Structure-Activity Relationships Defining the Cytotoxicity of Catechol Analogues against Human Malignant Melanoma

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INTRODUCTION

Malignant melanoma is a cancer with increasing worldwide incidence. The primary treatment for localized melanoma is surgery. Chemotherapy has no role in stage I disease and adjuvant chemotherapy has not improved survival for this group of patients. Prognosis worsens as the disease spreads. In one large series the median survival after diagnosis of metastases was less than 5 months (1). Surgery is of little benefit to patients with widespread disease and radiation therapy is generally ineffective against melanoma. The effectiveness of existing chemotherapy against disseminated melanoma is limited (2, 3). Surgery is of little benefit to patients with disseminated malignant melanoma are sorely needed.

The naturally occurring GHB, isolated from the mushroom Agaricus bisporus, has been shown to have antitumor activity (4). We evaluated these catechol-like compounds to determine if they exhibited selective cytotoxic activity against human malignant melanoma cell lines.

ABSTRACT

The cytotoxic activities of three new synthetic catechol analogues, \( \beta \)-[(p-hydroxyphenyl)amino]alanine (Compound 1), \( N^{2} \)-(p-hydroxyphenyl)ornithine (Compound 2), and \( N^{2} \)-(m-hydroxyphenyl)ornithine (Compound 3), were determined against 10 human melanoma and 5 nonmelanoma cell lines. Activities of \( L \)-DOPA and 3,4-dihydroxybenzylamine were also measured. Dose-response curves were obtained and concentrations in \( \mu \)g/ml required to give 90% inhibition of colony formation (IC\(_{90} \)) were calculated. Using a cutoff IC\(_{90} \) of <2.5 as a definition of activity, Compound 2 was active in 6 of 10 melanomas and 0 of 5 nonmelanomas cell lines while both Compound 1 and \( L \)-DOPA methyl ester were active in 1 of 10 melanomas and 0 of 5 nonmelanomas. Compound 3 was inactive in all cell lines and all IC\(_{90} \) values exceeded 100. 3,4-Dihydroxybenzylamine was active in 3 of 8 melanomas and 1 of 5 nonmelanomas. Regression analysis of IC\(_{90} \) values for Compound 2 and tyrosinase levels in the 15 cell lines yielded a correlation coefficient of 0.93 (\( P < 0.001 \)). By contrast, a similar analysis for 3,4-dihydroxybenzylamine gave a correlation coefficient of 0.17 (\( P > 0.05 \)).

The cytotoxic activities of three new synthetic catechol analogues, \( \beta \)-[(p-hydroxyphenyl)amino]alanine (Compound 1), \( N^{2} \)-(p-hydroxyphenyl)ornithine (Compound 2), and \( N^{2} \)-(m-hydroxyphenyl)ornithine (Compound 3), were determined against 10 human melanoma and 5 nonmelanoma cell lines. Activities of \( L \)-DOPA and 3,4-dihydroxybenzylamine were also measured. Dose-response curves were obtained and concentrations in \( \mu \)g/ml required to give 90% inhibition of colony formation (IC\(_{90} \)) were calculated. Using a cutoff IC\(_{90} \) of <2.5 as a definition of activity, Compound 2 was active in 6 of 10 melanomas and 0 of 5 nonmelanomas cell lines while both Compound 1 and \( L \)-DOPA methyl ester were active in 1 of 10 melanomas and 0 of 5 nonmelanomas. Compound 3 was inactive in all cell lines and all IC\(_{90} \) values exceeded 100. 3,4-Dihydroxybenzylamine was active in 3 of 8 melanomas and 1 of 5 nonmelanomas. Regression analysis of IC\(_{90} \) values for Compound 2 and tyrosinase levels in the 15 cell lines yielded a correlation coefficient of 0.93 (\( P < 0.001 \)). By contrast, a similar analysis for 3,4-dihydroxybenzylamine gave a correlation coefficient of 0.17 (\( P > 0.05 \)).

Abbreviations used are: GHB, \( \beta \)-l-glutaminyl-4-hydroxybenzene; IC\(_{90} \), concentration of drug in \( \mu \)g/ml resulting in 90% inhibition of colony formation.
CATECHOL ANALOGUES WITH TOXICITY FOR MELANOMA

Drug Stability Determinations. The techniques for measuring the stability and bioavailability of anticancer drugs under conditions of the assay have been reported previously (14). Briefly, drugs were incubated at 37°C on underlayers for 14, 7, 4, 2, and 1 day and 6, 5, 4, 3, 2, and 1 h prior to the addition of M14 cells. The cells were incubated for 72 h and incorporation of tritiated thymidine was used as the detection end point. DNA was precipitated by trichloroacetic acid and radioactivity was measured in a liquid scintillation counter (Model LS3801; Beckman Instruments, Irvine, CA) and compared to untreated controls.

Drug half-lives were determined from two graphs: a plot of percentage of survival versus drug concentration; and a plot of percentage of survival versus drug preincubation time. From these two plots a third graph was constructed relating residual drug concentration to the drug preincubation time. Half-lives were determined from this graph as the time required for the drug concentration to decrease by one-half. The in vitro half-lives of Compounds 1–5 were 24 h, 26 h, >7 days, 53 h, and 40 h, respectively.

Tyrosinase Assay. The method used was a modification of the procedure of Pomerantz (15). The medium was removed from 25-cm² Falcon culture flasks (Becton Dickinson, Oxnard, CA) containing cells in a log phase of growth and replaced with 2 ml of fresh culture medium containing 10 μCi l-[ring-3,5-3H]tyrosine (specific activity, 40–60 Ci/mmol; NEN Research Products, Boston, MA). The radiolabeled tyrosine had been reduced previously to dryness under a stream of nitrogen gas. Following incubation for 24 h at 37°C, 100-μl aliquots were added to 3 ml Dowex 50W (Bio-Rad Laboratories, Richmond, CA) columns equilibrated with 0.1 M citric acid buffer, pH 7.2. Trinitated water was washed off the column with 4 ml buffer. Aliquots of 100 μl were added to 5 ml Optifluor (Packard Instrument Co., Downers Grove, IL) and counted by liquid scintillation. All cultures were in duplicate and untreated controls were included in duplicate and aliquots were counted in triplicate. Flasks containing medium and labeled tyrosine but no cells served as background control to measure spontaneous release of tritiated water. Standard curves were prepared from flasks containing medium, radiolabeled tyrosine, and 0.1–1000 units/ml tyrosinase (Sigma). The limit of detection of the assay was 0.2 units/ml.

Relative Rates of Reaction. Reaction rates were measured in a cell-free system using 3-ml quartz cuvettes (Allied Fisher Scientific, Spring- field, NJ). Tyrosinase (50 units/ml) was added to the drug (100 μg/ml). The spectrum from 220 to 700 nm was scanned every 2 min in a Beckman DU-7 spectrophotometer (Beckman). Changes in absorbance of intermediate and final products were plotted and rates of reaction were determined. Maximum velocity was defined as the rate of reaction at the steepest part of the slope of the reaction curve. Relative reaction rates (Ri) were calculated relative to tyrosine (Ri = 1.00). Reaction half-time (Rm) was defined as the time required for one-half of final product to appear. Relative half-times were calculated relative to tyrosine (Rm = 1.00). Relative half-lives were calculated relative to tyrosine (Rm = 1.00).

RESULTS

Cytotoxicity Results. Dose-response curves for Compounds 1–5 were obtained against 10 human melanoma and 5 nonmelanoma cell lines. Typical curves are shown in Figs. 2 and 3. The M14 cell line was the highest producer of tyrosinase, and this cell line was one of the most sensitive to the catechol analogues. On the other hand, the M23 cell line was one of the more resistant lines and it did not synthesize detectable amounts of tyrosinase. From the dose-response curves IC50 values were calculated and these are listed in Table 1. A broad spectrum of activity was observed, but most IC50 values were either <25 or >100. Using the natural break in IC50 values of 25 as a definition of activity, Compounds 1 was active in 4 of 10 melanomas and in 0 of 5 nonmelanomas. Compound 2 was active in 8 of 10 melanomas and 4 of 5 nonmelanomas while Compound 3 was inactive in all cell lines. By contrast Compound 5 was active in all cell lines. Compound 4 (L-DOPA methyl ester) was active in 5 of 10 melanomas and in 0 of 5 nonmelanomas. These data are summarized in Table 2. Compound 2 was clearly more potent than Compound 1 as evidenced by its lower IC50 values and broad-spectrum activity. However, when the cutoff IC50 value was reduced by 1 log (2.5), Com-

Statistical Analysis. Correlations between tyrosinase activity of cell lines and IC50 values were calculated for Compounds 2 and 5. Lack of sufficient data points precluded a similar analysis for Compounds 1, 3, and 4, since the majority of IC50 values exceeded 100 μg/ml. Second order regression analyses were performed with Sigma-Plot (Version 3.0; Jandel Scientific, Sausalito, CA).

Fig. 2. Dose-response curves of Compounds 1–5 against M14 melanoma cells.

Fig. 3. Dose-response curves of Compounds 1–5 against M23 melanoma cells.
CATECHOL ANALOGUES WITH TOXICITY FOR MELANOMA

Table 1 IC₅₀ values of catechol analogues against human tumor cell lines and tyrosinase levels of cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tyrosinase (units/10⁷ cells)</th>
<th>Compound 1</th>
<th>Compound 2</th>
<th>Compound 3</th>
<th>Compound 4</th>
<th>Compound 5</th>
</tr>
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<tbody>
<tr>
<td>Melanoma 14</td>
<td>13.3</td>
<td>3.5</td>
<td>1.4</td>
<td>&gt;100</td>
<td>5.0</td>
<td>2.0</td>
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<td>Melanoma 20</td>
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<td>3.5</td>
<td>1.5</td>
<td>&gt;100</td>
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<td>2.1</td>
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<tr>
<td>Melanoma 12</td>
<td>12.7</td>
<td>3.8</td>
<td>2.2</td>
<td>&gt;100</td>
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<td>10.1</td>
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<tr>
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<td>5.4</td>
<td>1.8</td>
<td>0.3</td>
<td>&gt;100</td>
<td>2.0</td>
<td>2.0</td>
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<td>6.3</td>
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<td>&gt;100</td>
<td>4.1</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>13.5</td>
</tr>
<tr>
<td>Melanoma 32</td>
<td>1.9</td>
<td>&gt;100</td>
<td>2.1</td>
<td>&gt;100</td>
<td>15.0</td>
<td>3.8</td>
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<tr>
<td>Melanoma 23</td>
<td>&lt;0.2</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<td>Melanoma 40</td>
<td>&lt;0.2</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>Melanoma 28</td>
<td>0.8</td>
<td>&gt;100</td>
<td>4.9</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<td>Lung P3</td>
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<td>&gt;100</td>
<td>5.0</td>
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<tr>
<td>Breast 231</td>
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<td>&gt;100</td>
<td>2.5</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>1.9</td>
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<tr>
<td>Breast 157</td>
<td>0.5</td>
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<td>4.0</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>14.0</td>
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<tr>
<td>Breast 645</td>
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<td>20.0</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>3.8</td>
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<tr>
<td>Colon HT29</td>
<td>&lt;0.2</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>3.4</td>
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</table>

* NT, not tested.

Table 2 Summary of activity of catechol analogues against nonmelanoma and melanoma cell lines

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ &lt; 25 µg/ml</th>
<th>IC₅₀ &lt; 2.5 µg/ml</th>
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</thead>
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<tr>
<td></td>
<td>Melanoma cell lines</td>
<td>Nonmelanoma cell lines</td>
</tr>
<tr>
<td>1</td>
<td>4/10</td>
<td>0/5</td>
</tr>
<tr>
<td>2</td>
<td>8/10</td>
<td>5/5</td>
</tr>
<tr>
<td>3</td>
<td>0/10</td>
<td>0/5</td>
</tr>
<tr>
<td>4</td>
<td>8/10</td>
<td>5/5</td>
</tr>
<tr>
<td>5</td>
<td>8/10</td>
<td>5/5</td>
</tr>
</tbody>
</table>

Table 3 Rates of reaction in the presence of 50 units/ml tyrosinase in a cell free system

<table>
<thead>
<tr>
<th>Compound</th>
<th>Intermediate</th>
<th>Maximum</th>
<th>Rₘ</th>
<th>Rₚ</th>
<th>Final product</th>
<th>Maximum</th>
<th>Rₘ</th>
<th>Rₚ</th>
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</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>477</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>304</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Compound 1</td>
<td>486</td>
<td>0.15</td>
<td>1.51</td>
<td>0.10</td>
<td>351</td>
<td>0.10</td>
<td>9.06</td>
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<tr>
<td>Compound 2</td>
<td>511</td>
<td>0.15</td>
<td>1.48</td>
<td>0.10</td>
<td>336</td>
<td>0.10</td>
<td>11.72</td>
<td></td>
</tr>
<tr>
<td>L-DOPA</td>
<td>477</td>
<td>2.00</td>
<td>0.86</td>
<td>304</td>
<td>1.52</td>
<td>0.55</td>
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<tr>
<td>Compound 4</td>
<td>464</td>
<td>2.02</td>
<td>0.26</td>
<td>304</td>
<td>1.52</td>
<td>0.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound 5</td>
<td>383</td>
<td>0.09</td>
<td>0.09</td>
<td>314</td>
<td>0.82</td>
<td>1.06</td>
<td></td>
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<tr>
<td>3,4-Dihydroxybenzylamine</td>
<td>383</td>
<td>0.23</td>
<td>0.34</td>
<td>319</td>
<td>0.16</td>
<td>6.40</td>
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<td></td>
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</tbody>
</table>

**Fig. 4.** Mechanism by which β-(p-hydroxyphenyl)aminolalanine and analogues may be converted by tyrosinase to iminooquinones.

**Fig. 5.** Kinetics of the tyrosinase (50 units/ml) catalyzed oxidation of L-DOPA methyl ester (100 µg/ml). A, absorption spectra for the appearance and disappearance of a quinone intermediate (464 nm) and appearance of the dopachrome final product (304 nm); B, absorption spectrum of reaction mixture from A after 6 min. A few crystals of NaBH₄ completely eliminated the peak at 464 nm within seconds.

Compound 2 was active in 6 of 10 melanoma and 0 of 5 nonmelanoma cell lines. Tyrosinase levels for all cell lines are shown in Table 1. A correlation between IC₅₀ values and tyrosinase levels was made with a second order regression analysis. For Compound 2 a correlation coefficient of 0.93 (P < 0.001) was obtained. By contrast, for Compound 5, a similar analysis gave a regression coefficient of 0.17 (P > 0.05).

Rates of Tyrosinase Activation. The rates of activation of Compounds 1, 2, 4, and 5 by tyrosinase are shown in Table 3. Data for tyrosine, L-DOPA, and p-hydroxybenzylamine are included for comparison. Compound 3 was completely unreactive in the presence of tyrosinase. A transient intermediate with maximum absorption between 383 and 511 nm was observed for all other compounds. These data are consistent with the formation of a quinone intermediate. The higher wavelengths for the intermediates derived from Compounds 1 and 2 suggest rearrangement to iminoquinones; a scheme for their formation is shown in Fig. 4.

A final stable product with a maximum absorption between 304 and 351 nm was obtained for all compounds (except Compound 3). For tyrosine, L-dopa, and Compound 4, this wavelength was consistent with the formation of dopachrome. A typical kinetic curve for the tyrosinase catalyzed oxidation of Compound 4 is shown in Fig. 5. Profiles of the curves for Compounds 1, 2, and 5 were similar. The addition of a few crystals of NaBH₄ completely eliminated the peak at 464 nm within 15 s, suggesting that the intermediate was an easily reduced quinone. The rates of formation of the intermediate quinones for Compounds 1 and 2 were identical and were 7 times slower than the rate of activation of tyrosine. In contrast,
the rates of activation of Compound 4 and L-DOPA were double that of tyrosine, and the rate of activation of Compound 5 was 6 times that of tyrosine. The stability of the intermediate quinones derived from Compounds 1 and 2 was greater than that of the quinone formed from tyrosine. Quinones formed from Compounds 4 and 5 were considerably less stable. The rate of formation of the final product was generally proportional to the rate of formation of the intermediate and the fact that $R_{eq}$ and $R_k$ formed a nearly reciprocal relationship indicated that reaction sequences were similar for all compounds.

**Inhibition of Activation by Phenylthiocarbamide.** Kinetic curves for the oxidation of Compound 4 by tyrosinase in the presence of various concentrations of phenylthiocarbamide are shown in Fig. 6. The inhibition was clearly dose and time related. The reaction was completely stopped with $10^{-5}$ M phenylthiocarbamide, while $10^{-9}$ M was only marginally effective. A similar inhibition of activation of Compounds 1, 2, and 4 was observed when these drugs were tested against the M14 cell line. As shown in Table 4, IC$_{50}$ values were significantly increased for all three compounds in the presence of $10^{-5}$ M phenylthiocarbamide, a concentration that was nontoxic by itself to the M14 cells.

**DISCUSSION**

Although a number of enzymes undoubtedly play a role in melanin synthesis, only tyrosinase is unique to the melanocyte. Tyrosinase has several functions, including hydroxylation of tyrosine and oxidation of DOPA to dopaquinone. The presence of tyrosinase in human melanomas has suggested a rational approach to the development of anticancer drugs for this disease. If analogues of tyrosine and L-DOPA could be oxidized intracellularly in malignant melanoma to cytotoxic quinones, then malignant cells could be selectively killed while sparing normal tissues devoid of tyrosine.

Wick (16) described experiments in which L-DOPA methyl ester increased by 50% the life span of animals bearing the pigmented B16 melanoma. Dopamine was reported to have similar effectiveness in vivo against B16, and thymidine incorporation was inhibited in both the B16 and Cloudman S91 melanomas by 95% (17). Other studies indicated that L-DOPA was selectively toxic for pigmented melanoma cells as compared to amelanotic cells (18). Patients treated with 20 mg/kg/ml dopamine had a significant reduction in the labeling index of their tumor cells (19). However, the dose of dopamine was limited by severe cardiovascular side effects.

A natural catechol isolated from mushrooms (GHB) has antitumor activity in the Kent melanocarcinoma (5). The quinone resulting from the tyrosinase catalyzed oxidation of GHB interferes with a multitude of cellular functions, including inhibition of α-DNA polymerase (20), impairment of ribosomal protein synthesis, disruption of mitochondrial respiration (21), and inhibition of ribonucleotide reductase (22).

The five compounds studied here are analogues of GHB. A structure-activity analysis is instructive. The higher potency of Compound 2 compared to Compound 1 could be attributed to an increased lipophilicity due to a longer side chain. Enhanced transport across the cell membrane and/or more rapid intracellular accumulation of Compound 2 may account for this higher toxicity. The rates of oxidation of Compounds 1 and 2 by tyrosinase were identical and therefore cannot account for the difference in cytotoxicity. Differences in stability of the two compounds under conditions of the assay were also not great enough to be a significant factor. The structure of the inactive Compound 3 differs from that of Compound 2 only in that the hydroxyl group is in the meta position rather than the position para to the side chain.

The potency and selectivity of Compounds 1 and 4 were similar. The major structural difference between Compounds 1 and 4, the nitrogen adjacent to the phenyl ring in Compound 1 compared to the carbon in Compound 4, was apparently not a major determinant of potency or selectivity. The rate of conversion of Compound 4 to the quinone intermediate suggested that hydroxylation of the monohydroxy to the dihydroxy compound was the rate-limiting step (23). The fact that Compound 5 was more rapidly oxidized than its monohydroxy analogue (p-hydroxybenzylamine) confirms this. Compound 5 was the most potent of the compounds tested, but it exhibited no selectivity for melanoma. The greater reactivity (shorter half-life) of the intermediate quinone probably accounted for the lack of selectivity observed with Compound 5.

UV spectra of the products of tyrosinase catalysis of Compound 4 as well as L-DOPA and tyrosine were consistent with cyclization to dopachrome (24). Iminquinone intermediates derived from Compounds 1 and 2 are capable of cyclization; in the case of Compound 1 the product would be a tetrahydrodiquinonoxaline. Since the quinone derived from Compound 5 (as well as from p-hydroxybenzylamine) cannot cyclize, the final product was probably a dimer or polymer. This suggests that the quinones themselves possessed cytotoxicity and that subsequent conversion to the indole was not required for activity. That a cyclized product can exist as either the oxidized or reduced form (e.g., as cycldopa, dopachrome, or the quinone) may explain the greater stability of the intermediates derived from...
Compounds 1, 2, and 4 and consequently their increased selectivity for melanoma.

Evidence for the role of tyrosinase in the activation of these catechol analogues was provided by the strong correlation between IC50 values for Compound 2 and tyrosinase levels in the cell lines. The inhibition of activation of the catechol analogues by phenylthiocarbamide, both in cell-free and in cell line assays, confirm the key role of tyrosinase in the activation of these compounds.

That Compound 5 showed broad, nonselective antitumor activity suggests that its mechanism of action was different from that of the other compounds. It is possible that other enzymes, such as peroxidases (25), catalyzed the oxidation of Compound 5. On the other hand, a mere trace of intracellular tyrosinase may have been adequate to activate Compound 5. The very low but detectable levels of tyrosinase found in some nonmelanoma cell lines indicate that the tyrosinase phenotype was derepressed and may explain the previously observed activity of L-DOPA and dopamine in certain nonmelanoma cell lines (16).

All of the compounds tested in vitro in this study have been evaluated for antitumor activity in the P388 mouse leukemia system. It is not surprising that only Compound 5 showed significant antitumor activity in P388 (26) while Compounds 1 and 2 were inactive (6). The high 50% lethal doses for Compounds 1 and 2 obtained in mice (>200 mg/kg) suggest that these compounds are nontoxic to the animal (6). Tests in human melanoma xenografts will be required to further evaluate the usefulness of these compounds for the treatment of malignant melanoma.

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