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COMPETITIVE INHIBITION OF GLUTATHIONE REDUCTASE ACTIVITY BY CYCLOHEXYL-(4,5-DIHYDRO-1H-IMIDAZOL-2-YL)AMINE HYDROIODIDE AND BENZYL-(4,5-DIHYDRO-1H-IMIDAZOL-2-YL)AMINE HYDROIODIDE

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ABSTRACT

In this research, 0, 25, 50, 100, 200 and 400 mg/L (ppm) concentrations of 2-aminoimidazolines (cyclohexyl-(4,5-dihydro-1H-imidazol-2-yl)amine hydroiodide (CHX) and benzyl-(4,5-dihydro-1H-imidazol-2-yl)amine hydroiodide (BNZ)) were applied to baker's yeast (*Saccharomyces cerevisiae*) glutathione reductase (GR). Since the inhibition constant (Ki) (43 ± 2 nM) and 50 % inhibition concentration (IC_{50}) (947 mg/L =3.13×10^{-3} M) values of BNZ were found to be smaller than the Ki (59 ± 17 nM) and IC_{50} (1461 mg/L =4.95×10^{-3} M) values of CHX. At imidazoline concentrations of 400 mg/L, CHX reduced GR activity by 16.07 %, while BNZ reduced GR activity by 21.96 %. BNZ inhibited GR more than CHX. In addition, molecular docking results also support the experimental data. CHX and BNZ inhibited GR enzyme activity as competitively.

Keywords: Benzyl-(4,5-dihydro-1H-imidazol-2-yl)amine hydroiodide, Cyclohexyl-(4,5-dihydro-1H-imidazol-2-yl)amine hydroiodide, Enzymes, Inhibitors, Imidazoline.

1 INTRODUCTION

Imidazoles and their heterocyclic derivatives constitute many bioactive compounds [1]. In medicinal chemistry, iminoimidazolines are powerful pharmacophores. Clonidine, moxonidine and phentolamine are the best known of these compounds. Phentolamine, containing the imidazoline ring, is a known alpha1-adrenergic antagonist [2]. Guanidine compounds play a leading role in the prevention of metabolic disorders, cancer, cardiovascular

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diseases, and diabetes, antifungal, antimicrobial, antiviral, antioxidant, cytotoxic activity [3-8]. Since imidazoles are found in many drugs, the effects of these compounds on enzyme activity have been investigated.

Baker's yeast (*Saccharomyces cerevisiae*) glutathione reductase (EC 1.6.4.2) (GR) is an antioxidant enzyme consisting of two protein subunits, each with the flavin FAD in its active site. GR converts oxidized glutathione (GSSG) to two reduced glutathione (GSH) via β-nicotinamide adenine dinucleotide 2'-phosphate reduced (NADPH) [9]. The reaction:

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2\,GSH + NADP^+$$

In this study, the molecular docking and GR activity of amino imidazoline compounds which have an important place in medicine and pharmacology were studied and compared. It was investigated whether GR, an important enzyme that produces GSH and detoxifies oxidized species, is affected by amino imidazoline compounds.

2 MATERIAL AND METHOD

Cyclohexyl-(4,5-dihydro-1H-imidazol-2-yl)amine hydroiodide and benzyl-(4,5-dihydro-1H-imidazol-2-yl)amine hydroiodide (Figure 1) were received from Assoc. Prof. Dr. Murat Genc [10]. Baker's yeast (*Saccharomyces cerevisiae*) glutathione reductase was received from Sigma-Aldrich. L-glutathione oxidized ≥98 % was received from Serva. β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt ≥95 % was received from Roth.

The actual CHX concentration, determined at 233 nm maximum absorbance by UV-1800 spectrophotometer (Shimadzu Scientific Instruments) at Adıyaman University Central Research Laboratory Application and Research Center (ADYUMLAB), was 104.631 ± 1.090 mg/L of the nominal stock concentration of 100 mg/L. The limit of detection (LOD) and limit of quantification (LOQ) for CHX are 3.270 and 10.900 mg/L, respectively. The actual BNZ amount, determined at 232 nm maximum absorbance by spectrophotometer at ADYUMLAB, was 101.590 ± 0.385 mg/L of the nominal stock concentration of 100 mg/L. The limit of detection (LOD) and limit of quantification (LOQ) for BNZ are 1.155 and 3.850 mg/L, respectively.

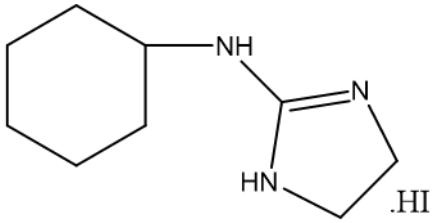
GR concentration was measured spectrophotometrically [11] at 750 nm. GR activities were measured with a UV-1800 UV-VIS incubation spectrophotometer (Shimadzu Scientific Instruments) at 340 nm and 37 °C. The convertion of NADPH to $NADP^+$ by GSSG was determined by reading the absorbances [12]. GR was calculated as U/mg protein. The description of the both methods is given by Karadag (2021) [13].

The interaction of enzyme and imidazoline derivatives was prepared as follows. 5000 mg/L CHX and BNZ stock solutions were prepared in 2 mL of 50 % ethanol by vortexing at room temperature. Then, 25, 50, 100, 200 and 400 mg/L of both derivatives were adjusted with 50% ethanol plus 700 µL GR solution in potassium phosphate buffer (100 mM, pH=7.6). The control or concentration of 0 mg/L was prepared with 300 µL of 50 % ethanol plus 700 µL GR solution. The volume of the enzyme and 50 % ethanol and the imidazoline derivatives was 1000 µL. The mixtures were incubated at room temperature for 5 minutes and GR activities were measured. These adjustments were made to measure the effect of the substances on the enzyme activity [13].

Types of GR inhibition were measured at different GSSG substrates (0.25, 0.5, 1 and 2 mM) and CHX concentrations (0, 200 and 400 mg/L) and BNZ concentrations (0, 200 and 400 mg/L) using the Lineweaver-Burk plot.

For statistics, Student-Newman-Keul test, one-way analysis of variance was used using SPSS.

a) Cyclohexyl-(4,5-dihydro-1H-imidazol-2-yl)amine hydroiodide



b) Benzyl-(4,5-dihydro-1H-imidazol-2-yI)amine hydroiodide

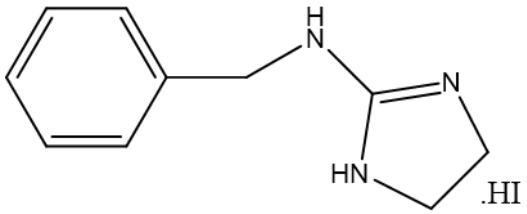


Figure 1. Structures of a) CHX and b) BNZ.

Docking

The crystal structure of the target enzyme GR (PDB ID: 2HQM) was obtained from the RCSB Protein Data Bank (www.rcsb.org/pdb/). The compounds were optimized using the 6-311+G(d,p) basis set in the Gaussian 09 software package, employing DFT/B3LYP technology to minimise their energy and determine stable conformers. A grid box measuring 25 Å × 25 Å × 25 Å was centred on the active site of the target to ensure the compounds were accurately docked. The compounds were then docked with the target using the PyRx 0.8 virtual screening tool, and the lowest energy conformer from each docked compound was selected for further analysis. Molecular visualisation of the interactions was performed using Discovery Studio Visualizer 3.5. Table 3 lists the interactions of GR with the synthesised compounds and their respective binding free energies (in kcal/mol). The binding energies for PDB ID: 2HQM were found to be −7.9 kcal/mol for BNZ and −7.2 kcal/mol for CHX.

3 RESULTS AND DISCUSSION

When CHX concentration was increased, a decrease in GR enzyme activity was observed compared to the control group, and this was statistically significant (N=3, P<0.05) (Table 1). Percentage changes in glutathione reductase enzyme activity under the influence of 25, 50, 100, 200 and 400 mg/L CHX were calculated as follows: –5.84; –9.58; –8.12; –11.04 and –16.07, respectively. The 50 % inhibition concentration (IC_{50}) of CHX was 1461 mg/L $=4.95\times10^{-3}$ M, $R^2 = 0.764$.

When the BNZ concentration was increased, a decrease in GR enzyme activity was observed at high concentrations (200, 400 ppm) compared to the control group, and this decrease was found to be statistically significant (N=3, P<0.05) (Table 1). Percentage changes in glutathione reductase enzyme activity under the influence of 25, 50, 100, 200 and 400 mg/L BNZ were calculated as follows: –4.05; –5.61; –5.92; –12.15 and –21.96, respectively. IC_{50} value of BNZ was 947 mg/L $=3.13\times10^{-3}$ M, $R^2 = 0.9776$.

Table 1. Effects of CHX and BNZ on GR activity.

Concentration (mg/L)	GR activities ± standard deviations (U/mg) for CHX	GR activities ± standard deviations (U/mg) for BNZ
0	616 ± 11^{a}	642 ± 7^{a}
25	580 ± 11^{b}	616 ± 25^{a}
50	557 ± 7^{c}	606 ± 12^{a}
100	566 ± 11^{bc}	604 ± 15^{a}
200	548 ± 8^{c}	564 ± 14^{b}
400	517 ± 4^{d}	501 ± 22^{c}

Values are given as arithmetic mean ± standard deviation. The letters "a, b, c, d" are used in the table to indicate the distinction between groups. Different letters indicate statistical distinction between activity levels (N=3, P< 0.05).

By examining Figure 2 and Figure 3, the Michaelis constant (Km), maximum reaction velocity (Vmax) and inhibition constant (Ki) of GR activities at different concentrations of CHX and BNZ were determined (Table 2).

According to Figure 2 and Figure 3, CHX and BNZ inhibited glutathione reductase enzyme activity as competitively.

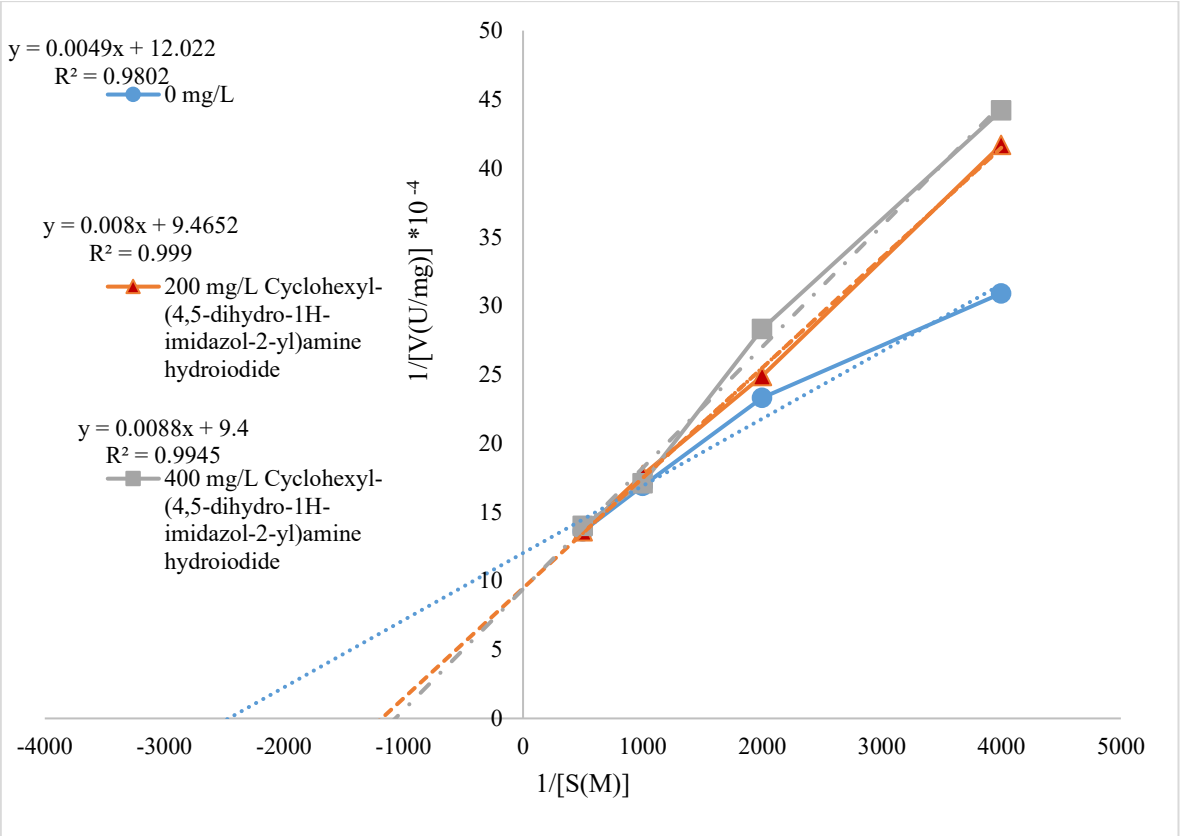


Figure 2. Lineweaver–Burk inhibition plot of CHX with GR activity.

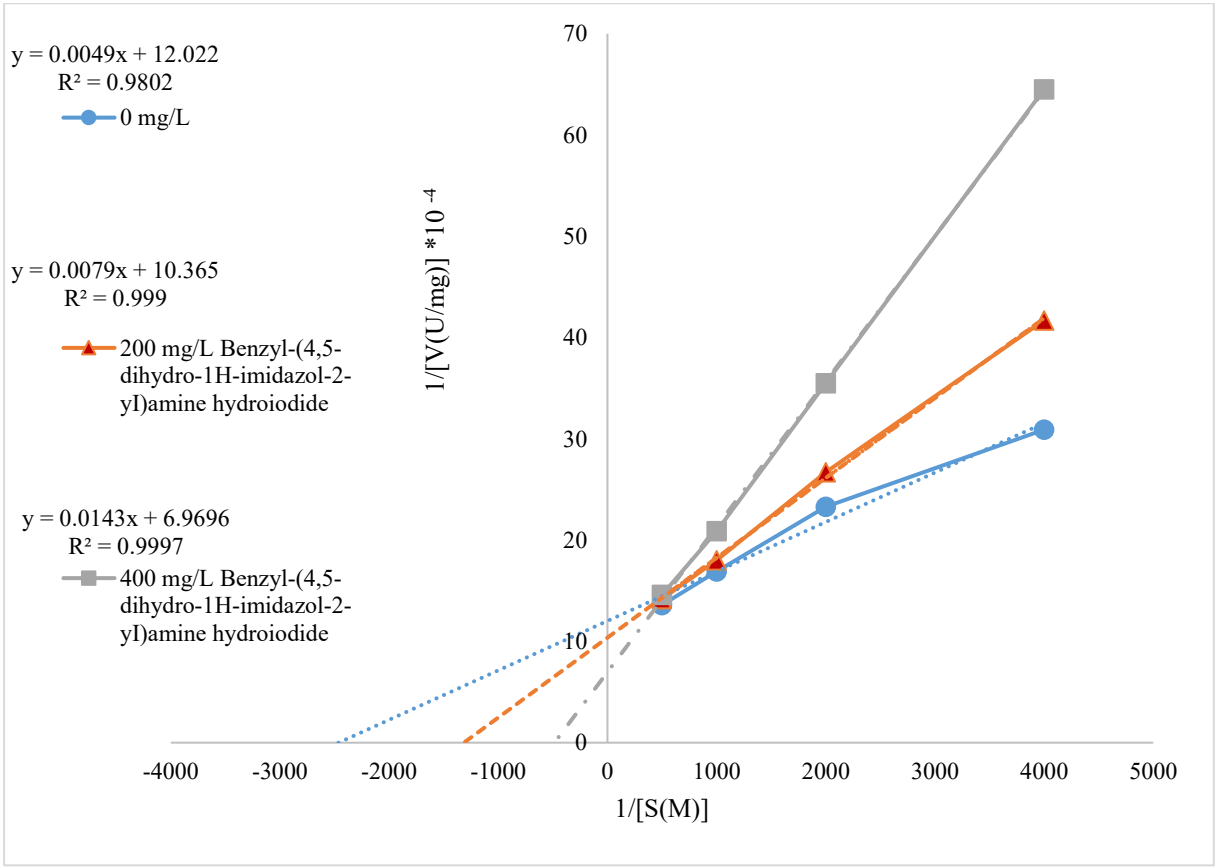
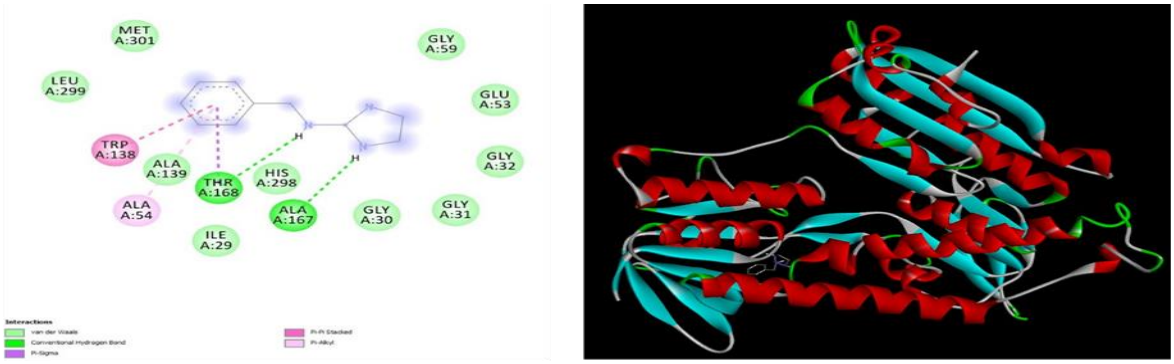


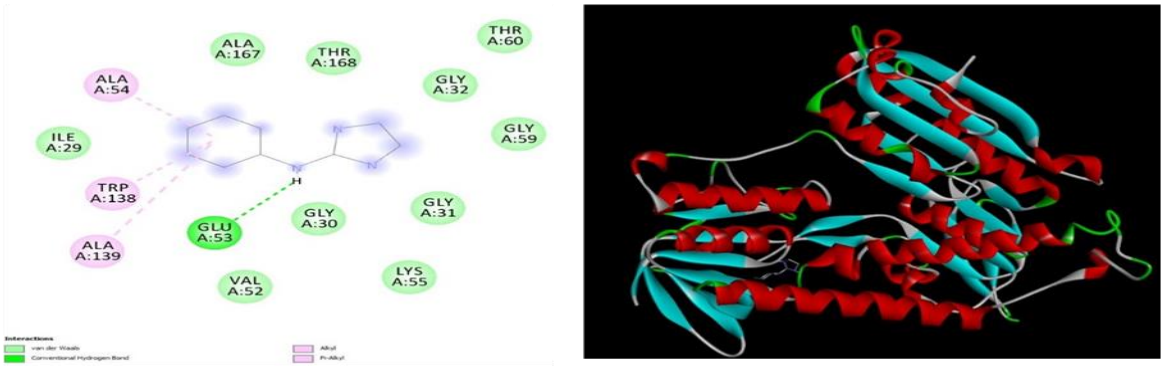
Figure 3. Lineweaver–Burk inhibition plot of BNZ with GR activity.

Table 2. Kinetic parameters of CHX and BNZ with GR activity.

Imidazoline concentrations (mg/L)	Vmax (U/mg)	Km (M, GSSG)	Ki (nM)
0	832	4.08×10^{-4}	0
200 CHX	1057	8.45×10^{-4}	59 ± 17
400 CHX	1064	9.36×10^{-4}	59 ± 17
200 BNZ	965	7.62×10^{-4}	43 ± 2
400 BNZ	1435	20.52×10^{-4}	43 ± 2



Benzyl-(4,5-dihydro-1H-imidazol-2-yl)amine hydroiodide



Cyclohexyl-(4,5-dihydro-1H-imidazol-2-yl)amine hydroiodide

Figure 4. Computed binding geometry of aminoimidazolines in the active site of 2HQM.

Table 3 and Figure 4 show that the NH groups of aminoimidazoline compounds form hydrogen bonds with GR amino acid residues THR A168, ALA A167 (for BNZ) and GLU A53 (for CHX). In addition, BNZ interacts with the receptor via pi-electrons and forms more hydrogen bonds than CHX, which is consistent with experimental studies.

Table 3. Interactions of GR with the synthesized compounds and binding free energies in Kcal/mol.

Name of compound	Amino acid residue	Type of interaction	Docking Score (Binding Free Energy) Kcal/mol	RMSD (A)
BNZ	THR A168, ALA A167	Conventional hydrogen	-7.9	2.591
	ALA A139, LEU A299, MET A301, GLY A59, GLU A53, GLY A32, GLY A31, GLY A30, ILE A29	Van Der Walls		
	TRP A138, ALA A54	Pi-Pi stacked, Pi-sigma		
CHX	GLU A53	Conventional hydrogen	-7.2	2.107
	ILE A29, ALA A167, THR A168, GLY A32, THR A60, GLY A59, GLY A31, GLY A30, LYS A55, VAL A52	Van Der Walls		
	ALA A139, TRP A138, ALA A54	Pi Alkyl		

When we look at the literature, there are few studies examining the effects of 2-aminoimidazolines on GR. It has been observed that the effects of compounds similar to 2-aminoimidazolines on enzyme activity have been studied. For example, Öztürk Kesebir (2021) [14] found competitive inhibition of GR isolated from fresh human erythrocytes by 2-amino-4-(2,4-difluorophenyl)thiazole. This compound also has -N- and -NH_2 groups. Similarly, we found that CHX and BNZ competitively inhibited glutathione reductase enzyme activity. Korkmaz (2022) [15] studied the effects of some amino thiazole derivatives on Lactoperoxidase (LPO) purified from bovine milk obtained from local dairy farms in Erzurum. These amino thiazole derivatives (2-Amino-4-(4-bromophenyl) thiazole, 2-Amino-4-(2,4-difluorophenyl) thiazole, 4-(3,4-difluorophenyl)-1,3-thiazol-2-amino, 2-Amino-4-(5,6,7,8-tetrahydro-2-naphthyl)thiazole, 2-Amino-5-(methyl)-4-phenyl) thiazole, 2- Amino-4-(4-chlorophenyl)thiazole) inhibited the LPO as competitively. The type of inhibition found was parallel to our results. Ascenzi et al. (2002) [16] concluded in their study the competitive inhibition of rat nitric oxide synthase type I and II and bovine trypsin by N-Amidino-2-hydroxypyrrolidine having guanidine group. The results found were in agreement with our results.

4 CONCLUSION AND SUGGESTIONS

As a result, CHX and BNZ have -N- and -NH- groups (guanidine groups). These -N- and -NH- groups compete with GSSG for binding to GR. Then, instead of reducing GSSG via NADPH to GSH, GR cannot reduce GSSG and as a result, no GSH is formed. GR is competitively inhibited by CHX and BNZ.

Excluding -N- and -NH- groups, BNZ has 3 double bonds in benzene ring while CHX does not have these 3 double bonds in cyclohexane ring. Instead of GR reducing GSSG with NADPH and converting it to GSH, the 3 double bonds of the benzene ring can bind to GSSG, therefore NADPH cannot reduce GSSG and as a result GSH is not formed. When the BNZ and the CHX are compared; the BNZ has more hydrogen bonds, pi interactions and higher binding energy than the CHX, which supports the experimental data. At imidazoline concentrations of 400 mg/L, CHX reduced GR activity by 16.07 %, while BNZ reduced GR activity by 21.96 %. BNZ inhibited GR more than CHX.

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Conflict of Interest Statement

There is no conflict of interest among the authors.

Statement of Research and Publication Ethics

The study is complied with research and publication ethics

Artificial Intelligence (AI) Contribution Statement

This manuscript was entirely written, edited, analyzed, and prepared without the assistance of any artificial intelligence (AI) tools. All content, including text, data analysis, and figures, was solely generated by the authors.

Contributions of the Authors

Hasan Karadag: Methodology, Experimentation, writing, review, editing. Murat Genc: Producing of synthetic materials, Molecular docking, writing, review, editing.

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