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Discovery of small-molecule inhibitors of tyrosinase

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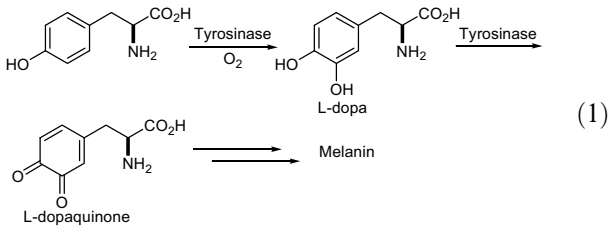
Abstract—To identify novel inhibitors of tyrosinase, a fluorescent assay was developed which is suitable for high-throughput screening. In the assay, oxidation of the substrate by tyrosinase leads to the release of a fluorescent coumarin. Several small molecules were identified that inhibited mushroom tyrosinase in vitro and human tyrosinase in cell culture. These compounds may represent lead structures for therapies targeted at disorders of hyperpigmentation.

The enzyme tyrosinase catalyzes the aerobic hydroxylation of L-tyrosine to L-dopa and the subsequent oxidation of L-dopa to L-dopaquinone (Eq. 1).[1] This sequence marks the initiation of melanin biosynthesis. For this reason, inhibitors of tyrosinase hold promise as treatments for disorders associated with overproduction of melanin, including cutaneous hyperpigmentation[2] and ocular retinitis pigmentosa.[3] Indeed, tyrosinase inhibitors are found in current treatments for melasma, postinflammatory hyperpigmentation, solar lentigo and Addison's disease.[4] However, currently available tyrosinase inhibitors suffer from toxicity and/or a lack of efficacy. For example, the most popular current medications include hydroquinone as a tyrosinase inhibitor.[5] Hydroquinone is a substrate analogue of tyrosine, and, like tyrosine, can undergo oxidation. The product of hydroquinone oxidation, benzoquinone, is a known mutagen[6] and can damage DNA.[7] It is cytotoxic to hepatocytes and melanocytes[8] and may be carcinogenic.[9] For these reasons, the use of hydroquinone in cosmetic products has been banned in the European Union[10] and is under increasing scrutiny by the United States Food and Drug Administration.[11]



(1)

Alternatives to hydroquinone include kojic acid[12] and arbutin.[13] Unfortunately neither substance offers a safe and effective replacement. In fact concerns regarding the carcinogenicity of kojic acid spurred Japanese officials to ban its use in skin treatments recently,[14] while arbutin is simply a hydroquinone glycoside.

Aware of the health and efficacy concerns related to kojic acid and hydroquinone-derived inhibitors, we considered possible approaches to discover alternative treatments for hyperpigmentation. To facilitate this search, we first focused our attention on the development of an assay suitable for high-throughput screening (HTS).[15] Known in vitro[16] or cell-based assays[17] can evaluate dozens of compounds, but present problems in the context of large screening campaigns.

To develop an assay of tyrosinase activity suitable for HTS, we planned to take advantage of the promiscuity of tyrosinase toward phenols and catechols.[18] As a general design feature, we sought a fluorogenic substrate

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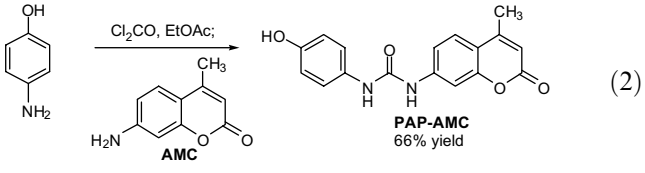
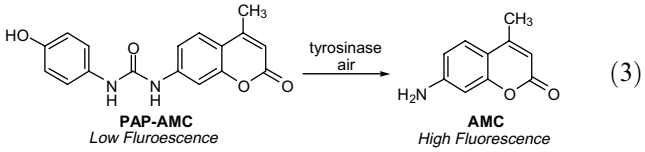
that, following tyrosinase-mediated oxidation, would release a detectable fluorophore. This process could be monitored spectroscopically, and small molecules that minimized fluorophore release could be considered potential enzyme inhibitors (Scheme 1). Inspired by a pro-drug strategy developed in the Osborn group,[19,20] we prepared several acylated amino coumarins as fluorogenic substrates for tyrosinase. Candidates were evaluated in terms of spectral characteristics, performance in enzyme assays, solubility, and ease of synthesis. From these initial studies urea **PAP-AMC** emerged as the most promising candidate for a reliable fluorescent assay. It is synthesized in a one-pot, two step sequence as shown in Eq. 2.

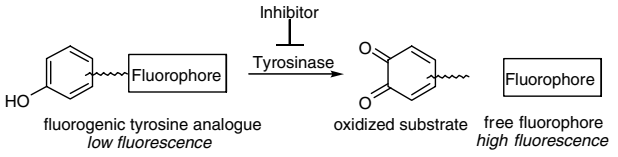


(2)



(3)

As anticipated, substantial differences in fluorescence are observed between the free coumarin and its acylated counterpart (Fig. SI1). Thus, if oxidation with tyrosinase promoted release of free coumarin, this transformation should be accompanied by a large increase in fluorescence (Eq. 3).[21] Indeed, monitoring a reaction mixture containing fluorogenic substrate **PAP-AMC** and mushroom tyrosinase revealed a steady increase in fluorescence as a function of time. Critically, in the presence of 0.2 mM kojic acid, a known tyrosinase inhibitor, the fluorescence increase was markedly attenuated (Fig. 1a).



Scheme 1. Strategy for fluorogenic reporter of tyrosinase activity.

Two studies of the kinetics of the reaction support the contention that the time-dependent fluorescence increase results from a tyrosinase-catalyzed oxidation of the fluorogenic substrate. First, the rate of fluorescence increase is linearly related to enzyme concentration (Fig. 1b). The reproducibility of the assay is also illustrated in Figure 1b. Second, the reaction shows saturation kinetics with respect to substrate **PAP-AMC** (Fig. 1c). Finally, free 7-amino-4-methyl-coumarin was observed by HPLC/MS analysis of the crude reaction mixtures. Taken together, the evidence shows that tyrosinase-catalyzed oxidation of **PAP-AMC** results in an increase in fluorescence signal, and that this signal is diminished in the presence of enzyme inhibitors. Accordingly, the assay conditions were optimized for a format involving robotic liquid handling and data acquisition in a 384-well plate format.

Using the fluorescence assay described above, an in-house library of compounds was screened at approximately 5 μM. By defining a potential hit as a well reading at least three standard deviations below the global mean, the assay yielded a hit rate of approximately 0.6% (1181 initial hits from 200,000 compounds). Initial hits were reevaluated at approximately 15, 5.0, and 1.7 μM. Thus we identified 421 small molecules showing a reproducible inhibition of mushroom tyrosinase. As a testament to the efficiency of the fluorescence assay, the entire library was screened in less than one month using less than 10 mg of substrate **PAP-AMC**.

In the next stage of analysis, the 421 candidates were evaluated for toxicity and their ability to decrease pigmentation in a human melanocyte cell line. Accordingly, 96-well plates were inoculated with 10,000 MNT-1 cells[22] and treated with compound (15 μM, in duplicate), DMSO (negative control), or phenylthiourea (250 μM, positive control). After incubation for 4 days at 37 °C, the wells were treated with CellTiter-Glo reagent and the absorbance of the mixture at 410 nm was determined. Absorbance at this wavelength was found to be proportional to melanin concentration. In addition,

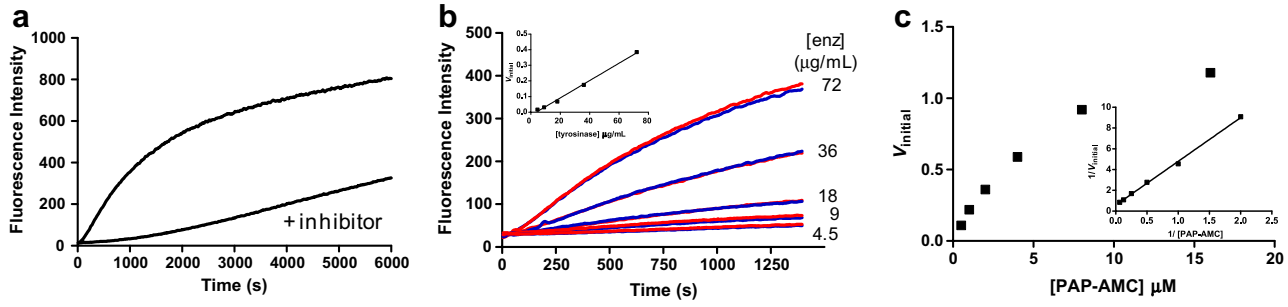


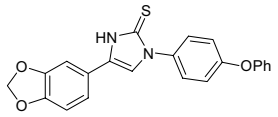
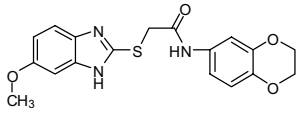
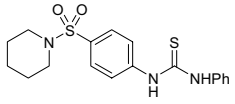
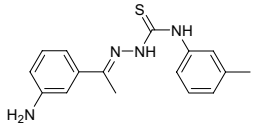
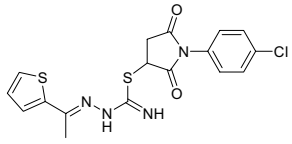
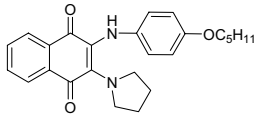
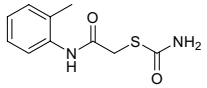
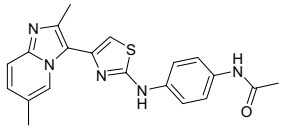
Figure 1. Fluorescence monitoring of the mushroom tyrosinase-catalyzed oxidation of **PAP-AMC**. (a) Oxidation of **PAP-AMC** in the presence or absence of 0.2 M kojic acid. (b) Reactions in the presence of varying concentrations of tyrosinase. Duplicate experiments are shown. Inset: plot of initial rate versus [tyrosinase]. (c) Initial rates as a function of [**PAP-AMC**]. Inset: Lineweaver–Burk plot. For all experiments, $\lambda_{excitation}$ = 350 nm; $\lambda_{emission}$ = 460 nm.

the luminescence of each well was measured to determine cytotoxicity.

Representative results from the in vitro and cell-based assays are shown in Table 1. A number of features of the data warrant comment. First, several classes of known tyrosinase inhibitors were identified. These compounds feature thiourea moieties (entries 1–4)[23] or could decompose under the assay conditions to yield thioureas (entry 5). Although no currently available treatment for hyperpigmentation includes these types of inhibitors because of toxicity concerns,[24] their identification validates the fluorescence assay. Second, the structures shown in Table 1 were not isolated hits; rather, multiple members of each class were identified.

Several classes of small molecules were potent inhibitors in the high-throughput screen, but upon closer inspec-

Table 1. Selected hits from high-throughput screening and cell-based assays

Entry	Compound	Related hits[b]	% Inhibition[a]		
			Mushroom tyrosinase		NMT-1 cells
			15 μM	1.7 μM	15 μM
1		21	54	41	44 (3)
2		21	43	40	34 (1)
3		46	48	23	56 (4)
4		16	52	44	38 (9)
5		6	40	25	19 (20)
6		17	51	47	48 (3)
7		3	66	47	−15 (10)
8		12	46	36	Toxic

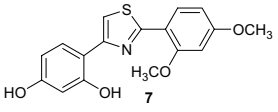
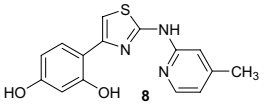
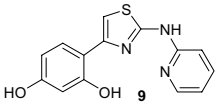
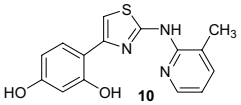
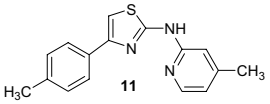
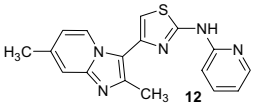
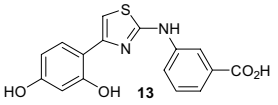
[a] Values are reported as $[(Fl^- - Fl^+)/(Fl^-) \times 100]$ (mushroom) or $[(Abs^- - Abs^+)/(Abs^-) \times 100]$ (MNT-1 cells), where Fl^- and Abs^- are fluorescence or absorbance in the absence of compound. Fl^+ and Abs^+ are fluorescence or absorbance in the presence of compound. Values in parentheses are standard deviations.

[b] Compounds identified in HTS assay with similar structures.

tion appeared less promising. For instance, a variety of quinones were identified, but these are likely non-specific anti-oxidants (entry 6). Other classes of initial hits did not inhibit pigmentation in cell culture, either due to low activity (entry 7) or toxicity (entry 8). The differential activity observed in the cell culture and the in vitro assays could arise from differences between mushroom and human tyrosinase, cell penetration, or metabolism.

Two inhibitors emerged from our screening experiments that warranted further study. A bis-resorcinol-substituted thiazole (**7**) was found to inhibit mushroom tyrosinase with an IC_{50} of around 2 μM using either the fluorescence assay (Fig. SI2) or the common Bestman's hyrazone assay.[16] Among compounds screened, **7** was the most potent inhibitor of human tyrosinase, with treatment at 15 μM resulting in an 86–97% reduction in pigmentation in MNT-1 cells (Table 2). An aminopyridyl-thiazole (**8**) was discovered that showed the most potent activity against mushroom tyrosinase ($IC_{50} \sim 0.1$ μM, Fig. SI2) and retained substantial potency in cell culture: a 78–88% reduction in pigmentation was observed in the presence of 15 μM compound.[25]

Table 2. Inhibition of pigmentation in MNT-1 cells with selected thiazoles[a]

Entry	Compound	Inhibition of pigmentation (%)
1	 **7**	91 (7)
2	 **8**	83 (7)
3	 **9**	54 (12)
4	 **10**	74 (5)
5	 **11**	75 (4)
6	 **12**	48 (10)
7	 **13**	−6 (3)

[a] Values are reported as $[(Abs^- - Abs^+)/(Abs^-) \times 100]$, where Abs^- and Abs^+ are absorbance in the absence or presence of compound, respectively. Values in parentheses are standard deviations.

The biological properties of selected 2-amino-4-arylthiazoles are summarized in Table 2. Methyl substitution of the pyridyl ring enhances inhibitory potency in cell culture, although the position is not critical (entries 2–4). While resorcinol rings occupied the 4-position of most thiazoles (entries 1–4), this substructure was not necessary for activity. For example, thiazoles **11** and **12** were identified by high-throughput screening and lack the resorcinol moiety. Inhibitory potency is lost relative to compound **8**, but certainly not abolished, by replacing the resorcinol ring with a toluene (**11**) or imidazopyridine ring (**12**). Further, thiazole **13** possesses the 4-(resorcinyl)-thiazole core, but is inactive in cell culture. Finally, resorcinol itself was found to inhibit mushroom tyrosinase, but with substantially decreased potency relative to **7** and **8** (IC_{50} ca. 20 μM).

As concerns regarding toxicity and efficacy compromise the future utility of current treatments for hyperpigmentation, we initiated a program to identify novel inhibitors of tyrosinase. We succeeded in identifying a fluorogenic tyrosine analogue that allowed us to develop an assay for tyrosinase activity. High-throughput screening revealed approximately 400 compounds that inhibited mushroom tyrosinase. Further tests using a human melanoma cell line revealed a collection of about 50 small molecules that minimized pigmentation in cell culture without displaying toxicity. Two of these compounds—both diaryl thiazoles—may represent valuable lead structures for the development of safer treatments for hyperpigmentation.

Acknowledgments

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Supplementary data

Experimental procedures for preparation of **PAP-AMC** and enzyme and cell-based assays; spectral data; dose–response data. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.10.014.

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