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Structure-Activity Relationship of 2-Substituted Hydroquinones as Tyrosinase Inhibitors for Topical Delivery

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ABSTRACT – In order to develop hydroquinone analogues for topical delivery, a structure-activity relationship study has been performed. A series of 2-substituted hydroquinones were tested for their ability to inhibit mushroom tyrosinase, alter melanin release and exert cytotoxicity in B6-F10 melanocytes. The electronic property of the 2-substituents did not affect the tyrosinase inhibition nor melanocyte toxicity. However, lipophilicity did affect to some degree the tyrosinase inhibition. The discrepancy in the structure-activity relationship may be due to the poor aqueous solubility of select analogues. Compounds with steric bulk at the 2-position seems to be less soluble, not enabling the analogue to interact effectively with the tyrosinase enzyme. Among the analogues tested, 2-isopropyl hydroquinone seems to be the most promising candidate for topical delivery, being the least toxic analogue with moderate melanin release inhibition.

Keywords – 2-substituted hydroquinone, Gentisic acid, Mushroom tyrosinase, Melanocyte, Structure-activity relationship

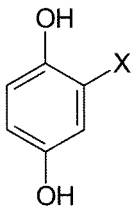
Melanins are produced in human skin to protect itself from environmental damages caused by ultraviolet radiation and high temperature. Although not essential for growth or development, melanins are known to be involved in serious aesthetic problems including melasma and senile lentigines. Tyrosinase is the key enzyme responsible for melanogenesis by catalyzing the rate-limiting step in melanin biosynthesis.[1] Tyrosinase inhibitors are, therefore, known to be useful for preventing hyperpigmentation.

A recent study reported on a patch type formulation of gentisic acid for topical delivery of the skin-whitening agent into the target site, the epidermis/dermis layer.[2,3] However, many of the skin-lightening agents including gentisic acid and arbutin are hydroquinone derivatives, which limit their use due to the cytotoxicity of hydroquinone to melanocytes.[4,5]

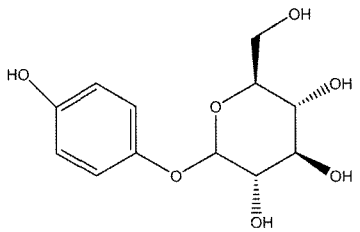
Hydroquinone (Figure 1) itself was used as a skin-lightening agent in the past, but its use has been limited due to its toxicity. Being a metabolite of benzene, it is known to transform into benzoquinone, which is a strong mutagen. Benzoquinone can induce lipid peroxidation, cytotoxicity and produce reactive oxygen species.[6]

Arbutin (Figure 1), on the other hand appears to be lower in toxicity compared to hydroquinone but its tyrosinase inhibition is lower as well. It is a glycosylated hydroquinone derivative, however, that cannot permeate through skin well due to its hydrophilic characteristics. Kojic acid is a safe and mild agent for hyperpigmentary disorders but has limited use due to its

Compound	-X
1	$-CH_3$
2	$-CH(CH_3)_2$
3	$-(CH_2)_3CH_3$
4	-Cl
5	-Br
6	-OH
7	$-OCH_3$
8(Hydroquinone)	-H
9 (Gentisic acid)	-COOH



(a)



(b)

Figure 1–Structures of (a) 2-substituted hydroquinones and (b) arbutin.

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instability in solution.[7] Thus, the choice of possible hydroquinone derivatives as skin-whitening products deliverable topically to the skin is limited.

Previous studies show that tyrosinase is a copper-containing monooxygenase, where the two coppers in tyrosinase are probably the active site.[5,8] It is speculated that the tyrosinase inhibition of gentisic acid is possibly related to its ability to form a complex with the coppers in the enzyme. Moreover, the electron withdrawing carboxylic acid moiety is probably responsible for the lower toxicity in melanocyte compared to hydroquinone.

In order to determine the more exact structural requirements for hydroquinone derivatives as a safer tyrosinase inhibitor for topical delivery, we, herein, report on a structure-activity relationship study on a series of 2-substituted hydroquinone derivatives.

Experimental

Materials

Gentisic acid, hydroquinone, arbutin and the 2-substituted hydroquinone derivatives were purchased from Aldrich Chemical Co. (St. Louis, MO, USA) and were reagent grade or better. Mushroom tyrosinase, L-tyrosine and L-Dopa were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Solvents and reagents for the MTT assay and tyrosinase inhibition assay were purchased from Sigma Chemical Co. (St. Loius, MO, USA). Ultraviolet spectra were recorded on an Optizen 2120 UV/Vis spectrometer. HPLC analyses were performed on a Waters 2690 Separations Module HPLC and Water 2487 Dual λ Absorbance Detector.

Selection of Compounds and Statistical Analysis

The analogues selected for this study were based on the Hansch π and σ values of the 2-substituent as shown in Table I. An even distribution of compounds to represent lipophilic and electronic characteristics has been taken into consideration. The statistical parameter used in the present study describing the results of linear regression is r^2, the square value of correlation coefficient.

HPLC Analysis of 2-Hydroquinone Derivatives

HPLC analyses were performed using a Merck C18 column (Lichrospher 125 * 4 mm, 5 μm particle size, RP-18). Mobile phase (methanol/water, 28:72, v/v with 1% phosphoric acid) was prepared by filtering through a membrane filter (47 mm, 0.2 μm) and degassing in an ultrasonicator (8510 Branson) before use. The flow rate was 1ml/min and injection volume was 20 μL.

Capacity Factors of 2-Substituted Hydroquinone by HPLC

Methanol solution of analogues (20 μg/mL) was prepared and analyzed by HPLC. The mobile phase was a mixture of methanol and water (28:72, v/v) containing 1% phosphoric acid at a flow rate of 1.0 mL/min. The variable wavelength ultraviolet detector was set at 292 nm. Injections of 20 μL were made for all solutions to be analyzed. Capacity factor, k', was calculated as follows:

$$k' = (t_R - t_0)/t_0$$

t_R : Retention time

t_0 : Dead time of methanol

Solubility of Select Hydroquinone Analogues

The calibration curve of every compound was constructed to determine their solubility by UV analysis. The saturated solution of each compound was made in 2 mL of 10% DMSO/water by adding excess amounts. These solutions were shaken until compounds no longer dissolved. They were then filtered and diluted to obtain UV absorbances.

Mushroom Tyrosinase Assay

Tyrosinase activity was estimated by measuring the rate of oxidation of L-Dopa by spectrophotometry as described by Pomerantz.[9]

Melanin Inhibition Assay

Melanin release inhibition was measured as described in literature.[10] B16-F10 melanoma cells were seeded at a density 1×10^4 cells per well in 200 μL medium in 96-well microtest tissue culture plates. After 24 hr, serial dilutions of MSH peptides were added in 50 μL of medium to the wells. Each concentration point was assayed in quadruplicates. The cells were incubated for 3 days at 37°C and then equilibrated to ambient temperature and atmosphere. The absorbance of each well was measured in a reader at 405 nm. The absorbance values were compared with a standard curve obtained with synthetic melanin (Sigma Co., MO. USA) which was dissolved in 0.85 N KOH, diluted in culture medium, and distributed in 96-well plates. The standard curve was linear in the range of the experimental values.

MTT Assay

Standard MTT assay as described in literature was used with slight modification.[11] MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-

Table I–*Physicochemical Properties of 2-Substituted Hydroquinone Derivatives*

Compound	Lipophilicity		Electronic parameter	Capacity factor
	π^a	logP[a]	$(\sigma)^b$	k'[c]
1	0.56	1.104 ± 0.207	-0.10	3.64
2	1.55	1.983 ± 0.214	-0.05	169.83
3	1.98	1.157 ± 0.238	-0.20	104.41
4	0.71	1.523 ± 0.230	0.37	6.53
5	0.86	2.009 ± 0.308	0.39	8.82
6	-0.67	0.058 ± 0.218	0.02	0.54
7	-0.02	0.467 ± 0.232	0.06	2.82
8	0.00	0.664 ± 0.203	0	1.33
9	-0.32	1.563 ± 0.258	0.25	11.69

a. From Hansch *et al.* (1995)
b. From SCI finder
c. Experimentally determined

diphenyl tetrazolium bromide (Sigma Co., MO. USA) was dissolved in isotonic phosphate buffer (IPB, pH 7.4) solution at 5 mg/mL and filtered to sterilize and remove insoluble residues. B16-F10 melanoma cells were cultured in 96-well plates and incubated for 4 h in DMEM (Dulbecco's Modified Eagle's Medium). Cell survival was assayed by measuring the conversion of yellow, water-soluble tetrazolium MTT to blue, water-insoluble formazan. The optical density (proportional to the number of surviving cells) was assessed with a microplate reader (Bio Rad) at 570 nm. The reference wavelength used was 630 nm.

Results and Discussion

Physicochemical Characteristics of 2-Substituted Hydroquinone by HPLC

The capacity factors (k') determined by reverse phase HPLC for the 2-substituted hydroquinones are shown in Table I. Good correlation ($r^2 = 0.9207$) was observed between the log k' values and Hansch π values[12] with the exception of the result for gentisic acid (**9**). However, even with gentisic acid included, the r^2 was 0.7409, which still indicates that the experimentally determined HPLC results can be used to predict the relative lipophilicity of these analogues (Figure 2). Capacity factors also correlated well with the calculated log P values ($r^2 = 0.9204$) except for the bulky analogues **2** and **3**.

Mushroom Tyrosinase Inhibition Assay

Mushroom tyrosinase inhibition was measured for the 2-substituted hydroquinone analogues (Table II). It is interesting to note that the chloro analogue **4** exceeded the activity of hyd-

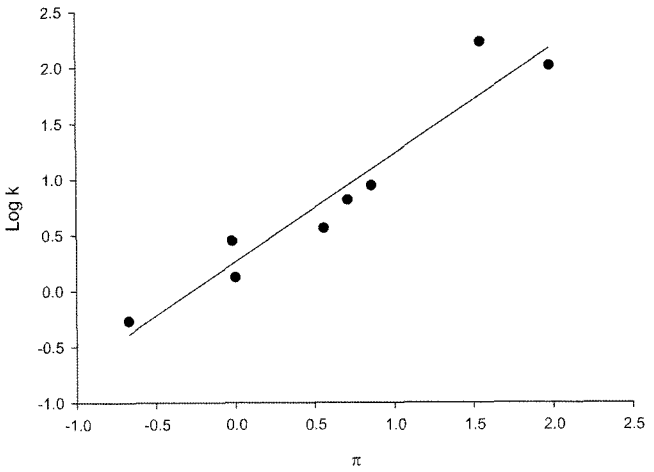

Figure 2–Correlation between capacity factor and relative lipophilicity of 2-substituted hydroquinone derivatives. Log k = $0.9644\pi + 0.2607$ ($r^2 = 0.9207$).

Table II–*The Tyrosinase Inhibition Activity and Solubility of 2-Substituted Hydroquinone Derivatives*

Compound	IC_{50} (μg/mL)	Solubility (mg/mL)
1	0.43	211.46
2	189.95	0.09
3	114.14	11.28
4	$7.73e^{-3}$	659.70
5	5.83	321.97
6	98.17	329.67
7	58.11	75.69
8	0.03	152.80
9	54.19	75.69
Arbutin	26.85	-

roquinone. However, considering that both arbutin and gentisic acid's activity is about 3 orders of magnitude less than hydroquinone, it may not be necessary to have a compound with an IC_{50} lower than 50 μg/mL. Except for compounds **2** and **3**, the tyrosinase inhibition seems acceptable to be considered as a skin-whitening candidate.

Structure activity relationship (SAR) study of the enzyme inhibition results are as follows. Tyrosinase inhibition did not correlate well with σ values ($r^2 = 0.1129$), indicating that the electronic effect at the 2-position did not affect the mushroom tyrosinase inhibition. The lipophilic effect (with π values as well as with log k') on enzyme activity was also not very significant as revealed by the correlation coefficient (r^2) of 0.2097 and 0.1451 respectively. However, when the bulky substituents, t-butyl, isopropyl and bromo analogues were removed from the regression analysis, a correlation coefficient (r^2) was approximately 0.6 while the additional removal of the unsubstituted hydroquinone gave an r^2 value of 0.8217 (Figure 3). This implies that for optimum tyrosinase inhibition, lipophilicity is important, yet a strict steric requirement exists. However, this steric requirement may be due to the bonding of the 2-substituent to the enzyme or may have to do with the decrease in solubility when a bulky lipophilic moiety exists. Thus, the relationship between solubility and enzyme activity was observed.

It was interesting to note that compounds **2** and **3** which showed higher IC_{50} values were less soluble in the buffer used in the enzyme inhibition assay. Although a direct correlation between solubility and biological activity have not yet been established, some of the discrepancies in the SAR study may be partly explainable by the fact that the *in vitro* assay is not always conducted in a homogeneous solution condition. In other words, solubility should be considered when biological data is interpreted.

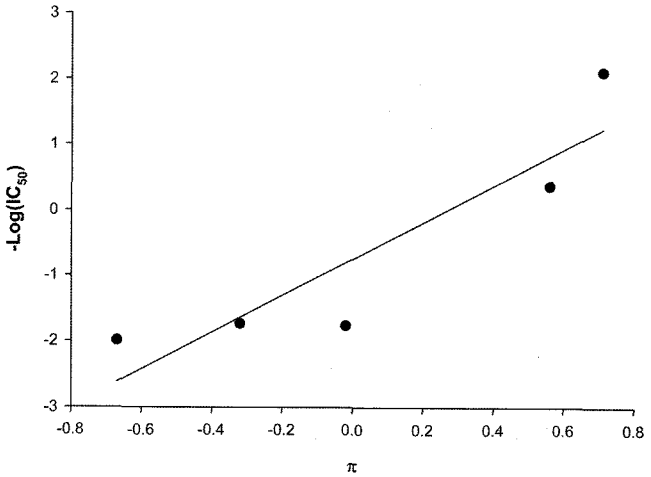

Figure 3–Correlation between tyrosinase inhibition activity and π values of 2-substituted hydroquinone derivatives excluding **2**, **3**, **5** & **8**. $-\text{Log}(IC_{50}) = 2.7927\pi - 0.7474$ ($r^2 = 0.8217$).

Table III–*Results of MTT Assay and Melanin Inhibition of 2-substituted Hydroquinones*

Compound	MTT assay IC_{50} (mg/mL)	Melanin inhibition IC_{50} (mg/mL)
1	7.09	2.12
2	86.79	0.51
3	9.66	0.14
4	5.43	1.70
5	5.51	1.44
6	19.46	55.56
7	5.16	0.41
8	16.88	0.0061
9	218.71	0.026
Arbutin	372.32	0.14

Melanin Inhibition Assay

Results of the melanin release inhibition are shown in Table III. None of the analogues exceeded the activity of hydroquinone or gentisic acid but compounds **2**, **3** and **7** were close in activity to the commercial product, arbutin, showing IC_{50} values of 0.51, 0.14 and 0.41 μg/mL, respectively. It is interesting to note that the results of the melanin inhibition assay do not necessarily correlate well with those of the tyrosinase inhibition assay. This may be partly due to the experimental conditions, where solubility is involved, or may be due to these compounds being involved in more than one mechanism. However, as observed with the tyrosinase inhibition assay, when the bulky substituents t-butyl, isopropyl and bromo analogues were removed from the regression, better correlation between lipophilicity (log k) and $-\log IC_{50}$ was observed ($r^2 = 0.7686$).

MTT Assay

Table III also shows results of cytotoxicity of the 2-substituted hydroquiones on B6-F10 melanoma cells. MTT assay results revealed that most of the analogues were quite cytotoxic. Except for compound **2**, all of the analogues tested showed more or equivalent toxicities to hydroquinone. Fortunately, the melanin inhibition of compound **2** was comparable to arbutin. Although the tyrosinase inhibition activity seemed to be low, results of its solubility indicates that the latter bioassay result may have been due to its insolubility, rather than its inability to inhibit enzyme activity. Therefore, compound **2** seems like a promising analogue to be further devel-

oped as a skin whitening topical agent by developing the appropriate formulation.

Acknowledgements

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