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Investigation of tyrosinase inhibition by some 1,2,4 triazole derivative compounds: in vitro and in silico mechanisms

Bazı 1,2,4 triazol türevi bileşiklerin in vitro ve in siliko mekanizmalarla tirozinaz inhibisyonunun incelenmesi

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Abstract

Background: Tyrosinase plays a central role in the biosynthesis pathway of melanin pigment. Melanin protects human skin against radiation and its unusual levels cause some skin disorders such as pregnancy scar, oldness spots and melanoma. Tyrosinase has also been linked to Parkinson’s and other neurodegenerative diseases. In addition, melanin plays a critical role as a defense molecule for insects during wound healing and is important for their life. Therefore, determination of inhibitor molecules for tyrosinase has a promising potential for therapies of some diseases and is an alternative method for keeping insects under control.

Material and methods: In this study, 1-heptyl-3-(4-methoxybenzyl)-4H-1,2,4-triazole-5-one derivative (A6, A8, A15) and 3-(4-chlorophenyl)-5-(4-methoxybenzyl)-4H-1,2,4-triazole (B5, B9, B13) derivative compounds were evaluated in terms of their potential for mushroom tyrosinase inhibition. IC$_{50}$ values of these six molecules were determined.

Results: It was seen that B9 molecule was the most effective inhibitor. Docking studies also nearly supported this end result. Tyrosinase inhibition type and Ki value were found to be uncompetitive and 370.7±0.3 μM, respectively, in the presence of B9 compound.

Conclusion: These results suggest that B9 compound is a potential tyrosinase inhibitor.

Keywords: Docking; Inhibition; Melanoma; Triazole; Tyrosinase.

Öz


Gereç ve Yöntem: Bu çalışmada, 1-heptyl-3-(4-methoxybenzyl)-4H-1,2,4-triazole-5-one türevi (A6, A8, A15) ve 3-(4-chlorofenil)-5-(4-methoxybenzyl)-4H-1,2,4-triazole (B5, B9, B13) türevi bileşikler, mantar tirozinaz inhibisyonu açısından potansiyel olarak değerlendirildi ve bu altı molekülün IC$_{50}$ değerleri belirlendi.


Sonuç: Bu sonuçlar, B9 bileşinin potansiyel bir tirozinaz inhibitöri olduğunu göstermektedir.

Anahtar Kelimeler: Doking; İnhibisyon; Melanoma; Triazol; Tirozinaz

Introduction

Besides the fact that enzymes and their activities are extremely necessary for life, the selective inhibition of critical enzymes is also considerably important for chemotherapeutic intervention in some diseases. Unregulated high enzyme activity results in the formation of reaction products at abnormal levels which can cause specific pathologies. Nowadays, the strategy of selective enzyme inhibition gets attention in modern pharmacy and enzymes have become interesting targets in drug therapies [1]. For this reason, many organic molecules have been synthesized as specific enzyme inhibitors and continue to be synthesized. Modeling methods for the three-dimensional structure and topology of the enzyme, active site and the organic molecules help to researcher to design new drug molecules or evaluate synthesized molecules for their enzyme inhibition potentials.

Tyrosinase (Polyphenol oxidase, PPO, E.C.1.14.18.1) which is a copper-containing metalloenzyme, catalyzes two major reactions in the biosynthesis pathway of melanin pigment: (1) the hydroxylation and oxidation of monophenols to o-quinones (monophenolase activity) and (2) the oxidation of o-diphenols to o-quinones (diphenolase activity) [2]. Melanin is an important pigment which is found in the eyes, hair and skin of animals and especially protecting human skin against radiation [3]. The melanin pigment is produced in the melanocyte cells located in the basal layer of the human epidermis and is called melanogenesis. However, excessive production and hyperpigmentation of melanin causes of dermatological disorders such as melasma, ephelides, chloasma, freckles, melanoderma and senile lentigines and can induce inflammation such as eczema, irritant and allergic eczema, contact dermatitis, which can result in critical and emotionally distressing trouble [4–6].

Melanoma resulted from abnormal accumulation of melanin is one of the fastest-spreading and deadly skin cancers among cancer types worldwide [7]. As the tyrosinase enzyme is found only in melanoma cells, inhibition of this enzyme will help to establish a highly specific treatment for skin cancer. So, in melanoma cells, a wide variety of inhibitors are used for the inhibition of tyrosinase enzyme [8]. However, it is expected that these inhibitors only target cancer cells and do not cause damage to DNA of healthy cells. Unfortunately, today’s cancer treatments are not long-lived. The greatest reason for this is that the chemotherapeutics applied for the treatment delay or prevent the definitive treatment of the disease by causing damage to the DNA of the healthy cells in the body [9]. This encourages the researchers in the world to design, synthesis and kinetically investigation of new organic molecules without side effect. Tyrosinase is not only associated with melanoma but also with Parkinson’s disease and some other neurodegenerative diseases [10]. The limited number of pharmacological agents used for Parkinson’s disease inhibitors are reported to show their effectiveness in reducing or preventing the progression of the disease. It is reported that the increased amount of enzyme also increases the amount of metabolites that are formed, leading to oxidative damage to the body in the long run. Therefore, synthesizing therapeutic drugs for Parkinson’s disease provides a vital way to treat this disease [8, 11].

Melanin plays a critical role as a defense molecule for insects during molting process and wound healing and is important for their survival. In addition, quinonoid intermediates generated by tyrosinase serve as defense molecules for insects. All of these make tyrosinase a potential target for developing effective inhibitors as insecticides for insect control [2].

Alkylbenzoic acids, tropolone, arbutin, aromatic acids, aromatic aldehydes and kojic acid, and lately, dialkylphosphorylhydrazones have been reported as well-known tyrosinase inhibitors. However, due to the low activity and cellular toxicity of these inhibitors in clinical use, effective and feasible new anti-tyrosinase agents should be developed [12, 13].

Many heterocyclic compounds containing 1,2,4-triazole ring is known to have important pharmacological properties such as anticonvulsant, antifungal, antimicrobial, analgesic, antiviral, anti-inflammatory, antioxidant, antitumor, anti-HIV and antihypertensive [14]. In addition, many compounds containing the 1,2,4-triazole ring are used as medicines in the market [15]. In recent year, synthesis of new thiosemicarbazones, phenylbenzoic acid, apocarotenoids, fumaric acid, 4H-chromene analogs, oxaloacetic acid as inhibitors of mushroom tyrosinase were reported. These molecules have been evaluated in terms of tyrosinase inhibition potentials by studying
kinetic mechanism and molecular docking [16–19]. In another study, some novel synthesized hydroxybenzaldehyde based kojic acid analogs as potential tyrosinase inhibitors were evaluated by performing biological activity and kinetic mechanism [20]. Several studies have also been conducted with new scaffold type of phenylbenzoic acid derivatives synthesized as potential tyrosinase inhibitor [18]. Also, *Lippia origanoides* essential oils and neorauflavane were isolated from *Campylotropis hirtella* supported by kinetic studies in terms of tyrosinase inhibitory potency [5, 21]. Addition to in vitro and in silico studies, in vivo studies have been carried out by the researchers for declaring tyrosinase inhibition potential of some newly synthesized molecules. As an example of recent studies on this perspective, chalcone derivative 1-(2-cyclohexylmethoxy-6-hydroxy-phenyl)-3-(4-hydroxymethyl-phenyl)-propenone and Na7PMo11CuO40 investigated in terms of tyrosinase inhibitor effect with in vivo anticancer studies [22].

However, due to the wide pharmaceutical properties of the triazole compounds is well known, the purpose of this study is to examine tyrosinase inhibition potentials of some newly synthesized 1,2,4-triazole derivatives (Table 1) by performing biochemical, kinetic and molecular modeling studies.

### Materials and methods

#### Chemicals

The main chemicals used in this study were ethanol (Merck, Germany), mushroom tyrosinase (Sigma, T3824, ≥1000 unit/mg solid, USA), *L*-tyrosine (Sigma, T3754, USA), *N,N*-dimethylformamide (DMF, Sigma, 227056, USA), 3-Methyl-2-benzothiazolinone hydrazide hydrochloride hydrate (MBTH, Sigma, 129739, USA), Kojic acid (Sigma, K3125, USA).

#### Tyrosinase assay, activity optimization and inhibition

Tyrosinase (polyphenol oxidase, PPO) activity was determined according to the method described by Espin et al. [26] with slight modification and kojic acid was used as a standard inhibitor. Briefly, 100 μL *L*-tyrosine solution (0.14 mM), 720 μL citrate-phosphate buffer solution (50 mM, pH 5.0), 100 μL MBTH solution (1 mM), 20 μL DMF (anhydrous), 10 μL pH 5.0 citrate-phosphate buffer solution (in inhibiton studies, it replaces with equal volume of inhibitor solution) and 50 μL tyrosinase solution (43.5 μg/mL in citrate-phosphate buffer solution) were added to the reaction mixture, respectively.

Optimum pH, temperature and reaction time, and *K*<sub>m</sub> and *V*<sub>max</sub> values were separately determined before inhibition studies for avoiding errors. For inhibition studies, 10 μL of each inhibitor solutions (in ethanol) were added onto 50 μL tyrosinase solution and preincubated at 25°C for 15 min. The activity was determined in the presence of *L*-tyrosine as substrate by measuring the increase in absorbance due to the formation of quinonoid compound as reaction product at 500 nm.

One unit of PPO activity was defined as 1 μmol of product formed per min in 1 mL of reaction mixture. The inhibition efficiencies of examined organic molecules were expressed as the concentration which inhibits 50% of the enzyme activity (*IC*<sub>50</sub>).

#### Determination of *K*<sub>m</sub>, *V*<sub>max</sub>, *K*<sub>i</sub> and inhibition type

After measuring tyrosinase activities in the absence and presence of inhibitor molecule (B9; 0 μM, 200 μM and 400 μM), Lineweaver-Burk graphics were prepared in the 50–700 μM *L*-tyrosine concentration range for the estimation of inhibition type, *K*<sub>i</sub> value and changes in *K*<sub>m</sub> and *V*<sub>max</sub> values.

#### Enzyme and compounds preparation for docking

Firstly, the inhibitor organic molecules given in Table 1 were optimized by using the Gaussian 03 program for docking studies. DFT 6-31G (d, p) was used as the optimization method and the most appropriate conformations of the compounds were identified [27, 28]. Crystalline form of the tyrosinase selected as the target receptor for which the docking of the compounds is to be performed was obtained in the PDB format (2Y9W for *Agaricus bisporus* tyrosinase) from the Protein Data Bank (PDB) website after crystalline form was found from literature [3]. Enzyme was purified using Discovery Studio 4.1 Client program and then ligand-protein interactions of the organic compounds in the binding pocket were investigated using AutoDock Vina 1.1.2 and PyMOL programs [29–32]. The binding energies of organic molecules were calculated and the inhibition potentials were theoretically monitored.
Table 1: Organic molecules evaluated for tyrosinase inhibition potentials.

<table>
<thead>
<tr>
<th>Code</th>
<th>Nomenclature</th>
<th>Formulas</th>
</tr>
</thead>
<tbody>
<tr>
<td>A6</td>
<td>2-[3-(4-Methoxybenzyl)-1-heptyl-5-oxo-1,5-dihydro-4H-1,2,4-triazol-4-yl]-N′-<a href="methylidene">5-chloro-2-hydroxyphenyl</a> acetohydrazide</td>
<td></td>
</tr>
<tr>
<td>A8</td>
<td>2-[3-(4-Methoxybenzyl)-1-heptyl-5-oxo-1,5-dihydro-4H-1,2,4-triazol-4-yl]-N′-<a href="methylidene">2-hydroxy-5-nitrophenyl</a> acetohydrazide</td>
<td></td>
</tr>
<tr>
<td>A15</td>
<td>2-heptyl-4-[(4-phenyl-4,5-dihydro-5-thione-1H-1,2,4-triazol-3-yl)methyl]-5-(4-methoxybenzyl)-2,4-dihydro-3H-1,2,4-triazol-3-one</td>
<td></td>
</tr>
<tr>
<td>B5</td>
<td>4-[(4-{(fluorophenyl)methyl}(amino)-5-[(3-(4-chlorophenyl)-5-(4-methoxybenzyl)-4H-1,2,4-triazol-4-yl)-methyl]-2,4-dihydro-3H-1,2,4-triazole-3-thione</td>
<td></td>
</tr>
<tr>
<td>B9</td>
<td>4-{(4-(fluorophenyl)methyl}amino)-2-(4-methylpiperazin-1-yl)-5-[(3-(4-chlorophenyl)-5-(4-methoxybenzyl)-4H-1,2,4-triazol-4-yl)methyl]-2,4-dihydro-3H-1,2,4-triazole-3-thione</td>
<td></td>
</tr>
</tbody>
</table>
Statistical analysis

Statistical analyses were performed using SPSS software (version 23), and the data were compared by one way ANOVA test. The differences between groups were analyzed by Post-hoc test. Tukey test p-values less than 0.05 were considered as significant.

Results

After optimization of tyrosinase activity (Table 2), inhibition studies were carried out in the presence of 0.14 mM ($K_m$) L-tyrosine substrate as described above. Percentage relative activities were separately plotted versus inhibitor concentrations for each organic molecule and the inhibitor concentration at which the relative activity is reduced by 50% determined as $IC_{50}$ value. Accordingly, B9 among the examined compounds was found as the most effective inhibitor for tyrosinase activity (Table 2). Changes in $K_m$ and $V_{max}$ values were observed by using Lineweaver-Burk graphs in the absence and presence of (0 μM, 200 μM and 400 μM) the B9 compound. The type of inhibition and $K_i$ value were also identified for B9 compound (Figure 1, Table 3). According to this, inhibition type was found to be uncompetitive and $K_i$ value was calculated as $370.7 \pm 0.3$ μM.

The compounds were investigated by performing docking studies with the crystal structure of mushroom tyrosinase to observe the interaction of these compounds with the enzyme. The top 9 predicted conformations of organic compounds generated by DFT 6-31G(d,p) were retained for analyzing the binding affinities of them. Accordingly, the results of the docking studies nearly support the experimental results and show that the B9 compound among the evaluated molecules binds to the enzyme (Figure 2A) efficiently as an inhibitor (Table 2).

importance of inhibitor-metal ion interactions in tyrosinase binding site which is a Cu$^{2+}$ containing metallo-enzyme and its highlights were discussed in detail with different perspectives [33, 34]. The interactions of different groups of the B9 molecule with the amino acid side chains in the appropriate position in the three-dimensional structure of the enzyme are also important in the formation of the enzyme-inhibitor complex (Figure 2B). Some of these highlights are listed as follows; (1) $\pi$-donor and $\pi$-alkyl interactions of chlorophenyl ring of B9 with Ser146 and Ala202, respectively, (2) $\pi$-anion, $\pi$-anion, $\pi$-alkyl
and conventional interactions of triazole ring with Asp99, Asp113, Ala110 and Ser96, respectively, (3) π-alkyl interaction of methoxyphenyl ring with Ala110.

For the detection of tyrosinase inhibition, in the light of these explanations, 1-heptyl-3-(4-methoxybenzyl)-4H-1,2,4-triazole-5-one derivative (A6, A8, A15) and 3-(4-chlorophenyl)-5-(4-methoxybenzyl)-4H-1,2,4-triazole derivative (B5, B9, B13) compounds shown in Table 1 were used and the inhibition studies were carried out in the presence of L-tyrosine substrate (0.14 mM). The IC$_{50}$ value in the presence of the B9 compound was determined to be 400 μM. In the presence of other inhibitor molecules, inhibition of tyrosinase was observed in the range of 20–55% even at the highest inhibitor concentrations tested (Table 2).

To determine the inhibition type of tyrosinase activity, biochemical kinetic studies were performed at two different concentrations of the B9 compound. Likewise, it has been reported that inhibition efficiency and mechanisms of the molecules derived from a particular starting molecule are likely to be similar. Graphs of 1/[S] – 1/V were plotted for each studied concentration of B9 compound.

Table 3: Type of tyrosinase inhibition in the presence of B9 compound and some kinetic parameters.

<table>
<thead>
<tr>
<th>[Inhibitor] (μM)</th>
<th>Apparent $K_m$ (μM)</th>
<th>$V_{max}$ (μmol/min)</th>
<th>Type of inhibition</th>
<th>$K_i$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>257.9 ± 1.4</td>
<td>149.3 ± 1.2</td>
<td>Uncompetitive</td>
<td>370.7 ± 0.3</td>
</tr>
<tr>
<td>200</td>
<td>192.8 ± 0.9</td>
<td>109.9 ± 0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>71.0 ± 0.7</td>
<td>41.2 ± 0.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Figure 2](image)

Figure 2: Predicted conformation of the compound inside the binding pocket of mushroom tyrosinase. (A) general projection (B) micro environment which shows various types of interactions of the compounds atoms with the amino acid residues.
Accordingly, as a result of activity measurements in the absence of inhibitor, $K_m$ and $V_{max}$ values were determined to be 2579 μM and 149.3 μmol/min, respectively. Whereas the concentration of B9 compound had an increase from 200 μM to 400 μM, both $K_m$ (192.8 μM and 71.0 μM, respectively) and $V_{max}$ (109.9 μmol/min and then 41.2 μmol/min) values decreased (Table 3).

Binding energies of inhibitor molecules were calculated in molecular docking studies for mushroom tyrosinase. The molecule with the lowest binding energy has the strongest interaction with the enzyme and thus it can be said that it is a potential molecule that causes inhibition. Accordingly, it was determined that among the studied six molecules, B13, B9 and A6 compounds were the most potent for tyrosinase inhibition (Table 3). But B9 had lowest $IC_{50}$ value than the others and so our study focused on B9 compound. This demonstrates that in vitro and in silico study results almost support each other. It has been identified that the B9-tyrosinase complex is mainly consisting some kind weak interactions such as π-alkyl interaction, π-anion interaction, π-donor interaction and conventional interaction.

**Discussion**

Currently, many compounds used as drugs possess five member heterocyclic rings such as 1,2,4-triazole, 1,3,4-thiadiazole and 1,3,4-oxadiazole derivatives having various isomers in terms of the position of the heteroatoms and a broad spectrum of biological activity particularly anticancer, antibacterial, antifungal anti-HIV, antiviral, antidepressant, antiinflammatory, antituberculosis, diuretic, analgesic [35, 36]. In addition, the easily synthesis of Schiff and Mannich base derivatives of 1,2,4-triazoles and using in a variety of applications have led these compounds to attract a great deal of attention in biology and chemistry in the last decade [37, 38]. It has also been reported that some 1,3,4-thiadiazole, 1,3,4-oxadiazole, 1,2,4-triazole and substituted hydrazide compounds have been synthesized as alternative inhibitors to hyperpigmentation for clinical usage [2]. Some new synthesized triazole compounds are used in the market but they are not indicated to be suitable for clinical use due to their mild activity and safety concerns [39, 40]. So, in this study, some of the synthesized 1,2,4-triazole derivative compounds by Bekircan et al. [23–25] have been examined in terms of tyrosinase inhibition potentials.

Prior to initiation of inhibition studies, tyrosinase activity was determined in accordance with the literature and then optimized. So, optimum temperature and pH value of tyrosinase activity and the amount of enzyme and substrate to be used in the reaction mixture for inhibition studies were determined. We paid attention to some ordered critical points when performing inhibition studies [1]: (i) to determine the $IC_{50}$ value of each inhibitor molecule, substrate concentration in reaction mixtures was adjusted to $K_m$ value calculated in optimization studies. Later, the type of inhibition and $K_i$ values were tried to be determined in the presence of the inhibitor molecule for which $IC_{50}$ value was found lowest. (ii) for this study, activity measurements were made at a series of substrate (in the concentration range of 0.2–5.0 $K_m$) and inhibitor concentrations (range caused 10–75% tyrosinase inhibition), (iii) after adding inhibitor solutions (in ethanol) to the reaction mixture, the final concentration of the organic solvent in the reaction mixture was adjusted to not exceed 1%. As declared before, organic solvents start to inhibit enzymes in the presence of their higher concentration than 3% [1]. It was also observed that tyrosinase partly inhibited by ethanol in its high concentration. For this reason, suitable blank mixtures were prepared considering the inhibitory effect of the solvent and relative activities are calculated according to this fact. Besides that even if the organic compounds are prepared at a given concentration in a suitable organic solvent, it has been observed that there is occasional collapse after the addition to the reaction medium. It can be attributed to the fact that the inhibitor organic molecules can precipitate in reaction mixtures containing largely buffer solution. Because of each organic molecule used in the study has a different behavioral profile as stated above, the concentration range of inhibitor molecules used in the inhibition studies for the tyrosinase activity was also different from the molecule to molecule (Table 2).

Kojic acid was used as the reference inhibitor molecule in this study and its $IC_{50}$ value was determined to be 18 mM. In this case, it can be said that the B9 compound used in the study is a more potent tyrosinase inhibitor than kojic acid. However, due to solubility problems, the B9 compound could not be studied for concentrations higher than 450 μM and a maximum inhibition (55.2%) was observed at this concentration. But, kojic acid that is a water-soluble molecule inhibited almost completely tyrosinase at a final concentration of 1 M. Oyama et al. [18] calculated $IC_{50}$ values for three different phenylbenzoic acid derivative molecules in terms of tyrosinase inhibition and reported the highest and lowest $IC_{50}$ values as >1000 μM and 6.97 μM, respectively. In another study, Gou et al. [17] observed tyrosinase inhibition in the presence of fumaric acid with the $IC_{50}$ and $K_i$ values 13.7 ± 0.25 mM and
12.64 ± 0.75 mM, respectively. Xie et al. [20] also found the highest and lowest IC₅₀ values of novel 14 synthetic compounds which were hydroxybenzaldehyde based kojic acid analogs as 17.50 ± 2.75 μM and 1.35 ± 2.15 μM, respectively. According to these results, the inhibitor potential of B9 compound is more effective than the molecules reported by Oyama et al. [18] and Gou et al. [17] but they are not when compared Xie’s molecules.

It was determined that tyrosinase inhibition type was uncompetitive in the presence of B9 compound (Figure 1). Therefore, it can be said that B9 is caused an inhibition for tyrosinase activity by binding enzyme-substrate complex reversibly with weak interactions at a site other than the active site of enzyme. In addition, Kᵢ value was determined as 370.7 ± 0.3 μM as a result of this study. However, Soares et al. [19] investigated the effect of thiosemicarbazones derivatives (thio-1) on tyrosinase inhibition and they reported the Kᵢ as varying values between 7.3 mM and 0.06 mM and the inhibition patterns as uncompetitive mixed (thio-1,5), uncompetitive (thio-6) and competitive (thio-2,4,7-8) inhibition. Brasil et al. [16] also found the highest and lowest Kᵢ values of 4H-Chromene analogs four synthetic compounds in order of 2.40 mM and 4.00 μM.

In conclusion, tyrosinase inhibition has a pharmacological importance. So, some newly synthesized triazole derivative molecules were evaluated in terms of their tyrosinase inhibition efficiencies as a first. It was determined that the B9 molecule among six molecules which was not studied before in terms of the tyrosinase inhibition potentials, caused tyrosinase inhibition at micromolar level. These in vitro studies have also been supported by molecular modeling studies. It can be speculated that further chemical and pharmacological studies on tyrosinase inhibition may perform for B9 molecule.

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Conflict of interest: The authors have declared no conflict of interest.

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