2-Hydroxypyridine-\(\text{N-oxide}\)-Embedded Aurones as Potent Human Tyrosinase Inhibitors

Romain Haudecoeur,†* Marcello Carotti,‡,† Aurélie Gouron,§ Marc Maresca,‡ Elina Buitrago,*,† Marie Boumendjel,‡,§ Elisabetta Bergantini,∥ Hélène Jamet,∥ Catherine Belle,∥ Marius Réglier,† Luigi Bubacco,⊥ and Ahcène Boumendjel‡

‘Univ. Grenoble-Alpes/CNRS, DPM UMR 5063, CS 40700, 38058 Grenoble, France
‡Department of Biology, University of Padova, Via Ugo Bassi 58b, Padova 35121, Italy
§Univ. Grenoble-Alpes/CNRS, DCM UMR 5250, CS 40700, 38058 Grenoble, France
∥Aix Marseille Univ, CNRS, Centrale Marseille, iSm2, Marseille, France

* Supporting Information

ABSTRACT: With the aim to develop effective and selective human tyrosinase inhibitors, we investigated aurone derivatives whose B-ring was replaced by a non-oxidizable 2-hydroxypyridine-\(\text{N-oxide}\) (HOPNO) moiety. These aurones were synthesized and evaluated as inhibitors of purified human tyrosinase. Excellent inhibition activity was revealed and rationalized by theoretical calculations. The aurone backbone was especially found to play a crucial role, as the HOPNO moiety alone provided very modest activity on human tyrosinase. Furthermore, the \(\text{in vitro}\) activity was confirmed by measuring the melanogenesis suppression ability of the compounds in melanoma cell lysates and whole cells. Our study reveals that HOPNO-embedded 6-hydroxaurone is to date the most effective inhibitor of isolated human tyrosinase. Owing to its low toxicity and its high inhibition activity, it could represent a milestone on the path toward new valuable agents in dermocosmetics, as well as in medical fields where it was recently suggested that tyrosinase could play key roles.

KEYWORDS: Human tyrosinase, aurones, human melanoma, transition state analogues, QM/MM calculations

Tyrosinases (Ty, EC 1.14.18.1) are type-3 copper-containing metalloenzymes spread across a wide range of organisms, especially mammals, fungi, bacteria, and plants. These include a binuclear active site, composed by two close copper centers (\(d_{\text{Cu}-\text{Cu}} \approx 2.9 \text{ to } 4.9 \text{ Å}\)) bridged by an aquo(hydroxo) ligand in a met state, coordinated by six histidine residues. Ty catalyzes the oxidation of phenols and catechols into catechols and \(\text{ortho}\)-quinones, respectively (Scheme 1).\(^1\) In mammals the transformations of Ty substrates, \(\text{l-tyrosine}\) and \(\text{l-DOPA}\), into reactive quinones are the first steps of melanogenesis.\(^3\) The resulting melanin pigments play a protecting role against UV radiation and free radicals. Despite similar dicopper active sites (see Supporting Information) and a common function, i.e., phenol/catechol oxidation, significant differences were pointed out at cellular and structural levels among the Tys from distinct sources, especially plant (TyP), mushroom (TyM), bacterial (TyB), and human (TyH).\(^3\) Indeed, while TyM and TyB are generally soluble oligomeric enzymes present in the cytosol, TyH exists as a highly glycosylated monomeric melanosomal transmembrane protein.\(^5,6\) Similarly, strong divergences are observed upon sequence comparison, a discrepancy that has been substantiated at a structural level in the past decade by the resolution of crystallographic structures of two TyB (from \(\text{Bacillus megaterium}\), TyB1\(^1\) and \(\text{Streptomyces castaneoglobisporus}\), TyB2)\(^8\) three TyM (from \(\text{Agaricus bisporus}\), TyM1\(^6,10\) and TyM2\(^11,12\) and \(\text{Aspergillus oryzae}\), TyM3)\(^13\) and very recently one TyP (from \(\text{Juglans regia}\))\(^14\) and one polyphenol oxidase exhibiting tyrosinase activity toward certain substrates (from \(\text{Coreopsis grandiflora}\)).\(^15,16\) More precisely, the overall identity is low (10–30%) between TyMs, TyBs, and TyH. Around the active site (58% identity within 6 Å from the metal center in TyM1 and TyB2), the environments are similar (see Supporting Information), despite some key features not shared among all Tys (e.g., a conserved CuA histidine ligand cross-linked to a cysteine residue in TyMs and TyP, a post-translational modification absent in TyBs and TyH).\(^17\) While preliminary crystallographic studies on TyH have been reported very recently, no structures are available to date at atomic resolution.\(^18\) However, a robust homology model built using structural data from TyB2 and \(\text{Ipomoea batatas}\) catechol oxidase has recently been published.\(^19\)
In humans, melanin-related disorders are known to cause cutaneous hyperpigmentation, skin lesions, or melanoma resistance to conventional therapies. As Ty inhibition is a well-established strategy for controlling melanin production in vivo, as demonstrated again recently, the development of TyH inhibitors raises considerable interest in dermocosmetics and cancer therapies fields. While the low homologies between the different forms of Ty exclude any extrapolation of inhibition studies performed on TyM to TyH, an abundant literature is dedicated to TyM inhibitors for human applications. As a result, only a handful of them are actually in use as depigmentation agents, such as hydroquinone, arbutin, or kojic acid (KA). Unfortunately, currently used inhibitors lack the affinity and selectivity required for TyH-targeting applications and harmful toxicity has often been reported. Therefore, there is an urgent need for novel selective TyH inhibitors that match efficacy and safety standards required for the development of products aimed to human use.

Over recent years, we reported that aurones (2-benzylidene-benzofuran-3(2H)-ones), naturally occurring flavonoids, act as inhibitors of melanin biosynthesis in human melanocytes, as effectors of TyM and as molecular probes for the investigation of the binding-site structural homology between TyM and TyB. In one of these previous studies, we suggested that these compounds bind to the Ty active site with their B-ring, whose substituents determined the behavior of diverse aurones toward both enzymes (i.e., inhibitor, activator, or substrate). Conversely, the A-ring hydroxylation pattern of aurones affected the activity range and selectivity for either TyM or TyB (TyB from Streptomyces antibioticus, which belongs to the same Streptomyces genus and shares 82% identity with TyB), sometimes considerably (aurones V in Figure 1). In parallel, we discovered and investigated the transition-state analogue HOPNO, a catechol-mimicking, non-oxidizable moiety (Scheme 1), as a potent inhibitor of TyM1 (K_{i} = 1.8 μM). Embedding this small moiety in an aurone backbone generated “hybrid” aurone 1a, which showed a TyM1 inhibition activity in the same range as HOPNO (Figure 1).

We report herein the synthesis of three hybrid aurones, whose A-ring substitution patterns were selected reminiscently of our previous results on human melanocytes, along with their biological evaluation through in vitro assays using (1) purified recombinant TyH (from Homo sapiens) and (2) human MNT-1 melanoma cells. The interactions of the most active hybrid aurone with TyH were then rationalized by combining QM/MM dynamics and noncovalent interaction (NCI) analysis, using the recent homology model of TyH mentioned above. Comparisons were made with the interactions of the HOPNO moiety alone on TyH.

As a whole, our recent studies highlighted a remarkable versatility of aurones as Ty-interacting agents, allowing us to gather valuable information on the relation between their substitution pattern and their activities. The B-ring of aurones, as it interacts directly with the active site, completely determined their general behavior toward TyM1 and TyB3. Indeed, aurones I and II act as alternative substrates, aurones III and IV as activators (for TyM1) or weak inhibitors (for TyB3), and aurones V as mixed inhibitors (Figure 1). We also
Table 1. Protonation Constants, Inhibition Constants (K<sub>i</sub>) on Purified TyH, IC<sub>50</sub> on Human MNT-1 Melanoma Cells, and Cytotoxicity Values (IC<sub>50</sub>) on MNT-1 Cells for Compounds 1a–c

<table>
<thead>
<tr>
<th>compound</th>
<th>protonation constants</th>
<th>purified TyH</th>
<th>MNT-1 lyase</th>
<th>MNT-1 whole cells</th>
<th>MNT-1 cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pK&lt;sub&gt;N-OH&lt;/sub&gt;</td>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
</tr>
<tr>
<td>1a</td>
<td>6.71 ± 0.05</td>
<td>0.35 ± 0.04</td>
<td>16.6 ± 0.3</td>
<td>85.3 ± 0.6</td>
<td>&gt;500</td>
</tr>
<tr>
<td>1b</td>
<td>7.57 ± 0.07</td>
<td>1.02 ± 0.04</td>
<td>30 ± 2</td>
<td>120 ± 10</td>
<td>80 ± 20</td>
</tr>
<tr>
<td>1c</td>
<td>7.2 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>34 ± 3</td>
<td>119 ± 1</td>
<td>&gt;500</td>
</tr>
<tr>
<td>HOPNO</td>
<td>6.07 ± 0.02</td>
<td>128 ± 2</td>
<td>1300 ± 100</td>
<td>150 ± 20</td>
<td>&gt;200</td>
</tr>
<tr>
<td>KA</td>
<td>8.3 ± 0.1</td>
<td>350 ± 70</td>
<td>2800 ± 800</td>
<td>15000 ± 2000</td>
<td>&gt;80000</td>
</tr>
</tbody>
</table>

*pSee ref 35. *See ref 4.*

There was an improvement in the HOPNO moiety in terms of TyM1 inhibition activity (e.g., IC<sub>50</sub> = 1.5 µM for 1a versus >1000 µM for the analogous 6-hydroxyaurone V). To further support the potential of this group, we defined the ionization state involved in the binding. Protonation constants for 1a–c have thus been determined by spectrophotometric titrations in water/DMSO (90/10, w/w, see Supporting Information). Protonation constants corresponding to the HOPNO moiety (log K<sub>N-OH</sub> range 5.4–5.8) embedded on aurones indicate that at physiological pH, HOPNO moiety in 1a–c exists exclusively in an anionic form, thereby facilitating the binding on dicopper center (Table 1). These values are lower than that of free HOPNO (6.07), and lower than the protonation constants for hydroxyl groups at position 4′ of aurones I (R<sub>4</sub> = H or OH, R<sub>6</sub> = OH) and II (R<sub>4</sub> = R<sub>6</sub> = OH), in the range 8.3–8.9 (corroborated by classical values found for 4′-hydroxy-groups of flavonoids in the literature), indicating that these moieties are fully protonated at physiological pH. These data reinforced the potential of HOPNO contribution vs phenolic derivatives, for interacting with the copper ions.

All these elements provided the rationale to design and produce the reported HOPNO-embedded aurones. The synthesis of aurones 1a–1c was performed according to Scheme 2 from benzofuran-3(2H)-one derivatives 2a–2c (see Supporting Information). We then comparatively studied the mode of inhibition, consistent with a direct link to the active site. The inhibition constants were then determined by nonlinear regression and curve fitting (see Supporting Information).

Compound 1b and 1c shared a similar inhibition potency (K<sub>i</sub> = 1.02 and 1.2 µM, respectively, Table 1), whereas analogue 1a was found 3.5 times more active. This possibly indicates a detrimental effect of the presence of a hydroxyl group at position 4 of aurones. Interestingly, the evaluation of the HOPNO moiety in the same conditions provided very different results. Indeed, considering TyH inhibition, HOPNO was found as a very weak inhibitor, with a K<sub>i</sub> of 128 µM, approximately 350 times higher than the value measured for compound 1a, whereas it was previously found as a rather potent inhibitor of TyM1 (K<sub>i</sub> = 1.8 and 7.7 µM, respectively). These data emphasize the crucial role of the whole aurone backbone, and not only of the embedded HOPNO moiety, in activities reported herein. Additionally, a correlation could be observed between pK<sub>N-OH</sub> values and K<sub>i</sub> values against TyH, the inhibition potency of tested compounds (including HOPNO) enhancing as deprotonation of the N–OH moiety increases.

In order to understand the reactivity of aurones with TyH and more precisely the role of the aurone backbone, theoretical studies were conducted. We focused our attention on the most...
active hybrid aurone 1a and comparisons were done with the interactions of the HOPNO moiety on TyH. QM/MM dynamics followed by a NCI analysis were performed. For the initial position, we made the hypothesis that 1a and HOPNO would adopt the monodentate binding mode (Scheme 1) obtained in our previous work on TyB2\(^{40}\). In which the oxygen atom of the nitrosyl group of HOPNO is fixed only on one copper atom (see Supporting Information).

The NCI method calculates an index based on the electron density and the electron density gradient.\(^4^1\) This index presents singularities at low density when a weak interaction appears between two fragments. The strength of these interactions can also be calculated and isosurfaces can be plotted to visualize the domains of noncovalent interactions. Thus, the blue surfaces represent strong and attractive interactions (as hydrogen bonds), whereas the green ones indicate weak interactions (as van der Waals interactions). Figure 2 shows an average position with NCI surfaces from the TyH–HOPNO and TyH–1a complexes extracted from their QM/MM dynamics trajectories. If HOPNO appeared as firmly linked to one of the two copper centers in a monodentate mode, only few interactions were observed with the surrounding residues of the second coordination sphere. On the contrary, compound 1a revealed an extended interaction pattern involving hot spot residues relatively far from the coppers, such as Ile368, Arg308, Lys306, and His304, and allowing a robust fit of 1a inside the active site pocket. These observations are in good agreement with the aforementioned, experimentally measured \(K_i\) values.

Finally, the ability of aurones 1a–c to suppress melanin biosynthesis in a human integrated cellular model (human melanoma MNT-1 cells) was evaluated. Results from cell lysates confirmed the potency of hybrid aurones to prevent melanogenesis in a human complex cytoplasmic environment, with an order of inhibition potency similar to that obtained from the isolated TyH inhibition assay (Table 1). Indeed, compound 1a reached a lower \(IC_{50}\) (\(IC_{50} = 16.6\ \mu\text{M}\)), compared to compound 1b and compound 1c, which obtained \(IC_{50}\) values about two times higher (\(IC_{50} = 30\) and 34 \(\mu\text{M}\), respectively). The contribution of the aurone scaffold remained very significant, as HOPNO alone had a very low activity on lysates (\(IC_{50} = 1300\ \mu\text{M}\)). The “hybrids” revealed a limited capacity to hinder melanogenesis in MNT-1 whole cells. The recorded \(IC_{50}\) values ranged from 85.3 (for 1a) to 120 \(\mu\text{M}\) (for 1b), with a concomitant low cytotoxicity of the compounds at such concentrations, except for 1b (Table 1). These values were thus found 3.5–5 times higher than those from lysates. These results suggest a scarce ability of hybrid compounds 1a–c to cross MNT-1 cell membranes that may originate from the partial zwitterionic nature of the HOPNO group, as this moiety is probably experiencing a tautomeric equilibrium between the N-hydroxy-2-pyridinone and 2-hydroxyoxypyridine-N-oxide forms. Nevertheless, HOPNO-based compounds have already demonstrated very good ability to act in cell-based and physiological contexts.\(^4^2,4^3\) Finally it is worth noting that, at the tested conditions, the reference KA was found almost completely inactive both on lysate and whole cells.

In conclusion, a series of HOPNO-embedded aurones have been synthesized and evaluated to determine their inhibition potency against TyH and to elucidate the added value of the aurone backbone compared to the HOPNO moiety alone. If the described aurones are excellent inhibitors of TyH, a considerable gap exists in terms of inhibition potency between these compounds and the HOPNO moiety, indicating an undeniable beneficial effect of the aurone backbone, confirmed by theoretical calculation. Finally, it is worth noting that, to the best of our knowledge, compound 1a is the most active TyH inhibitor ever reported (\(K_i = 0.35\ \mu\text{M}\)). Its lower efficiency in suppressing melanogenesis in MNT-1 human whole cells remains to be addressed. We thus shed light on the great potential of these compounds for human-directed applications and as tools for deciphering tyrosinase role in pathologies such as melanoma.\(^3^5\)

### ASSOCIATED CONTENT

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.6b00369.
Synthesis and characterizations of compounds, TyH inhibition assays, MNT-1 cellular assays, cytotoxicity assays, determination of protonation constants, computational methods, and QM/MM calculations (PDF)

**AUTHOR INFORMATION**

**Corresponding Author**
*E-mail: romain.haudcoeur@univ-grenoble-alpes.fr*

**ORCID**
Romain Haudcoeur: 0000-0002-6271-4717

**Author Contributions**
†These authors contributed equally to this work.

**Notes**
The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**
The authors are grateful to ANR (Agence Nationale pour la Recherche) for financial support (Labex ANR-11-LABX-0003-01 and Blanc program 2Cu-TargMelanin ANR-09-BLAN-0028-01/02/03), ICMG (Institut de Chimie Moléculaire de Grenoble) for computer, analysis, and characterization facilities, and the European COST Program in the framework of which this work was carried out (COST action CM1003 WG 2). This work has been financed by the grant “Human recombinant tyrosinase: a testing bench for new drugs to treat melanin-related disorders” (Progetto di Ricerca di Ateneo CPDA110789/11) from the University of Padova to E.B.

**ABBREVIATIONS**
DOPA, 3,4-dihydroxyphenylalanine; HOPNO, 2-hydroxyproline,N-oxide; KA, kojic acid; NCI, noncovalent interaction; TFAA, trifluoroacetic anhydride

**REFERENCES**


