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Integrated kinetic studies and computational analysis on naphthyl chalcones as mushroom tyrosinase inhibitors

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ABSTRACT

Melanin helps to protect skin from the damaging ultraviolet radiation of the sun. Tyrosinase, the key enzyme in melanogenesis is responsible for coloration of skin, hair and eyes. This enzyme is considered to have a critical role in governing the quality and economics of fruits and vegetables, as tyrosinase activity can lead to spoilage through browning. Development of tyrosinase inhibitors is a promising approach to combat hyperpigmentation conditions like ephelides, lentigo, freckles and post-inflammatory hyperpigmentation. In the present study, we have used a docking algorithm to simulate binding between tyrosinase and hydroxy-substituted naphthyl chalcone oxime compounds and studied the inhibition of tyrosinase. The results of virtual screening studies indicated that the estimated free energy of binding of all the docked ligands ranged between −19.29 and −9.12 kcal/mol. Two of the oximes synthesized were identified as competitive tyrosinase inhibitors and were found to be twice as potent as the control kojic acid with their IC_{50} values of 12.22 μM and 19.45 μM, respectively. This strategy of integrating experimental and virtual screening methods could give better insights to explore potent depigmentation agents.

The quantity of melanin is an important determinant of skin color in humans but an excessive amount of it could be detrimental. Abnormal pigmentation is related to a variety of cosmetic and clinical conditions including melasma, lentigo, age spots, inflammatory hypermelanosis and trauma-induced hyperpigmentation.[1–3] Melanin is formed by a combination of enzymatically catalyzed chemical reactions. The major rate limiting step in melanin biosynthesis involves the enzyme tyrosinase [EC 1.14.18.1] that catalyzes two different reactions of melanin biosynthesis, the hydroxylation of L-tyrosine to L-DOPA (L-3,4-dihydroxy phenylalanine) and oxidation of L-DOPA to DOPA quinone.[4] In addition, tyrosinase is responsible for undesired enzymatic browning of fruits and vegetables that take place during senescence or damage in post-harvest handling, which makes the identification of novel tyrosinase inhibitors extremely important.[5] Tyrosinase or polyphenol oxidase (PPO) inhibitors have been used as herbicides to control weeds.[6] It has also been suggested that tyrosinase may contribute to the neurodegeneration associated with Parkinson's disease.[7] Thus, studies on tyrosinase inhibitors have gained immense significance mainly due to its wide range of applications in cosmetics as a depigmentation agent and in agriculture for controlling the quality and economics of fruits and vegetables.

From a structural perspective, tyrosinase belongs to a type-3 copper protein family known as the oxidoreductases harboring a catalytic center.[8–10] Tyrosinase has two copper ions in its active site which play a vital role in its catalytic activity. At the active site of tyrosinase, a dioxygen molecule binds in side-on coordination between two copper ions. Each of the copper ions is coordinated by three histidines in the protein matrix.[11] The copper atoms participate directly in hydroxylation of monophenols to diphenols (cresolase activity) and in the oxidation of *o*-diphenols to *o*-quinones (catechol oxidase activity) that enhance the production of the brown color.[12] Therefore, chelation of tyrosinase Cu^{2+} by synthetic compounds or agents from natural sources has been targeted as a way to inhibit or block tyrosinase catalysis.[13] An alternative solution to inhibit tyrosinase catalytic activity would be by effectively blocking access to the active site of enzyme. Several natural and synthetic tyrosinase inhibitors have been reported, including aromatic aldehydes and acids, tropolone, arbutin, flavonoids, and kojic acid.[14,15] However, many popular depigmenting compounds either lack potency or produce undesirable side effects. Kojic acid is currently applied as a cosmetic skin whitener and food additive to prevent enzymatic browning.[16] However, its use in cosmetics has been limited, because of its instability during storage.[17] In addition, kojic acid has been shown to promote thyroid and liver

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carcinogenicity in rodent models, leading to its ban in Switzerland.[18] Although hydroquinone has been the mainstay treatment for hyperpigmentation, its clinical potential has been complicated due to its cytotoxic and mutagenic properties.[19,20] This necessitates the need for further investigation of potential tyrosinase inhibitors that could be applied during food processing to curb enzymatic browning and for the treatment of hyperpigmentation disorders.

Chalcone, also known as phenyl styryl ketone is a privileged structure with immense therapeutic potential. The ease of synthesis, feasibility of oral administration and safety favor chalcones as promising therapeutic agents. Chalcones are open-chain flavonoids in which two aromatic rings are joined by a three carbon α,β-unsaturated carbonyl system (1,3-diphenyl-2-propen-1-ones).[18] The presence in chalcones of a conjugated double bond and a completely delocalized π electron system reduces their redox potentials and makes them prone to electron transfer reactions. The α-β unsaturated bond enables the chalcone to act as a Michael acceptor for nucleophilic species including glutathione (GSH) or cysteine residues on proteins.[21] As a part of our drug discovery programme, we have hypothesized that the hydroxyl group of the ligand may block the mushroom tyrosinase activity by binding to the Cu atoms in the tyrosinase active site based on the fact that previous findings had shown the role of hydroxyl groups in tyrosinase inhibition.[22] Oximes form an important class of organic compounds having the general formula RR′C═N—OH. They are important precursors to functional groups such as amines, nitro compounds and amides and also act as important ligands in the formation of mono- and polynuclear metal complexes.[23–26] Also, oximes upon deprotonation, can act as strongly coordinating ligands in metal-coordination chemistry.[27,28]

Classically, oximes are prepared by refluxing an alcoholic solution of a carbonyl compound with hydroxylamine hydrochloride and pyridine.[29] The excessive use of organic solvents, long reaction times, high temperatures, and extensive work-up procedures, make this solution-based synthetic method environmentally stressful and expensive. This has been circumvented by employing the use of a solvent free reaction that is not just eco-friendly but also found to be much less time consuming with improved selectivity and yields.[30] The method makes use of local heat generated by simply grinding the reactants and catalyzed by cheap and commercially available CaO for driving the chemical reaction at room temperature. Previously, several hydroxy-substituted 2-phenylnaphthalene derivatives were reported as potent tyrosinase inhibitors. Among them, 4-(6-hydroxy-2-naphthyl)-1,3-bezendiol (HNB) and 5-(6-hydroxy-2-naphthyl)-1,2,3-benzentriol (5HNB) were found to inhibit mushroom tyrosinase activity.[31,32] Hence, in our continuous effort to search for potential tyrosinase inhibitors and to develop a new template, we have attempted to design and synthesize a series of novel hydroxy naphthyl substituted chalcone oximes for application as depigmentation agents and as anti-browning food additives. To confirm our hypothesis, we simulated the docking between the ligands and tyrosinase and conducted kinetic studies. The docking score for the ligand with receptor is composed of various energy terms such as electrostatic energy, van der Waals energy, and solvation energy.[33] From the docking results, we checked for possible hydrogen-bonding and non-bonding interactions with the amino acid residues. For the control simulation, the docking simulation of kojic acid, a well-known tyrosinase inhibitor, with tyrosinase was also performed (Fig. 1).

Chalcones were synthesized by the base catalyzed Claisen–Schmidt condensation of an aldehyde and an appropriate ketone in a polar solvent like methanol (Scheme 1). In the second step, these naphthyl chalcones on reaction with hydroxylamine hydrochloride were converted into their corresponding oximes which were isolated and recrystallized from ethylacetate. This method emphasizes the effectiveness of CaO in oxime synthesis (Scheme 2) under grinding conditions without any rearrangement of α,β-double bond. The structures of the compounds were confirmed by ^{1}H NMR, ^{13}C NMR, FTIR and HRMS. The signals for phenolic hydroxyl protons appeared between 11 and 13 ppm and were indicated by ^{1}H NMR spectra. The signals for aromatic hydrogens are between 6.85 and 8.75 ppm. The signals for vinylic protons appeared between 9.5 and 13 ppm which confirms their *trans* conformations. Chemical shift values further confirm an anti-isomer conformation of the oximes. Assays were performed with L-DOPA as the substrate, using kojic acid, a well-known tyrosinase inhibitor as the positive control.

The parent naphthyl chalcones (**1a**–**1g**) showed poor or low tyrosinase inhibitory activities (Table 1). However, it was interesting to note that the oxime derivatives synthesized (**2a**–**2e**) had better tyrosinase inhibitory activities when compared with the positive control kojic acid, whereas the oxime compounds **2f** and **2g** had slightly decreased inhibition. In particular, compounds **2b** and **2c** exhibited the greatest inhibition of L-DOPA oxidase activity of mushroom tyrosinase with their IC_{50} values of 12.22 μM and 19.45 μM, respectively. These compounds were found to be more potent than the positive control, kojic acid (IC_{50}; 23.72 μM). Some possible structure–activity relationships could be inferred from tyrosinase inhibitory assay results: The pyridinyl nitrogen atom in compounds **2a**–**2c** can possibly get protonated at physiological pH or might be available to coordinate the copper atom existing in the tyrosinase active site. Additionally, some research groups have reported hydroxamate derivatives to possess tyrosinase inhibitory activities.[35] The structures of the non-cyclic moieties of our molecules have similar features to those of hydroxamic acids and hydroxamates which are good chelating agents. There is a possibility of the oxime moiety to coordinate with the copper metal at the active site of mushroom tyrosinase thereby preventing electron transfer by the metal ion. This could decrease the enzymes ability to oxidize the substrate subsequently leading to an inhibition in mushroom tyrosinase activity. This was in agreement with the experimental data. It has been suggested that the presence of a hydroxyl group and of an electron donator group in the phenol ring is a primary requirement for effective action as an alternative substrate of tyrosinase. The hydroxyl groups in compounds carry out the nucleophilic attack on the coppers of the tyrosinase active site and are directly involved in transferring protons during catalysis, which resulted in inactivation of tyrosinase. Also, the presence of an *ortho–para* directing methoxy group in compound **2d** could be accounted to its better tyrosinase inhibitory potential. The electron-donating groups increase the electron density of ring B through a resonance donating effect and higher electron density binds copper ions more effectively in the active site of enzyme. Thus, the inhibition mechanism of novel substituted hydroxy chalcone oximes **2a**–**2g** might involve binding to the copper active site of mushroom tyrosinase.

Accelrys Discovery studio 4.5 suite was utilized to simulate binding between the active site of mushroom tyrosinase and substituted hydroxy naphthyl chalcone oximes. To model the tyrosinase structure, we used the crystal structure of *Agaricus bisporus* (mushroom) tyrosinase (PDB ID: 2Y9X) A chain. The results of virtual screening studies indicated that the estimated CDOCKER energy of all the docked ligands ranged between −19.29 and −9.12 kcal/mol. Figure 1 shows selected docked conformations of compounds **2a**–**2g** along with the positive control, kojic acid in the tyrosinase binding site. Additionally, we searched for hydrogen bonding interactions between mushroom tyrosinase and the inhibitor compounds or kojic acid (Table 2). Among all the compounds docked, compound **2b** showed the lowest estimated free energy of binding which correlated well with the experimental results. We searched for tyrosinase residues that might bind to compound **2b**