Toward New Depigmenting Agents through Repurposing Existing Drugs: Substituted Hydroxyquinolines as Melanogenesis Inhibitors

**REFERENCES**


Langbein L, Rogers MA, Praetzel-Wunder S, Helmke B, Schirmacher P, Schweizer J, K25 (K25irs1), K26 (K25irs2), K27 (K25irs3), and K28 (K25irs4) represent the type I inner root sheath keratins of the human hair follicle.


Supplementary Material is linked to the online version of the paper at www.jidonline.org, and at http://doi.org/10.1016/j.jid.2022.07.010.
### Table 1. Inhibitory Activity of Hydroxyquinoline Drugs

<table>
<thead>
<tr>
<th>X</th>
<th>Y</th>
<th>Name</th>
<th>$K_i$ (micrometer)</th>
<th>IC$_{50}$ (micrometer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO$_2$</td>
<td>H</td>
<td>Nitroxoline (1)</td>
<td>0.9</td>
<td>0.1</td>
</tr>
<tr>
<td>Cl</td>
<td>Cl</td>
<td>Chloroxine (2)</td>
<td>12</td>
<td>1.0</td>
</tr>
<tr>
<td>Cl</td>
<td>I</td>
<td>Clioquinol (3)</td>
<td>367</td>
<td>n.d</td>
</tr>
<tr>
<td>I</td>
<td>I</td>
<td>Iodoquinol (4)</td>
<td>1,290</td>
<td>n.d</td>
</tr>
</tbody>
</table>

Abbreviations: Cl, chlorine; H, hydrogen; I, iodine; IC$_{50}$, half-maximal inhibitory concentration; $K_i$, inhibitor constant; n.d., not determined; NO$_2$, nitrogen dioxide.

---

**Figure 1. Characteristics of tyrosinase inhibition by hydroxyquinoline drugs.**

(a) In vitro fluorescence assays using mushroom tyrosinase were performed in 50 mmol/l sodium phosphate buffer at pH 7.4 with PAPAMC as substrate. Varying concentrations of inhibitors were used as indicated. Data represent the mean ± SD of three independent experiments. (b) Lineweaver–Burk analysis of tyrosinase inhibition by nitroxoline 1 was performed at concentrations noted (in micrometer). The experiment was performed in triplicate at pH 7.4, and data were plotted according to Lineweaver–Burk plot. Data represent the mean ± SD of three independent experiments. (c) Inhibition of melanin production in human MNT-1 cells by nitroxoline 1 and chloroxine 2 was assessed by incubation of cells with inhibitor for 3 days, harvesting and quantifying melanin as described in Supplementary Materials and Methods. Data represent the mean ± SD of three experiments. *$P < 0.05$ between control and inhibitor-treated cells. (d) Positions of metal-binding functional groups and lone pairs of kojic acid and nitroxoline 1. (e) View of nitroxoline 1 (magenta) within the active site of A bisporus tyrosinase when overlayed with the analogous atoms of tropolone (green) in the tyrosinase–tropolone cocrystal structure. OH, hydroxyl; PAPAMC, $p$-aminophenol aminomethylcoumarin.
recently been severely restricted (Liu et al., 2021).

Hence, demand exists for new efficacious drugs that hinder the overproduction of melanin in the skin. Similar to hydroquinone, most depigmenting agents inhibit the enzyme tyrosinase, a copper-containing oxidase found within melanocytes that catalyzes the rate-determining initial step of melanin synthesis (Sánchez-Ferrer et al., 1995). New tyrosinase inhibitors have been identified by high-throughput screening of small-molecule libraries (Germanas et al., 2007; Mann et al., 2018). Mechanistically, they act mostly as competitive inhibitors, vying for the active site with Y (Pillaiyar et al., 2017). Another strategy of drug discovery is drug repurposing or finding new applications for existing drugs for diseases for which they were not originally developed (Pushpakom et al., 2019). Repurposing-established drugs represent an expedient route to an effective therapy that may stabilize an epidemic until a more targeted drug is discovered (Wang et al., 2020) or for treatment of a rare disease (Cho et al., 2016; Lee et al., 2022). In this study, we characterize the potent depigmenting activity of hydroxyquinolines (Table 1), a class of approved antibiotics (Prachayasittikul et al., 2013). Although never previously tested against tyrosinase, we report that hydroxyquinolines are effective inhibitors and show that they act through a unique mechanism of action: metal chelation and extraction.

The compounds used in this study were hydroxyquinolines, a drug class previously approved by the United States Food and Drug Administration as antimicrobial agents. None had ever been studied as pigmentation inhibitors. Four specific drugs were chosen for their diverse array of substituents (Table 1). Initial screening of the hydroxyquinoline drugs for inhibitory activity was performed with mushroom tyrosinase (Espín et al., 2000). The previously described fluorescence-based tyrosinase assay using the substrate p-aminophenol aminomethylcoumarin was used to assess the inhibition of enzyme activity by the hydroxyquinoline drugs (Germanas et al., 2007). None of the compounds displayed a fluorescence signal at the wavelengths used that would interfere with the assay. All the four compounds exhibited a dose-dependent inhibition of the monophenolase activity of tyrosinase but to starkly varying degrees (Figure 1a). The 5-nitro–substituted derivative nitroxoline 1 was the most potent inhibitor, with an inhibitor constant value of 0.9 μmol/l (Table 1). On the other hand, iodoquinol 4 was the least active with a high micromolar inhibitory constant. At higher concentrations, the heavier-molecular-weight compounds clioquinol 3 and iodoquinol 4 were less soluble in an aqueous solution. The smaller compounds nitroxoline 1 and chloroxine 2 did not show evidence of insolubility under the assay conditions.

The specific type of enzyme inhibition of tyrosinase by hydroxyquinolines was next investigated through kinetics assays with the drug nitroxoline 1. Initial velocities of the reaction of p-aminophenol aminomethylcoumarin were measured with varying concentrations of substrate and nitroxoline and subsequently visualized in a series of Lineweaver-Burk plots. The intersection of the individual plots on the y-axis of the plot clearly shows that nitroxoline behaves as a competitive inhibitor (Figure 1b).

To assess the mode of action of the hydroxyquinoline drugs, residual enzyme activity after separation of the drug was measured to assess reversibility. Separation of nitroxoline 1 from the reaction mixture by ultracentrifugation revealed <10 % of the original activity after the addition of fresh p-aminophenol aminomethylcoumarin (Supplementary Figure S1). However, if the resulting enzyme concentrate was incubated with exogenous copper sulfate and then assayed with p-aminophenol aminomethylcoumarin, substantial recovery of original enzyme activity was observed. These observations suggest that nitroxoline reversibly extracts copper from the enzyme active site through the formation of a drug–copper complex.

Next, the ability of hydroxyquinolines to inhibit melanin production in cells was determined by exposing human melanoma MNT-1 cells to varying concentrations of the compounds (Hah et al., 2012). As positive and negative controls, cells were incubated with either the well-characterized tyrosinase inhibitor phenylthiourea or solvent DMSO, respectively. The hydroxyquinoline drugs caused different effects on the MNT-1 cells. At higher concentrations, the iodo-substituted compounds 3 and 4 were toxic, with decreased cell counts in the viability assay. On the other hand, nitroxoline 1 and chloroxine 2 were not toxic at chosen concentrations. Nitroxoline and chloroxine displayed a dose-dependent ability to block melanin production within MNT-1 cells (Figure 1c). Half-maximal inhibitory concentration values for blocking melanin synthesis by 1 and 2 were estimated at approximately 0.1 and 1.0 μmol/l, respectively (Table 1).

To gain greater insight into the structure-activity relationships of hydroxyquinolines with tyrosinase, molecular modeling studies were undertaken. A striking structural similarity is revealed when 8-hydroxyquinoline is compared with the well-known copper chelator and tyrosinase inhibitor, kojic acid (Lachowicz et al., 2015). When the important pharmacophore groups of these two structures, the hydroxyl and heteroatom lone pairs, are overlaid, an exact alignment and orientation of these groups are found (Figure 1d). Because these atoms are critical in the interaction between kojic acid and the active site of tyrosinase, it stands to reason that the analogous functional groups of 8-hydroxyquinoline would align similarly.

This hypothesis was tested by superimposing the structure of nitroxoline 1 onto that of tropolone (a molecule that has the same functional group orientations as kojic acid) in the crystal structure of the mushroom tyrosinase-inhibitor complex (Ismaya et al., 2011). A fit without adverse steric interactions in the active site resulted (Figure 1e). The 5-nitro group of nitroxoline faces outward, whereas the amine and hydroxyl groups orient toward the copper atoms. This fit explains why hydroxyquinolines with large 7-position substituents, such as 3 and 4, were less active inhibitors, owing to adverse steric interactions with the active-site periphery.

In conclusion, the demonstration in this study that 8-hydroxyquinoline antibiotics are potent inhibitors of melanin formation shows that repurposing is an effective route to the discovery of potential antipigmentation drugs. The clinical efficacy of select hydroxyquinolines to treat disorders of hyperpigmentation is being actively investigated.
SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2022.06.026

REFERENCES


SUPPLEMENTARY MATERIALS AND METHODS

Materials
Drugs and analogs were obtained from commercial suppliers. Mushroom tyrosinase was obtained from Worthington Biochemicals (Lakewood, NJ). Fluorescent tyrosinase substrate p-aminophenol aminomethylcoumaran (Germanas et al., 2007) was obtained from BioVision (Milpitas, CA). MNT-1 cells were obtained from ATCC (Manassas, VA) and were cultured and divided according to the recommended protocol.

Database search
The identification of potential inhibitors was pursued through a database search of approved drugs for those whose structure contained a phenol moiety. A phenol substructure was targeted owing to the frequent presence of this functionality in many known tyrosinase inhibitors as well as its presence within the natural substrate Y. We focused also on lower-molecular-weight molecules that would be of similar size to Y and would therefore fit in the enzyme’s active site. A substructure search of the Cheminfo database of Food and Drug Administration–approved drugs resulted in finding the class of antibiotics, aminoquinolines (Table 1).

Enzyme kinetics measurement
Enzyme activity was measured using the fluorescent substrate p-aminophenol aminomethylcoumarin with Agaricus (mushroom) tyrosinase (Germanas et al., 2007). Fluorescence spectroscopy was carried out on a Cary Eclipse spectrophotometer. Fluorescence intensity was measured at 6-second intervals in quartz cuvettes kept at 37 °C. Prospective inhibitors were tested to ensure that they had no fluorescent activity at the wavelengths used to reduce spectrophotometric artifacts.

The use of mushroom tyrosinase is justified by its ready availability and substrate specificity to the mammalian enzyme. In addition, inhibitors found to have a strong affinity for the mammalian enzyme possessed very close structural similarity to those that potently blocked the mushroom enzyme (Hornyak, 2018). In a 3-ml cuvette, buffer (total volume of 2.0 ml of 50 mM phosphate, pH 7.4) and DMSO (total DMSO in an assay of 0.050 ml) were heated to 37 °C for 2 minutes. Enzyme solution (final concentration of 0.25 mg/ml), and L-dihydroxyphenylalanine (0.002 ml of 5 mM) were added. After 5 minutes of incubation, the inhibitor was added as a solution in DMSO. After an additional 2 minutes of incubation, a DMSO solution of p-aminophenol aminomethylcoumarin (0.02 ml of 1 mM solution) was added, and after thoroughly mixing, the reaction was monitored by fluorescence (λexcitation = 350 nm, λemission = 440 nm). Initial rates were calculated using the slope of the curve for the first ca. 10% of the reaction. The first 1 minute of the reaction was not used owing to the usual induction period observed with tyrosinase. Inhibition assays were performed in triplicate trials. Nonlinear regression and curve fitting were done using GraphPad PRISM (GraphPad Software, San Diego, CA).

To assess the reversibility of enzyme inhibition, a reaction mixture containing 5 µm inhibitor was placed into Amicon Ultra centrifugal filters (10K molecular weight cutoff), concentrated at 4,000 r.p.m. for 10 minutes, followed by dilution with sodium phosphate buffer for a total of three cycles. To the remaining solution was added exogenous copper sulfate to a final concentration of 0.1 mM, if reconstitution of enzyme activity was being evaluated. Then fresh L-dihydroxyphenylalanine and p-aminophenol aminomethylcoumarin substrate were added, and fluorescence change was measured as previously described.

Melanogenesis assay in cells
To determine melanogenesis inhibition within cells, the human melanoma MNT-1 cell line (CRL-3450 from ATCC) was used. Frozen stocks (3.8 × 106 cells) of cells were thawed in a 37 °C water bath and then plated into three 10-cm plates containing 10 ml of MDEM + 10% fetal bovine serum and 100 µl of 100× penicillin/streptomycin. When the cells became confluent, the medium was aspirated and cells were rinsed with sterile PBS and then treated with 1 ml of 0.25% trypsin with 0.02% EDTA at 37 °C for 3 minutes. When the cells became loose, 4 ml of medium was added to neutralize the trypsin.

To assess inhibition of melanin synthesis, freshly passaged cells were seeded into six-well plates at a density of 30,000 cells per well, cultured in MDEM medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% carbon dioxide in the air. Inhibitor compounds were added in a DMSO stock solution to final concentrations of 20–200 µM. The well-characterized tyrosinase inhibitor phenylthiourea was used as a positive control. After 3 days, the cells were washed with PBS and dissolved in 250 µl of 1 N sodium hydroxide for 1 hour at 80 °C and transferred to 96-well plates. The melanin content was determined by measuring absorbance at a wavelength of 405 nm. Percent inhibition for each concentration of each test compound was calculated according to the following equation:

\[
\text{Percent inhibition} = \left(1 - \frac{A_{\text{cmpd}} - A_p}{A_n - A_p}\right) \times 100
\]

Where \(A_{\text{cmpd}}\) is the absorbance value for the test compound, and \(A_p\) and \(A_n\) are the absorbance values of the positive and negative controls, respectively.

The compounds were tested for toxicity by measuring viability with the WST-1 assay (MilliporeSigma, Burlington, MA). With the exceptions noted in the manuscript, tested derivatives did not display toxicity at the concentrations reported.

Statistical analysis
Data are expressed as mean ± SEM. Statistical comparisons were performed using GraphPad PRISM (GraphPad Software). Statistical comparison between two groups was analyzed by Student’s t-test.

Molecular modeling
Structures of hydroxyquinolines were obtained from PubChem (Bethesda, MD). The crystal structure of...
A. bisporus (mushroom) tyrosinase complexed with the inhibitor tropolone (PDB: 2Y9X) was retrieved from the Protein DataBank (www.rcsb.org). Overlay and visualization of protein–inhibitor complexes were assessed with PyMOL, version 2.5.0. Specifically, the pair-fitting algorithm of PyMOL was used to align the analogous atoms of nitroxoline (O1, C8, C8a, and N1) with those of tropolone (O2, C2, C1, and O1), respectively.

Supplementary Figure S1. Enzymatic activity after ultrafiltration of nitroxoline. Bars represent the percentage of initial activity (a) without inhib, (b) with 5 µM of inhib before ultrafiltration, (c) with 5 µM of inhib after ultrafiltration and 30-minute incubation with 0.1 mM final concentration Cu sulfate, (d) 10-minute incubation with 0.1 mM final concentration Cu sulfate, and (e) after ultrafiltration and no exogenous copper sulfate. Cu, copper; inhib, inhibitor; min, minute.

SUPPLEMENTARY REFERENCES