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Aryl boronic acid inhibition of synthetic melanin polymerization

Jason M. Belitsky *

Department of Chemistry and Biochemistry, Oberlin College, Oberlin, OH, USA

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ABSTRACT

Inhibitors of melanin formation are sought after for a range of applications. Boronophenylalanine is known to inhibit melanogenesis via boronic acid–catechol interactions. A spectroscopic assay was developed to study the polymerization of L-dopa to synthetic melanin in the presence of *para*-substituted aryl boronic acids. The best inhibition was observed for aryl boronic acids with electron-withdrawing substituents. The IC_{50} values exhibit a correlation with the Hammett σ_p parameter ($\rho = 0.97$, $r^2 = 0.92$).

The relationship between boronophenylalanine (BPA, Fig. 1) and melanogenesis is a fascinating and relatively unheralded chapter in bioorganic chemistry.[1–4] Melanin is produced in melanocytes, which are also the precursor cells for malignant melanoma. The enzyme tyrosinase[5] carries out the initial steps of melanogenesis[6] by catalyzing two successive oxidations of the amino acid tyrosine: a hydroxylation of the phenol ring to provide L-dopa followed oxidation to the quinone form (dopaquinone). Tyrosinase activity and melanin production typically increase in the initial stages of malignant transformation. Beginning in 1980, Mishima and co-workers investigated the ^{10}B isotopomer of the tyrosine/dopa mimic BPA as a candidate for boron neutron capture therapy (BNCT) treatment of malignant melanoma.[1–4,7] As hypothesized, BPA accumulates in melanocytes, yet the predominant target does not appear to be the enzyme tyrosinase. Instead, BPA binds directly to melanogenesis intermediates via boronic acid–catechol interactions (Fig. 1).[2–4]

Boronic acids are well known to form reversible covalent adducts with neighboring hydroxyls in sugars[8–14] and catechols.[12–20] These interactions have been widely explored for materials science[17] and bio-analytical applications, including chromatography[8] and sensors for diols,[21] carbohydrates,[9–11] catecholamines,[18,19] and multivalent catechols.[20] Recently, green tea has been suggested as a dietary modulator of the boronic acid-containing proteasome inhibitor bortezomib, via the interaction of its boronic acid with catechol-containing components of green tea.[22]

Mishima and co-workers identified the late stage intermediates 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA), as well as DHI/DHICA oligomers as key binding partners of BPA.[2–4] DHI and DHICA are considered the primary monomers of eumelanin, the black to brown pigment.[6] In addition to reversible covalent bond formation, BPA adducts can breakdown irreversibly to release borate, which can form additional adducts.[3,4] Covalent trapping of BPA/borate concentrates ^{10}B within the melanoma cell, as desired for BNCT. Mishima and co-workers performed BNCT clinical trials[1–4,7] and also leveraged their discovery by using BPA as a tool for studying the melanogenesis pathway and treating hyperpigmentation disorders.[2–4]

We have been inspired to revisit boronic acid–melanogenesis interactions as a tool for probing the natural system and a modifier for generating 'synthetic melanin' analogs. There is a growing interest in exploiting the properties of melanins and related synthetic analogs for non-biological applications.[23–26] As materials applications are developed, precise control over the rate of

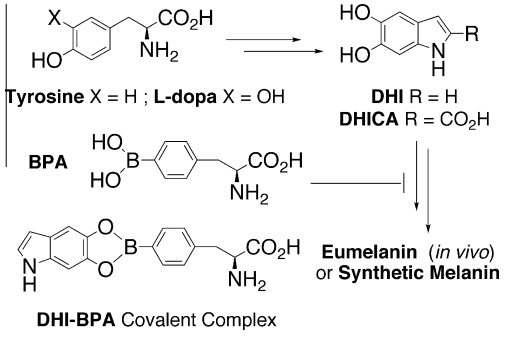

Figure 1. BPA, a mimic of tyrosine and L-dopa, has been found to form reversible covalent adducts with late stage melanogenesis intermediates including DHI, DHICA, and DHI/DHICA oligomers.

* Tel.: +1 440 775 8303.
E-mail address: Jason.Belitsky@Oberlin.edu

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polymerization will be increasingly desirable. To the extent that these polymerizations mimic melanogenesis and/or utilize catechol-containing intermediates, boronic acids could be effective polymerization modulators. Along with ^{11}B NMR and in vivo studies, Mishima and Kondoh had used a synthetic polymerization assay with a visual readout to observe inhibition by BPA and a few other boron-containing species.[3] Among their findings, phenylboronic acid and BPA inhibited polymerization to a similar degree while tetraphenylborate was non-inhibitory. We reasoned that a spectroscopic assay might allow for finer discrimination among similar boronic acids. We were particularly interested to see if a Hammett correlation would emerge from the inhibition of synthetic melanin polymerization by a series of *para*-substituted aryl boronic acids.

We monitored the polymerization of L-dopa to synthetic melanin initiated by the chemical oxidant sodium periodate ($NaIO_4$) by UV–vis spectroscopy at 600 mm. Absorbance at 600 nm is often taken as a proxy for light scattering indicative of particulate matter. In this polymerization, black, macroscopically particulate material forms on the time scale of hours. Both scattering and the broad absorbance of the melanin-like material may contribute to OD_{600}. We found that OD_{600} increases linearly during an extended phase of the polymerization. Polymerizations were initiated by mixing L-dopa (4.0 mM) with $NaIO_4$ (3.0 mM), in 50 mM sodium phosphate buffer pH 7.0 at room temperature. Sodium periodate has been used by Mishima and Kondoh[3] and Canovas et al.[27] to rapidly oxidize L-dopa, initially to dopaquinone. The solution turns red quickly, indicating the brightly colored eumelanin intermediate dopachrome (ε_{max} at 475 nm) is a predominant species in solution at this stage. After 5 min at room temperature, aliquots of the red L-dopa/$NaIO_4$ solution are added to wells of a 96-well plate, containing the same phosphate buffer and DMSO, with or without boronic acids. We refer to the twofold dilution of the initial mixture, for example, as [L-$dopa_{total}$] = 2 mM, where L-$dopa_{total}$ refers to L-dopa itself and all derived polymerization intermediates. All wells have a final volume of 300 μL including 15 μL DMSO (5%). The 96-well plate is placed in a microplate reader equipped with an incubator/shaker (Bio-Rad Microplate Plus) at 25 °C, and OD_{600} monitored every 30 s with 10 s shaking intervals. After ~15 min, we observe an increase in OD_{600} that is linear for at least an hour for [L-$dopa_{total}$] = 2 mM. At lower [L-$dopa_{total}$] there is a longer induction time to reach a linear ΔOD_{600}/min and within the linear phase ΔOD_{600}/min decreases with decreasing [L-$dopa]_{total}$ (Fig. 2). The measured ΔOD_{600}/min values correlate with differences in solution darkening and ultimately visible particulate matter as observed by eye.

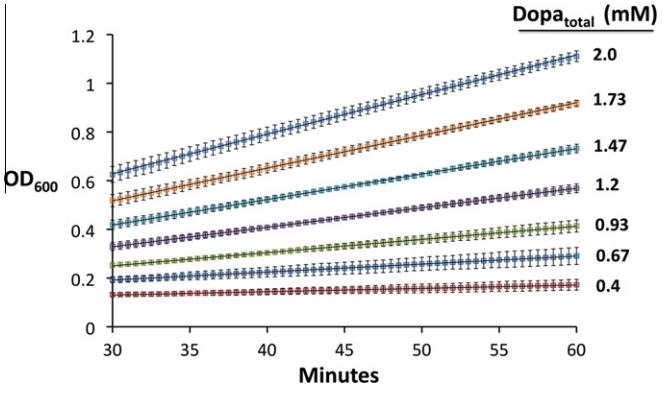

Figure 2. Plot of OD_{600} for synthetic melanin polymerization 30–60 min after placement in the microplate reader at 25 °C. Each well contains the indicated concentration of L-$dopa_{total}$ (prepared as described in the text), 50 mM sodium phosphate buffer, pH 7.0 and 5% DMSO. Average values from three independent titrations shown, error bars represent one standard deviation. Best fit lines shown in black.

With a fixed [L-$dopa_{total}$] value of 2 mM, increasing concentrations of boronic acids increase the length of the induction period and decrease ΔOD_{600}/min in the linear phase. Boronic acid concentrations were adjusted such that all trials enter the linear phase prior to t = 30 min from placement in the microplate reader (25 °C). OD_{600} readings from t = 30 min to t = 60 min were graphed and ΔOD_{600}/min determined. In all included trials, slopes were linear ($r^2 \geqslant 0.98$). Seven concentrations per boronic acid were tested per titration, as well as a L-$dopa_{total}$-only control. Independent titrations were performed in triplicate. A series of ten *para*-substituted aryl boronic acids with substituents –OMe (methoxy), $-CH_3$ (methyl), –H, –F, –Cl, –Br, –Ac (acetyl), $-CF_3$ (trifluoromethyl), –CN (cyano), and $-NO_2$ (nitro) were tested, as well as sodium borate. Representative titrations for 4-cyanophenyl boronic acid and 4-methoxyphenyl boronic acid are shown in Figure 3A and B, respectively. The relative rate (ΔOD_{600}/min with boronic acid/ΔOD_{600}/min without) has an empirical linear correlation with log ([boronic acid]) ($r^2 \geqslant 0.98$ for all boronic acids tested, seven concentrations each, tested in triplicate). Representative data is shown in Figure 3C. Using this empirically determined relationship the point of 50% inhibition was calculated. These IC_{50} values are presented in Table 1, along with the percentage rate (100 × relative rate) observed when [boronic acid] = [L-$dopa]_{total}$ = 2 mM. It is apparent that the aryl boronic acids with electron-withdrawing substituents are more effective inhibitors than those with electron-donating substituents. Indeed, as shown in Figure 3D, there is a reasonable Hammett correlation[28,29] evident in the plot of $-\log(IC_{50}/IC_{50,H})$ versus the Hammett σ_p parameter (r^2 = 0.92), with a slope (ρ) of 0.97.

Increased inhibitory potency for electron-withdrawing substituents is consistent with general trends in boron acid–diol binding interactions.[8,10–15] Aryl boronic acids with electron-withdrawing substituents have lower pK_a's and generally have stronger interactions with diols near neutral pH, although there are exceptions to the latter point.[12,13] Independent studies of the acidity of aryl boronic acids under diverse experimental conditions find ρ values of ~2.1.[13,28,30] Compared to the present results, this indicates that the observed inhibition is less sensitive to the nature of the *para* substituent than the acidity of these same compounds. Wang and co-workers have shown that the relationship between aryl boronic acid pK_a's and diol binding constants is complicated.[13] Optimal complex formation depends on a pK_a match between boronic acid and diol relative to the pH, as well as other factors. Here, the aryl boronic acids could be interacting with a range of different, related species, including the DHI/DHICA monomers and soluble oligomers, remaining earlier intermediates including L-dopa itself, and/or with catechol groups on the surface of nascent particles. In each case the catechol group will have a somewhat different pK_a and steric environment. Thus, we suspect that the Hammett correlation reflects boronic acid–catechol binding but further computational and experimental work will be necessary to differentiate between potential interactions.

We considered two other possibilities for the inhibitory potency of the aryl boronic acids. Control experiments with strong acid (HCl) and base (NaOH) ruled out the significance of indirect, through-pH effects, as expected for a buffered system. (However, once the buffer capacity is overcome there is pH dependence to ΔOD_{600}/min, with slower rates at high [H^+].) We also considered the role of borate. Using $_{11}$B NMR, Mishima and co-workers had observed irreversible decomposition of BPA–DHI complexes to release borate, on a time-scale of hours to days.[4] For some of the aryl boronic acids tested here, the reaction could be accelerated such that it occurs to a significant extent during the assay. Borate can cross-link catecholic species which could have an uncertain effect on the OD_{600}. Cross-linked aggregates might inhibit the normal polymerization and/or be large enough to increase scattering. In

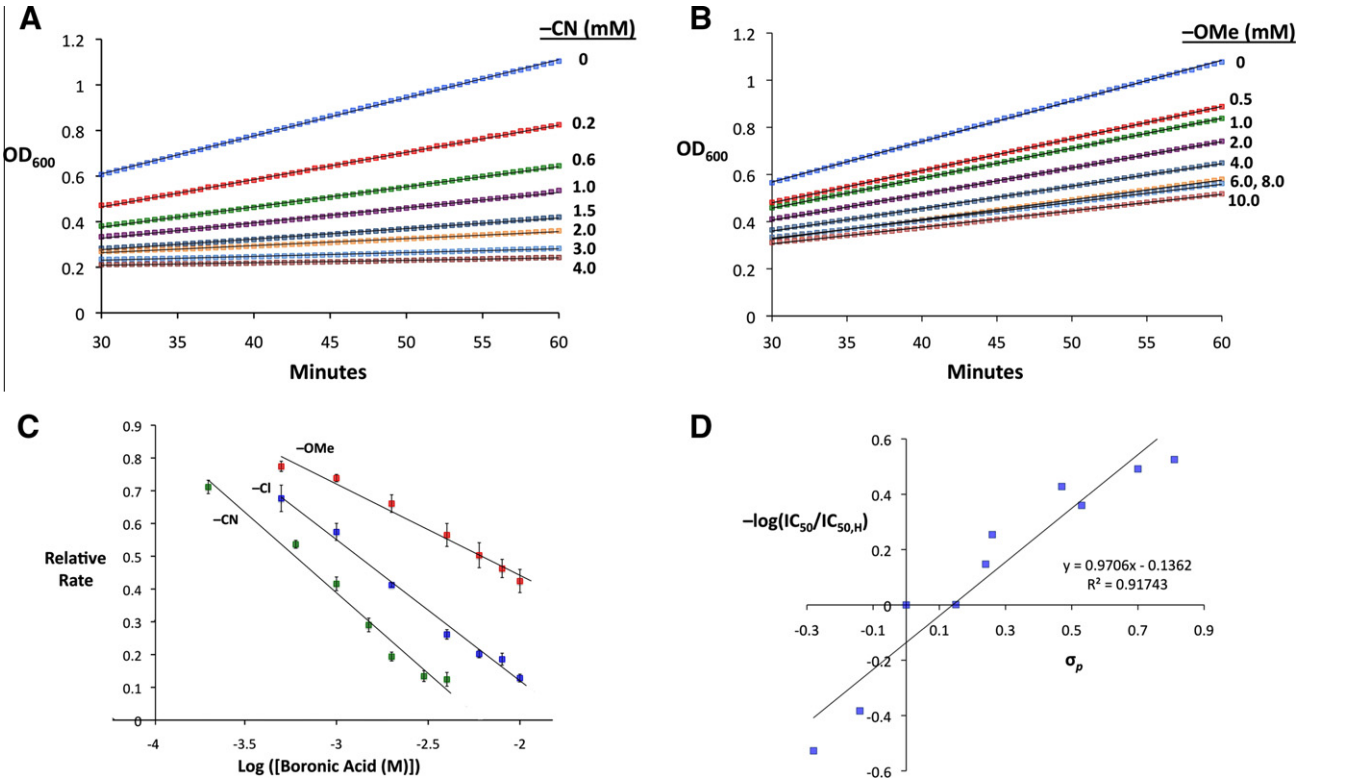

Figure 3. Aryl boronic acid inhibition of synthetic melanin polymerization. (A and B) Individual titrations of 4-cyanophenyl boronic acid (A) and 4-methoxy-phenyl boronic acid (B). Plots of OD_{600} 30–60 min after placement in the microplate reader at 25 °C. Each well contains 2 mM L-$dopa_{total}$ (prepared as described in the text), the indicated concentration of boronic acid, 50 mM sodium phosphate buffer, pH 7.0, and 5% DMSO. Best fit lines shown in black. (C) Plot of relative rate (ΔOD_{600}/min with boronic acid/ΔOD_{600}/min without) versus log ([boronic acid]) and for representative *para*-substituted aryl boronic acids (–OMe, –Cl, –CN). Three independent titrations each, error bars represent one standard deviation, best fit lines shown in black. (D) Hammett correlation between the empirical IC_{50} calculated from the best fit lines of plots such as shown in 3C for 10 *para*-substituted aryl boronic acids.

Table 1
Boronic acid inhibition of synthetic melanin polymerization

X=	σ_p[a]	IC_{50}[b] (mM)	% Rate[c] with 2 mM boronic Acid
–OMe	–0.28	6.20 (±0.32)	66.1 (±2.7)
$-CH_3$	–0.14	4.45 (±0.21)	62.8 (±2.0)
–H	0	1.84 (±0.11)	51.7 (±1.1)
–F	0.15	1.84 (±0.11)	49.7 (±2.1)
–Cl	0.24	1.31 (±0.08)	41.2 (±0.7)
–Br	0.26	1.03 (±0.08)	37.7 (±1.3)
–Ac	0.47	0.69 (±0.04)	30.5 (±1.5)
$-CF_3$	0.53	0.80 (±0.06)	26.9 (±1.0)
–CN	0.70	0.59 (±0.04)	19.4 (±1.3)
$-NO_2$	0.81	0.55 (±0.05)	21.5 (±1.3)
Sodium borate		1.36 (±0.06)	44.3 (±1.3)

[a] $\sigma_p$values from Ref. 29.
[b] Calculated using the empirical linear relationship between log ([boronic acid]) and relative rate (ΔOD_{600}/min with boronic acid/ΔOD_{600}/min without).
[c] % rate = 100 × relative rate.

the event, sodium borate has a net inhibitory effect on ΔOD_{600}/min, with an IC_{50} similar to 4-chlorophenyl boronic acid (Table 1). Since many of the aryl boronic acids had lower IC_{50}'s, this suggests that the production of borate, should it occur, is not the dominant inhibitory factor.

In summary, we have found that aryl boronic acids and sodium borate inhibit the polymerization of L-dopa to synthetic melanin as measured by a simple spectroscopic assay. Aryl boronic acids with electron-withdrawing substituents are more inhibitory than those with electron-donating substituents, which we attribute to formation of stronger boronic acid–catechol complexes. In recent years there has been an explosion in the number of commercially available aryl boronic acids. This study suggests that they may be useful to modify melanin-related polymerizations for materials applications, in vitro models of melanin biochemistry, and potentially in vivo pharmacological intervention, as with BPA.[1–4] Tyrosinase inhibitors[5,31] are widely sought after for cosmetic, therapeutic, and agricultural applications; boronic acids might be alternatives to, or act synergistically with, tyrosinase inhibitors. Finally, a three-component version of the assay with added sugars may be a useful reporter for carbohydrate analysis.

Acknowledgments

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