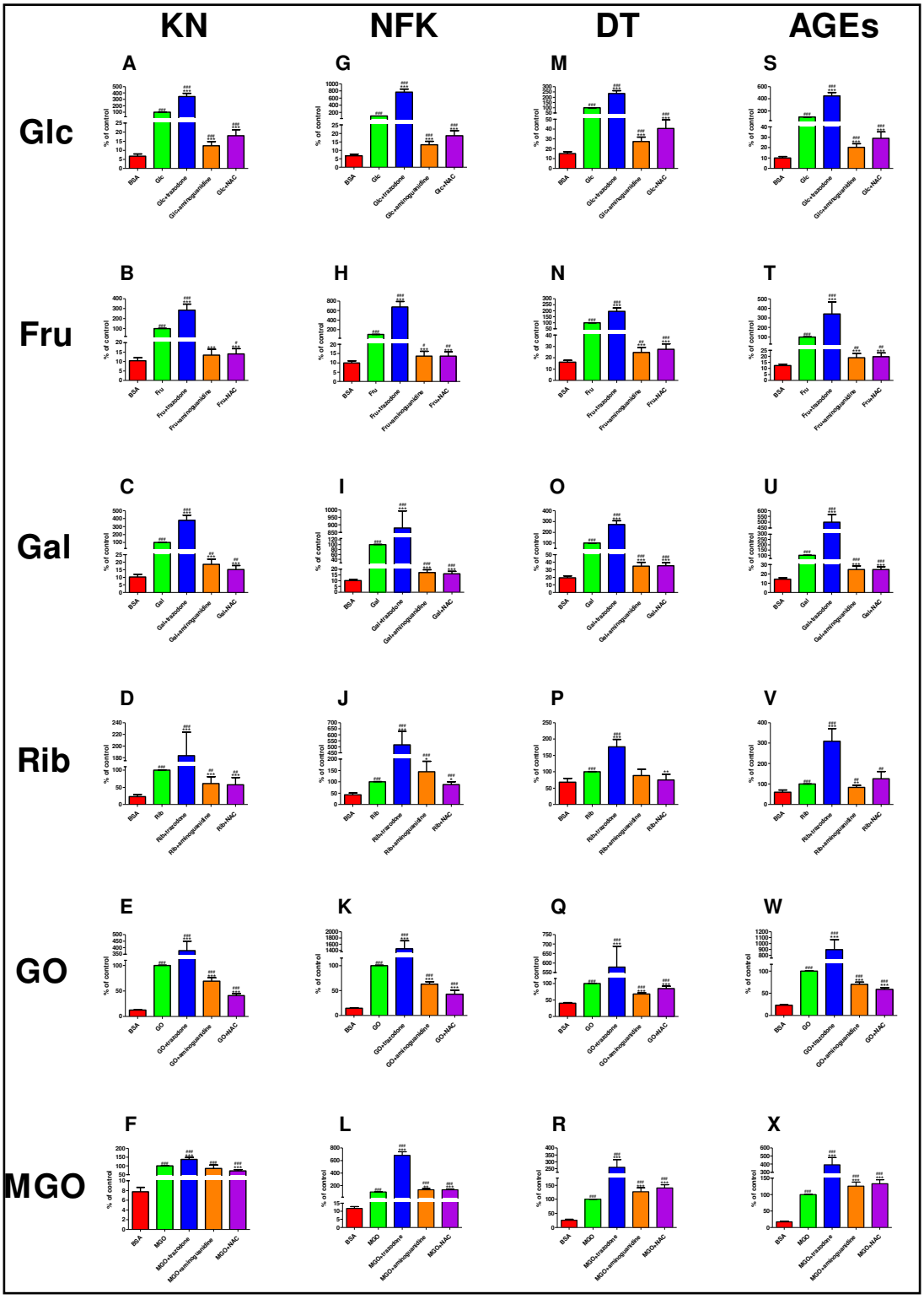

Fig. 5. The effect of trazodone, protein glycation (aminoguanidine) and free radical (N-acetylcysteine, NAC) inhibitors on kynurenine (KN, Fig. 5A–F), N-formylkynurenine (NFK, Fig. 5G–L), dityrosine (DT, Fig. 5M–R) as well as advanced glycation end products (AGEs, Fig. 5S–X) fluorescence in bovine serum albumin (BSA) glycated with glucose (Glc), fructose (Fru), galactose (Gal), ribose (Rib), glyoxal (GO) and methylglyoxal (MGO). *p<0.05 vs. positive control (glycoxidation agent); **p<0.01 vs. positive control (glycoxidation agent); ***p<0.001 vs. positive control (glycoxidation agent); #p<0.05 vs. negative control (BSA); ##p<0.01 vs. negative control (BSA); ###p<0.001 vs. negative control (BSA)

Glc+NAC (−73% and −59%, respectively) versus Glc. The content of DT was substantially elevated in Glc (+564%), Glc+trazodone (+1465%), Glc+aminoguanidine (+82%) and Glc+NAC (+170%) compared to BSA (Fig. 5M). The parameter was effectively higher in Fru+trazodone (+95%) but markedly lower in Fru+aminoguanidine and Fru+NAC (−75% and −73%, respectively) than in Fru alone. The level of DT was significantly augmented in Fru (+518%), Fru+trazodone (+1105%), Fru+aminoguanidine (+52%) as well as Fru+NAC (+70%) versus BSA (Fig. 5N). The marker was significantly enhanced in Gal+trazodone (+172%) in comparison with Gal. The fluorescence of DT was markedly decreased both in Gal+aminoguanidine and Gal+NAC (both −65%) versus Gal alone. This parameter was substantially increased in Gal (+413%), Gal+trazodone (+1294%), Gal+aminoguanidine (+78%) and Gal+NAC (+81%) compared to BSA (Fig. 5O). The fluorescence of DT was relevantly elevated in Rib+trazodone (+76%); however, the biomarker was notedly reduced in Rib+NAC (−25%) versus Rib. The content of DT was markedly enhanced in Rib and Rib+trazodone versus BSA (+47% and +159%, respectively) (Fig. 5P). The parameter was considerably increased in GO+trazodone (+478%) versus GO alone. Nevertheless, DT content was significantly lowered in GO+aminoguanidine (−32%) as well as GO+NAC (−15%) in comparison with GO. The marker was considerably elevated in GO (+147%), GO+trazodone (+1329%), GO+aminoguanidine (+69%) and GO+NAC (+110%) versus BSA (Fig. 5Q). The fluorescence of DT was markedly higher in MGO+trazodone, MGO+aminoguanidine and MGO+NAC (+161%, +27% and +40%, respectively) than in MGO alone. This parameter was significantly increased in MGO (+289%), MGO+trazodone (+915%), MGO+aminoguanidine (+394%) as well as MGO+NAC (+447%) compared to BSA (Fig. 5R).

Advanced Glycation End Products (AGEs)

The content of AGEs was considerably higher in Glc+trazodone (+348%) but much lower in Glc+aminoguanidine (−80%) and Glc+NAC (−71%) than in Glc. AGEs fluorescence was significantly elevated in Glc, Glc+trazodone, Glc+aminoguanidine as well as Glc+NAC in comparison with BSA (+893%, +4344%, +101% and +188%, respectively) (Fig. 5S). The biomarker was markedly enhanced in Fru+trazodone (+242%) over against Fru alone. Nevertheless, the fluorescence of AGEs in Fru+aminoguanidine (−81%) as well as Fru+NAC (−80%) was relevantly diminished versus Fru. This parameter was relevantly elevated in Fru (+710%), Fru+trazodone (+2668%), Fru+aminoguanidine (+53%) and Fru+NAC (+59%) compared to BSA (Fig. 5T). The content of AGEs was substantially higher in Gal+trazodone (+401%) but significantly lower in Gal+aminoguanidine as well as Gal+NAC than in Gal alone (−75% in both). The marker was considerably increased in Gal, Gal+trazodone, Gal+aminoguanidine and Gal+NAC versus BSA (+604%, +3425%, +75% and +74%, respectively) (Fig. 5U). The content of AGEs was markedly boosted in Rib+trazodone (+210%) when compared to Rib. This parameter was significantly lowered in Rib+NAC (−16%) versus Rib alone. The fluorescence of AGEs was relevantly improved in Rib (+67%), Rib+trazodone (+418%), Rib+aminoguanidine (+40%) as well as Rib+NAC (+111%) versus BSA (Fig. 5V). The biomarker was substantially higher in GO+trazodone (+796%) mut significantly lower in GO+aminoguanidine and GO+NAC than in GO (−30% and −41%, respectively). The level of AGEs was considerably enhanced in GO (+336%), GO+trazodone (+3802%), GO+aminoguanidine (+206%) and GO+NAC (+156%) versus BSA (Fig. 5W). This parameter was markedly increased in MGO+trazodone (+292%), MGO+aminoguanidine (+26%) as well as MGO+NAC (+33%) versus MGO alone. The content of AGEs was substantially elevated in MGO, MGO+trazodone, MGO+aminoguanidine and MGO+NAC compared to BSA (+479%, +2171%, +628%, and +671%, respectively) (Fig. 5X).

Validation of Results by the Enzyme-Linked Immunosorbent Assay (ELISA)

The additives used may have an impact on the glycoxidation process of BSA assessed fluorometrically. Therefore, the content of AGEs was also marked by means of the ELISA test. It was demonstrated that fluorometrically-assayed AGEs content was equivalent to the data obtained via the reference method, i.e., ELISA (Fig. S1) [25].

Products of Protein Oxidation

The BSA molecule contains 35 thiol groups, only one of which does not form a disulfide bridge. This thiol group is involved in the oxidation of the protein as well as attachment of various ligands to it. Thus, oxidative damage was measured based on the degree of decline in TTs. Oxidative stress also leads to an increase in the concentration of PCs which are formed from the oxidation of amino acids containing free hydroxyl amine as well as amide groups. End products of these transformations are AOPPs [42–44].

Total thiols (TTs)

The concentration of TTs was considerably lower in Glc+aminoguanidine (−33%) but significantly higher in Glc+NAC (+744%) than in Glc alone. The marker was notedly reduced in Glc+aminoguanidine (−39%) versus BSA. TTs level was markedly increased in Glc+NAC (+664%) in comparison with BSA (Fig. 6A). The parameter was significantly diminished in Fru+aminoguanidine (−27%) but relevantly enhanced in Fru+NAC (+272%) versus Fru. The concentration of TTs was meaningfully suppressed in Fru, Fru+trazodone as well as Fru+aminoguanidine (−59%, −59% and −70%, respectively) compared to BSA. This biomarker was pointedly augmented in Fru+NAC (+53%) over against BSA (Fig. 6B). The level of TTs was substantially attenuated in Gal+trazodone and Gal+aminoguanidine (−22% and −35%, respectively) versus Gal alone. This parameter was relevantly potentiated in Gal+NAC (+549%) in comparison with Gal. TTs concentration was markedly lowered in Gal+trazodone (−47%) as well as Gal+aminoguanidine (−55%) but considerably increased in Gal+NAC (+344%) versus BSA (Fig. 6C). The marker was significantly improved in Rib+aminoguanidine (+142%) and Rib+NAC (+290%) compared to Rib. Nevertheless, the level of TTs was notedly reduced in Rib as well as Rib+trazodone (−58% and −50%, respectively) versus BSA. This parameter was significantly enhanced in Rib+NAC when compared to BSA (+63%) (Fig. 6D). The content of TTs was considerably improved in GO+trazodone (+16%) and GO+NAC (+569%) in comparison with GO. This biomarker was markedly elevated in GO (+27%), GO+trazodone (+46%), GO+aminoguanidine (+38%) and GO+NAC (+747%) in comparison with BSA (Fig. 6E). The concentration of TTs was substantially higher in MGO+NAC (+624%) than in MGO alone. This parameter was significantly inhibited in MGO, MGO+trazodone as well as MGO+NAC versus BSA (−45%, −43% and −43%, respectively). TTs level was relevantly enhanced in MGO+NAC compared to BSA (+295%) (Fig. 6F).

Protein Carbonyls (PCs) level

The level of PCs was significantly decreased in Glc+NAC versus Glc (−50%) (Fig. 6G). This parameter was markedly lowered in Fru+aminoguanidine (−53%) compared to Fru alone (Fig. 6H). The concentration of PCs in Gal+NAC was substantially mitigated (−37%) versus Gal (Fig. 6I). PCs level was significantly reduced in Rib+trazodone, Rib+aminoguanidine as well as Rib+NAC over against Rib alone (−45%, −25% and −12%, respectively). The marker was considerably increased in Rib (+70%) and Rib+NAC (+50%) versus BSA (Fig. 6J). The concentration of PCs was relevantly lower in GO+trazodone, GO+aminoguanidine as well as GO+NAC (−49%, −73%, and −63%, respectively) than in GO. This parameter was markedly elevated only in GO compared to BSA (+128%) (Fig. 6K). The concentration of PCs was substantially enhanced in MGO+trazodone, MGO+aminoguanidine and MGO+NAC versus MGO alone (+97%, +57% and +39%, respectively). The marker was significantly improved in MGO (+133%), MGO+trazodone (+358%), MGO+aminoguanidine (+266%) and MGO+NAC (+223%) versus BSA (Fig. 6L).

Advanced Oxidation Protein Products (AOPPs)

The level of AOPPs was substantially attenuated in Glc+NAC (−48%) versus Glc alone. The biomarker was effectively augmented in Glc, Glc+trazodone, Glc+aminoguanidine as well as Glc+NAC compared to BSA (+535%, +627%, +536% and +228%, respectively) over against BSA (Fig. 6M). The concentration of AOPPs was relevantly reduced in Fru+aminoguanidine (−33%) and Fru+NAC (−45%) versus Fru alone. This parameter was considerably

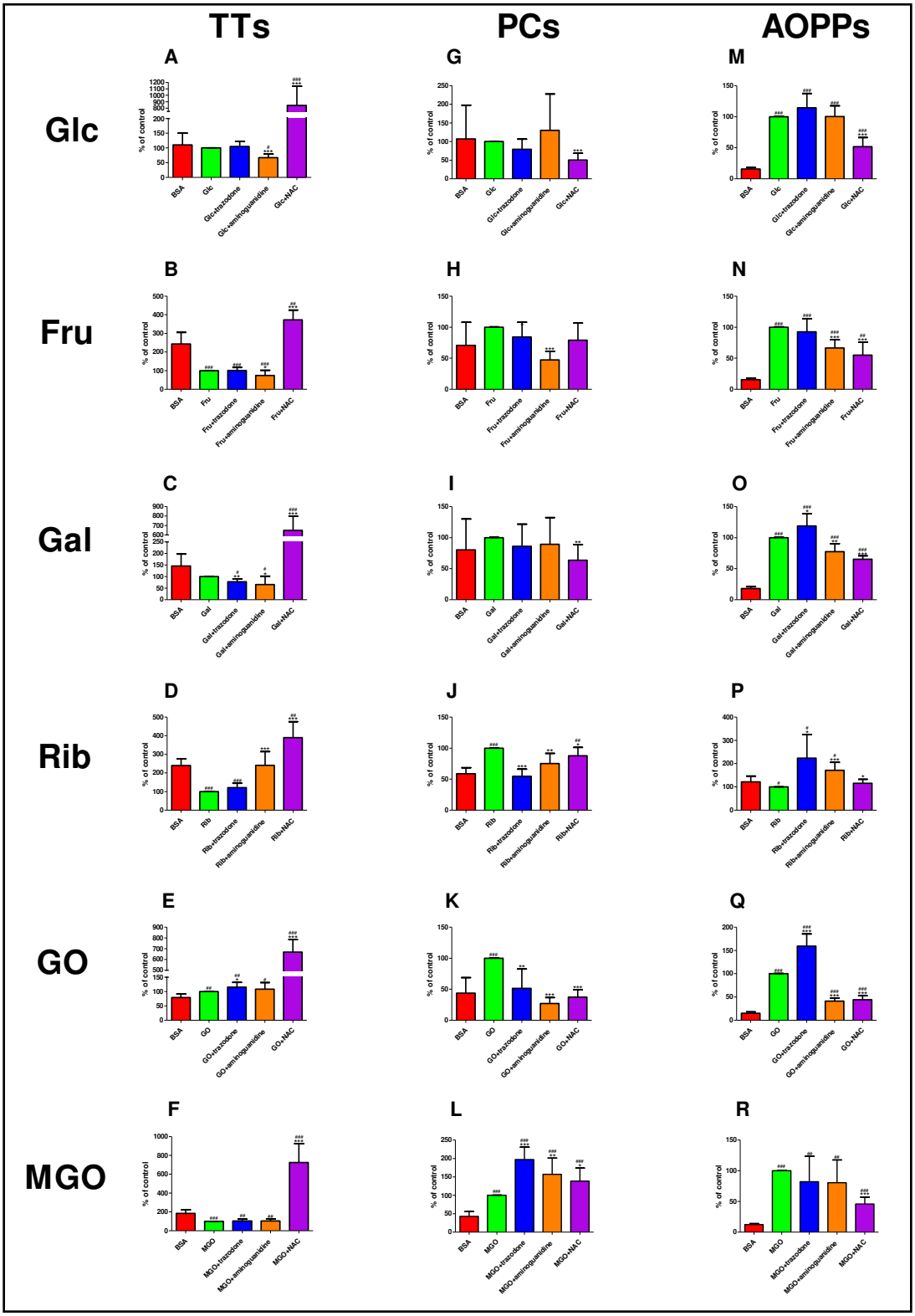

Fig. 6. The effect of trazodone, protein glycation (aminoguanidine) and free radical (N-acetylcysteine, NAC) inhibitors on total thiols (TTs, Fig. 6A–F), protein carbonyls (PCs, Fig. 6G–L) as well as advanced oxidation protein products (AOPPs, Fig. 6M–R) concentration in bovine serum albumin (BSA) glycated with glucose (Glc), fructose (Fru), galactose (Gal), ribose (Rib), glyoxal (GO) and methylglyoxal (MGO). ∗p<0.05 vs. positive control (glycoxidation agent); ∗∗p<0.01 vs. positive control (glycoxidation agent); ∗∗∗p<0.001 vs. positive control (glycoxidation agent); #p<0.05 vs. negative control (BSA); ##p<0.01 vs. negative control (BSA); ###p<0.001 vs. negative control (BSA)

elevated in Fru (+552%), Fru+trazodone (+504%), Fru+aminoguanidine (+334%) and Fru+aminoguanidine (+259%) in comparison with BSA (Fig. 6N). The content of AOPPs was significantly higher in Gal+trazodone (+19%), but markedly lower in Gal+aminoguanidine (−22%) and Gal+NAC (−35%) than in Gal. The marker was considerably increased in Gal, Gal+trazodone, Gal+aminoguanidine and Gal+NAC (+453%, +558%, +329% and +260%, respectively) versus BSA (Fig. 6O). The level of AOPPs was markedly potentiated in Rib+trazodone (+124%), Rib+aminoguanidine (+71%) and Rib+NAC (+16%) in comparison with Rib alone. The parameter was substantially diminished in Rib versus BSA (−18%). The level of AOPPs was largely enhanced in Rib+trazodone as well as Rib+aminoguanidine (+83% and +40%, respectively) compared to BSA (Fig. 6P). The biomarker was significantly higher in GO+trazodone (+60%) but substantially lower in GO+aminoguanidine (−59%) and GO+NAC (−56%) than in GO. The concentration of AOPPs was considerably elevated in GO (+552%), GO+trazodone (+940%), GO+aminoguanidine (+168%) as well as GO+NAC (+188%) over against BSA (Fig. 6Q). This parameter was relevantly decreased in MGO+NAC (−54%) versus MGO alone. The concentration of AOPPs was markedly raised in MGO, MGO+trazodone, MGO+aminoguanidine and MGO+NAC in comparison with BSA (+699%, +557%, +544% and +267%, respectively) (Fig. 6R).

Molecular Docking Analysis

The molecular docking simulation revealed a very weak (≥ −10 kcal/mol) affinity of trazodone hydrochloride to a particle of BSA [45], amounting to −9.3 kcal/mol. Merely three modes of docking sites presented root-mean-square deviations of atomic positions (RMSD) below 3 (Table 3) [46]. Two of them exhibited a polar contact with a side chain of a BSA particle, and in both the ligand interfered with lysine (Lys) residue in the position of 136. Fig. 7 presents mode 1.

Discussion

The association of psychiatric disorders with abnormalities of the KN pathway has been thoroughly investigated [47]. Tryptophan (Try) is an exogenous amino acid that serves as a major source of neurotransmitters such as serotonin and melatonin [48]. In the KN pathway, enzyme indoleamine-2, 3-dioxygenase (IDO) converts Try to NFK, which is then converted to KN [49]. Excessive IDO activation (e.g., due to mental stressors, inflammation or oxidative stress) reduces Try availability, resulting in insufficient neurotransmitter synthesis [49, 50]. In addition, KYN may be converted to 3-hydroxyquinurenine that plays a special role in the pathogenesis of depression. 3-hydroxykynurenine is responsible for overproduction of ROS, which contribute to neuronal apoptosis and the dysfunction of serotonergic and noradrenergic receptors [9, 50, 51]. Interestingly, disturbances in the KN pathway are also accompanied by abnormal carbohydrate metabolism [52]. Increased glycation of proteins, particularly those containing arginine (Arg) or Lys, has been noted in patients with depression [6, 22, 53]. Another amino acid prone to protein glycation is Tyr [40]. Increased levels of plasma DT and AGEs were found in adults with

Table 3. Results of a molecular docking analysis of trazodone hydrochloride to BSA particle. K: tyrosine; R: arginine; RMSD: root-mean-square deviations of atomic positions; Y: lysine

Mode	Affinity (kcal/mol)	RMSD (lower bond)	RMSD (upper bond)	Amino acid residues
1	−9.3	0.000	0.000	K-136
2	−8.6	1.527	2.297	K-136
3	−8.6	1.183	1.777	
4	−8.4	21.542	24.485	
5	−8.1	3.942	10.138	Y-160
6	−7.7	21.341	24.176	
7	−7.5	2.955	4.307	
8	−7.5	4.467	10.555	Y-137, Y-160, R-185
9	−7.3	29.436	31.722	

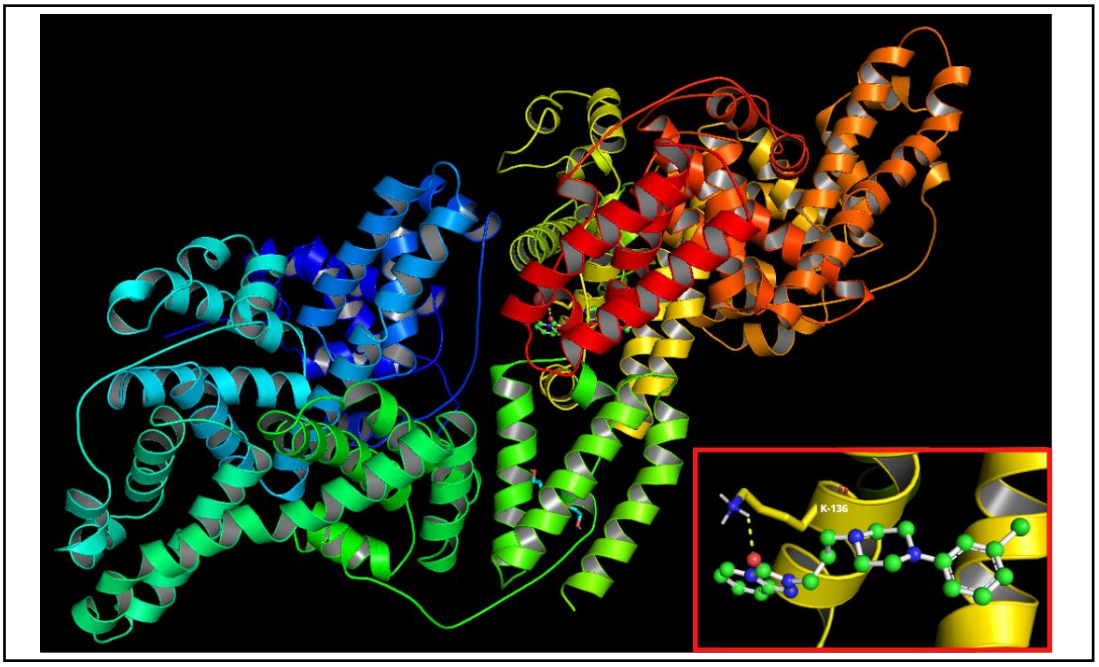

Fig. 7. Visualization of a docking site (mode 1) of trazodone hydrochloride in a BSA particle.

depression, which indicates increased glycation of brain proteins in the course of the disease [22, 54]. Given the important role of protein glycation in the pathogenesis of depression, it is not surprising that antidepressants with additional antiglycation activity are being sought [55, 56]. In this study, we comprehensively evaluated the antiglycation properties of trazodone in various *in vitro* and *in silico* models. Trazodone is an N-arylpiperazine in which one nitrogen is substituted by 3-chlorophenyl and the other one – by 3-(3-oxo [1, 2,4]triazolo [4, 3-a]pyridin-2(3H)-yl)propyl [57]. Trazodone is used for treatment of depression, especially with accompanying insomnia, anxiety or sexual dysfunction [14, 15, 58]. Trazodone exerts an antidepressant effect by inhibiting the reuptake of serotonin into synapses. Acting as an antagonist of the excitatory 5-HT_2 receptors, it enhances the binding of serotonin to the inhibitory 5-HT_{1A} receptor. Trazodone also counteracts the suppressive effect of the 5-HT_{2A} receptor on the 5-HT_{1A} receptor. Nevertheless, other mechanisms of the drug's antidepressant action have also been postulated [14, 16, 17].

To evaluate the antiglycation activity of trazodone, we used BSA treated with various glycation agents [25, 26, 59]. The use of albumin should come as no surprise, since it constitutes 50–70% of all plasma proteins [60]. Albumin is fundamental in maintaining the oncotic pressure of the blood, acts as a pH buffer, is involved in CO_2 transport as well as transporting some drugs, hormones, fatty acids and bile pigments [60, 61]. The BSA molecule contains 583 amino acids, while human serum albumin (HSA) contains 585 of them. Both proteins show 76% homology of polypeptide chains, which is about 67% in the α-helix form. The spatial structure of BSA/HSA is heart-shaped, stabilized by 17 disulfide bridges forming 9 loops. Binding sites I and II have a similar location and the same functions for both albumins. BSA and HSA have one free thiol group located at position 34. Despite the slight difference in albumin structure (BSA contains threonine in the place of alanine), the N-terminus of both albumins has the same function – it binds transition metal ions (Cu^{2+}, Co^{2+}, Ni^{2+} or Zn^{2+}) [60, 62–65]. Thus, BSA is a frequently used model protein in *in vitro* studies. This is also evidenced by its wide availability, low cost, good stability, binding properties and structural similarity to HSA. Kinetic studies comparing diabetic-derived HSA with BSA confirm the analogous glycoxidation mechanism of both. The resemblance demonstrates the usefulness of the glycated albumin model for assessing antiglycation and antioxidant properties [25, 26, 66–68]. Furthermore, with regard to brain cells, proteins are the most sensitive to oxidation

and glycation. The brain, compared to other organs, is characterized by very high oxygen consumption, unfavorable surface-to-volume ratio, lower activity of antioxidant enzymes and higher content of prooxidant metal ions [69–71].

In our study, BSA was effectively glycated by all sugars (Glc, Fru, Gal, Rib) and aldehydes (GO and MGO). This is evidenced by a significant increase in protein glycoxidation products in BSA+sugar/aldehyde samples compared to the controls (BSA). Glycation is a multiphase process of post-translational modification of proteins called the Maillard reaction. It begins with the non-enzymatic reaction of carbonyl groups of the glycation agent with amino groups of the protein, forming Schiff bases. During the Amadori reaction, the Schiff base is regrouped to APs. Schiff bases and APs are the early products of glycation. Their degradation leads to the formation of intermediate products of the Maillard reaction – dicarbonyl compounds characterized by significantly higher reactivity, like GO or MGO. The final stages of protein glycation are oxidation, polymerization, dehydration and condensation with other amino groups. These irreversible reactions result in the formation of persistent AGEs [40, 41, 67]. Thus, it is not surprising to find an increase in early (↑KN, ↑NFK, ↑DT) and late (↑AGEs) glycation protein products under the influence of all sugars and aldehydes. The content of glycation products was assessed by the fluorimetric method, as some of the modified amino acids (KN, NFK and DT) and AGEs demonstrate fluorescent properties [32, 33]. AGEs were also assessed via the reference ELISA method, as additives can hinder determinations made via spectrofluorometry [33]. However, there were no statistical differences between both techniques. The process of protein glycation is inextricably linked to protein oxidation referred to as glycoxidation [72]. In our study, the concentration of protein oxidation markers was also statistically different compared to the controls (↓TTs, ↑PCs, ↑AOPPs).

The mechanisms of protein glycation vary, depending on the agent used [59, 73, 74]. Thus, we applied different *in vitro* models to objectively assess the effect of trazodone on BSA glycation. Trazodone caused a significant increase ($p < 0.001$) in all the assessed glycation products (↑KN, ↑NFK, ↑DT and ↑AGEs) in the presence of both sugars (Glc, Fru, Gal, Rib) and aldehydes (GO and MGO). Lys, Arg and cysteine (Cys) are susceptible to glycation due to their strong nucleophilicity [75]. Of the 29 albumin Glc-binding sites, 18 are Lys residues. The main site is Lys-525, responsible for 33% of glycation with Glc. Other Lys residues important in the Glc attachment are located at positions 199 (5%), 281 and 439. Smaller contributions to glycation are made by Lys-12, Lys-51, Lys-205, Lys-233, Lys-276, Lys-317 and Lys-538 [76, 77]. On the other hand, MGO glycates mainly Arg residues of albumin. The most important binding site of MGO is Arg-410 (characterized by 89% reactivity), while Arg-114, Arg-186, Arg-218 and Arg-428 demonstrate lower contribution in this regard. MGO can react with Cys residue at position 34 (the only one that does not form a disulfide bridge) and the site exhibits reactivity as high as 80% [78, 79]. Interestingly, the synthesis of early glycation protein products boosts the formation of free radicals [80–82]. This occurs by increasing the affinity of modified Lys for prooxidants such as Cu^{2+} and Fe^{2+} ions [83–86]. However, the formation of ROS does not necessarily occur in the Fenton or Haber-Weiss reactions [79]. In high concentrations, Glc and other sugars undergo autooxidation which also leads to ROS overproduction [87]. In addition, albumin exposed to oxidative stress gains prooxidant activity itself by cross-reacting with other proteins [88–90]. In our study, trazodone increased the production of PCs and AOPPs, which are major post-translational protein modifications through oxidation [44]. We also observed depletion of TTs in BSA samples incubated with glycation agents and trazodone. This explains the reduced antioxidant activity of albumin treated with trazodone. The thiol group of Cys-34 is a major free radical scavenger due to its ability to specifically bind ROS [91]. The weak antiradical and antioxidant properties of trazodone were also confirmed in other studies [92–96]. Indeed, we showed that trazodone poorly scavenges OH, NO and H_2O_2, and has low antioxidant activity in the DPPH assay. The drug also poorly chelates transition metal ions in the FIC test.

The effects of trazodone were compared to substances with documented antiglycation (aminoguanidine) and antioxidant (NAC) properties [25, 26]. Aminoguanidine counteracts carbonyl stress due to the guanidinium group in its structure. The drug displaces glycation

factors from binding sites on the protein, traps α-dicarbonyls via nucleophilic transformation and acts as an antioxidant [97]. On the other hand, NAC is an acetylated precursor of L-cysteine. As a source of a free thiol group, NAC reduces ROS by donating one electron or acts as a nucleophile by donating one or two electrons [98]. In our study, the concentrations of early and late glycation products as well as protein oxidation products were significantly higher in BSA samples incubated with trazodone compared to aminoguanidine and NAC. In BSA treated with trazodone, we also showed enhanced carbonyl and oxidative stress compared to BSA+glycation agent. This indicates the proglycation properties of trazodone. Concentrations of trazodone and other additives were selected in kinetic studies of BSA glycation, in a manner proportional to high concentrations of glycation agents [25, 26, 59]. Nevertheless, it should be remembered that in a different range of concentrations, trazodone may exhibit different activity.

Why does trazodone demonstrate proglycation properties? This may be explained by the molecular docking analysis, which, in our study, revealed a very weak affinity of trazodone to BSA of above −10 kcal/mol. Merely three modes of docking sites presented RMSD below 3. Additionally, two of them exhibited a polar contact with a side chain of the BSA particle [27, 37, 45, 46]. However, ligand binding by albumin can alter its spatial conformation, preventing the attachment of other substances or strengthening the binding already formed. Ligand attachment at one binding site can change the structure of other binding sites or even their number [99, 100]. A two-stage course of ligand-protein interaction is postulated. In the first phase, the substance molecule approaches the hydrophobic cavity of albumin due to the hydrophobic effect. Then it binds to the protein through short-range interactions (hydrogen bonds, van der Waals forces as well as spherical or electrostatic interactions). Specific binding is characterized by very high affinity and low binding capacity, while non-specific binding presents low affinity and unlimited ligand binding capacity [101–103]. Trazodone (which demonstrated very low affinity to the albumin molecule) may bind to this protein non-specifically. A possible effect could be facilitating BSA glycation by sugars or aldehydes. Nonetheless, this hypothesis requires further research in both *in silico* and *in vitro* models.

Protein glycation together with oxidative stress mutually induce their adverse effects on the body [72]. In the course of a literature review, we found only one paper on the effects of trazodone on carbonyl stress [19]. Trazodone counteracted the formation of ROS-mediated tau fibrils in SHSY5Y cells. The authors reported that trazodone had inhibited tau protein oligomerization, which increased cell survival [19]. However, no study to date has evaluated the effects of trazodone on typical biomarkers of protein glycation and glycoxidation. Additionally, all the other *in vitro* studies showed prooxidant action of trazodone [92–94]. The drug increased ROS fluorescence, enhanced MDA formation and lowered mitochondrial membrane potential. Trazodone increased GSSG and decreased GSH levels. In addition, trazodone was demonstrated to be involved in oxidative stress-induced hepatocytotoxicity [92–94]. On the other hand, significant number of animal studies indicate that trazodone inhibits oxidative and nitrosative stress [14, 104–109]. The drug boosted antioxidant enzyme activity (catalase [CAT], superoxide dismutase [SOD], as well as glutathione-S-transferase [GST]) in the brain. Trazodone had positive effects on cerebral GSH level, glutathione redox ratio (GSH/GSSG) and mitochondrial function. It also presented alleviating effect on MDA and nitrite concentrations in the brain. The study drug reduced NO concentration in plasma and inducible nitric oxide synthase (iNOS) activity in lymphocytes [14, 104–109]. Therefore, trazodone can induce an adaptive response of the body. Moreover, the drug can stimulate defense mechanisms and strengthen the antioxidant barrier against carbonyl stress [110]. Therefore, further studies are needed to evaluate the effects of trazodone on protein glycation *in vivo*. Also, it cannot be ruled out that additional effects of trazodone are demonstrated by its metabolites. In the liver, trazodone undergoes biotransformation processes involving N-oxidation and hydroxylation reactions by the CYP3A4 isoenzyme of cytochrome P450. The metabolite m-chlorophenylpiperazine exhibits antidepressant activity (similar to trazodone), but might also present additional effects [111]. Since a systematic review of the literature indicates potential antioxidant properties of trazodone *in vivo*, further investigations are required to assess the biological activity of the drug's metabolites as well.

Conclusion

In conclusion, we demonstrated weak antiradical and antioxidant activity of trazodone in *in vitro* and *in silico* studies. We were the first to demonstrate that the drug has strong proglycation effects, making further studies in animal models as well as in humans necessary. A systematic review of the literature indicates a prooxidant nature of trazodone *in vitro*, in contrast to its protective effect against oxidative stress in animal models. Thus, trazodone may induce an adaptive response by stimulating antioxidant mechanisms at the tissue/organ level. Further studies, both molecular and clinical, are required.

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Author Contributions

M.N. performed laboratory determinations, interpreted the data, prepared the graphic part of the manuscript, wrote the manuscript and granted final approval of the version to be published. M.Z.P. interpreted the data. J.R.L. interpreted the data. A.Z. conceptualized and reviewed the article, and gave final approval of the version to be published. M.M. conceptualized the article, performed laboratory determinations, interpreted the data, prepared the graphic part of the manuscript, wrote the manuscript, reviewed the article and granted final approval of the version to be published.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

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