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Research paper

Analogues of ethionamide, a drug used for multidrug-resistant tuberculosis, exhibit potent inhibition of tyrosinase

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ABSTRACT

Tyrosinase catalyzes two distinct sequential reactions in melanin biosynthesis: the hydroxylation of tyrosine to DOPA followed by the oxidation of DOPA to dopaquinone. The central roles of melanin in living species have motivated researchers to maintain constant efforts to discover new agents that modulate tyrosinase activity. In this study, we report on the inhibition of tyrosinase by ethionamide and its analogues. Ethionamide, 2-ethylpyridine-4-carbothioamide, is a second-line antituberculosis drug used for the treatment of multidrug-resistant tuberculosis. The chemical similarity of ethionamide to phenylthiourea, a well-known tyrosinase inhibitor, led us to investigate its inhibitory effects on mushroom tyrosinase and the IC_{50} was calculated as 4 μM. Five analogues of ethionamide, including another antituberculosis drug, prothionamide, were also inhibitory, with values for IC_{50} in the range of 3–43 μM. Fluorescence quenching experiments supported a mechanism of direct binding. In contrast, isoniazid, a structural analogue and first-line antituberculosis drug, was a poor inhibitor of tyrosinase. We also tested the effects of ethionamide and its analogues on melanin content in B16F10 cells. At a concentration of 50 μM, the molecules, pyridine-2-carbothioamide and thiobenzamide substantially decreased the melanin content by 44% and 37%, respectively. In addition to identifying other interactions, docking simulations showed that the carbothioamide groups of the molecules make essential contacts with the catalytic di-copper atoms. Our results suggest that carbothioamide can be a central moiety for the development of new and potent tyrosinase inhibitors.

1. Introduction

Type-3 copper proteins are a family that includes tyrosinase, catechol oxidase, and hemocyanin. These enzymes share an evolutionarily conserved motif of six histidines, three of which chelate with a copper atom, forming a di-copper (Cu_A and Cu_B) catalytic center. Hemocyanin carries oxygen, while tyrosinase and catechol oxidase catalyze the oxidation of polyphenols. Tyrosinase also facilitates the hydroxylation of monophenols. Thus, tyrosinase can catalyze two distinct sequential reactions, the hydroxylation of tyrosine to DOPA followed by the oxidation of DOPA to dopaquinone, which leads to melanin via eumelanin. The skin color of humans depends on the quantity of melanin, which is associated with the colors of eyes and hair and the browning of food. Abnormal activity of tyrosinase is related with disorders including albinism, a congenital disorder where the body synthesizes very little melanin because of a deficiency or absence of tyrosinase. Overactivity of tyrosinase, resulting in increased amounts of melanin, also causes skin disorders. Abnormal activity of tyrosinase has been reported in other diseases including cancer and Parkinson's disease [1–3].

Tyrosinase has four possible oxidation states of the copper at the active center: oxy-, met-, deoxy- and deact-states [4]. In the oxy-state the two copper atoms form a planar structure with two oxygen atoms, [Cu(II)–O_2^{2-}—Cu(II)], whereas in the met- and deoxy-forms they exist as [Cu(II)–Cu(II)] and [Cu(I)–Cu(I)], respectively. In the met-state two Cu(II) atoms are bridged by one or two hydroxide molecules while the coordinates around the copper are disrupted in the deact-state [4]. Crystal structures of two complexes, one between tropolone and mushroom tyrosinase (PDB ID: 2Y9X) [5] and the other between phenylthiourea (PTU) and sweet potato catechol oxidase (PDB ID: 1BUG) [6], revealed that the

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enzyme inhibitors bind to the proteins in the met-state, and not in the oxy-state. Crystal structures prepared with zinc in place of copper atoms could capture the substrates, L-tyrosine and L-DOPA, bound at the active site of tyrosinase from *Bacillus megaterium* [7]. The structures provide a snapshot of the subtle changes in the orientation required for the oxidation of monophenol and catechol, providing a clue to understanding the common and distinct features between tyrosinase and catechol oxidase.

The desire to treat skin disorders or develop skin-lightening cosmetics has fueled the continuous efforts of researchers to discover new agents that modulate tyrosinase activity [8–15]. *In vitro* enzyme- and cell-based assays have identified numerous natural and synthetic compounds as inhibitors of tyrosinase, including the natural phenolic compound arbutin, which has been used as a skin-lightening agent [8–15]. Another well-known major class of tyrosinase inhibitors includes PTU and its synthetic analogues [16–29].

Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis*. The World Health Organization reports that one third of the world population has been infected with *M. tuberculosis* [30]. The first-line drugs for the treatment of such patients are isoniazid (INH), rifampin, ethambutol, pyrazinamide, and streptomycin. The emergence of multidrug-resistant strains of *M. tuberculosis* has necessitated the development of second-line drugs, including fluoroquinolone, ethionamide, cycloserine, *p*-aminosalicylic acid, capreomycin, kanamycin, and amikacin [31,32]. Ethionamide (2-ethylpyridine-4-carbothioamide, ETH) is a structural analogue of INH, and both INH and ETH are pro-drugs that require activation by mycobacterial enzymes to exert their therapeutic effects. Interestingly, whereas both drugs target InhA, the enoyl acyl carrier protein reductase of mycobacterium, to exert their antibacterial activities, their activators are distinct. EthA enzyme modifies ETH, while INH is activated by KatG [32].

In this study, we retrieved compounds chemically similar to PTU from FDA-approved drugs. We identified ETH and its analogues and confirmed their abilities to inhibit tyrosinase activity by enzyme- and cell-based assays. This manuscript will also discuss the modes of binding predicted by a docking simulation for tyrosinase with ETH and its analogues.

2. Materials and methods

2.1. Chemoinformatics

We screened FDA-approved drugs for chemicals with a structural similarity to PTU. In total, 3180 molecules registered in the ZINC database [33] were screened and the similarity of each molecule to PTU was digitized using the Tanimoto coefficient (Tc) with Morgan circular fingerprints implemented in RDKit (http://www.rdkit.org). Tc has the values between 0 and 1, where 0 and 1 correspond to no and perfect overlap of two chemicals, respectively. To quantify the similarity between a test molecule and known tyrosinase inhibitors, we extracted the 483 known direct binding molecules deposited in the BindingDB database [34]. The ChEMBL database was also used to verify the bioactivities of the molecules in this study [35,36]. In-house written scripts, ALIS-PP (Automated LIgand Search for PolyPharmacology), automated the chemoinformatics procedures.

2.2. Enzyme activity assays and kinetics with inhibitors

All the reagents in this study were purchased from Tokyo Chemical Industry (Tokyo, Japan) or Sigma–Aldrich (St. Louis, MO, USA). The final reaction mixture (100 μL) for enzymatic assays included 200 nM mushroom tyrosinase and 500 μM substrate (L-tyrosine), along with test inhibitors in phosphate-buffered saline (PBS). All the solutions for measuring enzyme activity contained 5% dimethyl sulfoxide to solubilize organic molecules. The reaction mixture containing enzyme and inhibitor was incubated at 30 °C, and the change in absorbance at 475 nm after addition of substrate was measured in a time-dependent manner. After confirming the presence of inhibition at a single concentration of 50 μM, the IC_{50} was calculated by using the inhibitor at a series of different concentrations. For enzyme inhibitory kinetics, substrates at concentrations of 0.25, 0.33, 0.4, 0.5, 0.67, and 1 mM were chosen. The range of concentrations for each inhibitor was adjusted to include the IC_{50} value. The simultaneous nonlinear fitting of the Michaelis–Menten equation with the data for all concentrations allowed extraction of the kinetic parameters (V_{max}, K_m, K_{ic}, and K_{iu}) for four models, with minimized χ^2 values [37]. Here, V_{max}, K_m, K_{ic}, and K_{iu} indicate the maximum velocity, Michaelis constant, and dissociation constants between the substrate-free enzyme and inhibitor, and the substrate-bound enzyme and inhibitor, respectively. The χ^2 value is defined as the sum of the squared deviations between experimental and fitted values. The four models are competitive, uncompetitive, non-competitive, and mixed inhibitions [38]. Comparison of χ^2 values between the four models by means of *F*-statistics enabled selection of the proper model [37,39]. All fitting and statistical analyses in this study were done with MATLAB® (MathWorks, Natick, MA, USA).

2.3. Fluorescence quenching experiment

The changes in fluorescence intensity (excitation (λ_{ex}) and emission (λ_{em}) wavelengths set to 290 and 340 nm, respectively) in tyrosinase were recorded following the addition of ligands. Initially the fluorescence profiles around 340 nm were scanned while varying the concentrations of tyrosinase and ligands in order to identify the range for linear responses. A concentration of 0.25 μM tyrosinase, and 0–130 μM ligands were chosen. Neither red- nor blue-shifted fluorescence was observed for tyrosinase, however direct interaction of an inhibitor with tyrosinase resulted in fluorescence quenching. The Stern–Volmer equation was used to interpret the decrease in fluorescence intensity (F) on binding to a series of ligand concentrations ($[L]$) as binding affinity in:

$$\frac{F_0}{F} = 1 + k_q \tau_0 [L] = 1 + K_{SV}[L]$$

where F_0 and τ_0 are the fluorescence intensity and the fluorophore lifetime respectively of a protein in the absence of the ligand, k_q is the quencher rate constant, and K_{SV} is the Stern–Volmer quenching constant. We measured the intensities of fluorescence at concentrations of ligand of 0, 12, 24, 35, 45, 56, 65, 74, 83, 92, 100, 108, 115, 123, and 130 μM. All measurements were repeated three times for each concentration

2.4. Cell-based activity assays with inhibitors

B16F10 murine melanoma cells purchased from the Korean Cell Line Bank (Seoul, Korea) were used for cell-based assays. The cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin under 5% CO_2 at 37 °C. Cell viability was determined using the MTT assay. Extracellular melanin release was measured by protocols described as follows. B16F10 cells were grown to a density of 1×10^5 cells in 96-well plates. Test inhibitors (10, 20, 50 μM) and IBMX (3-isobutyl-1-methylxanthine, 100 μM) were added and the cells were transferred to 96-well plates and incubated for 48 h. The amount of melanin was measured at