In Vitro Growth Inhibition of S-91 Mouse Melanomas by Tyrosinase Substrate Analogs with and without L-Cysteine

PHILLIP S. DUKE, TED G. H. YUEN, AND HARRY B. DEMOPOULOS

Department of Biochemistry, The University of Nebraska, College of Medicine, Omaha, Nebraska 68105; Department of Pathology, University of Southern California, School of Medicine, Los Angeles, California 90033; and Department of Pathology, New York University, School of Medicine, New York, New York 10016

SUMMARY

Tyrosinase, the enzyme responsible for melanin biosynthesis in normal tissues and malignant melanomas, has been reported to be a vital respiratory enzyme of melanizing tissue. In the present study, S-91 mouse melanoma explant growth inhibition in vitro was evaluated quantitatively for 7 substrate analogs of tyrosine, alone and in the presence of L-cysteine. DL-β-Phenyllactate (PLA) was the most effective inhibitor, alone and in the presence of L-cysteine. PLA alone and in the presence of L-cysteine does not inhibit the in vitro growth of hearts of newborn albino mice explants. L-Cysteine facilitates specific inhibition by PLA in the same manner that transient tissue chilling or transient anoxia plus PLA does, suggesting a related mechanism. The facilitated inhibition of S-91 mouse melanoma growth by PLA plus L-cysteine in vitro suggests the possibility of such powerful specific inhibition in vitro.

INTRODUCTION

The enzyme tyrosinase is responsible for melanin biosynthesis in normal animal tissues and in malignant melanomas. Tyrosinase catalyzes the conversion of tyrosine to DOPA and the conversion of DOPA to DOPA quinone. Nonenzymatic polymerization of DOPA quinone then forms melanin (2,3). Tyrosinase requires copper for its activity, and in the melanosome it is believed to function as a terminal oxidase, somewhat similar to cytochrome oxidase in the mitochondrion (4,5,7,8). Ultrastructural changes due to phenylalanine and tyrosine restriction and the administration of penicillamine over a period of years, further means were sought to inhibit melanoma growth.

The amino acid L-cysteine has been shown to produce selective inhibition of S-91 mouse melanoma growth in vitro (1). Its possible mechanisms of action include scavenging of DOPA-related semiquinone free radical intermediates, reduction and chelation of tyrosinase copper, and 1,4 addition to DOPA quinone (6,10,14). In the present work, in vitro inhibition of S-91 mouse melanoma growth is quantitatively measured for 7 tyrosinase substrate analogs alone and in the presence of L-cysteine. Factors of cell penetration are examined by means of the alcohol and water solubilities of 5 substrate analogs. PLA was found to be the best inhibitor tested, alone and in the presence of L-cysteine. Results are presented in Table 1. PLA has been shown previously to selectively inhibit respiration of pigmented human metastatic malignant melanoma and S-91 mouse melanoma explant takes in vitro, with a period of transient chilling or hypoxia being required to produce these strong, selective inhibitions by PLA (4,5). As a result of the present study, it was found that L-cysteine greatly increases the effectiveness of PLA-induced S-91 mouse melanoma explant growth inhibition without transient chilling or anoxia. L-Cysteine, therefore, mimics the effect of chilling or transient anoxia, producing what may be termed facilitated inhibition of S-91 mouse melanoma growth in vitro by PLA. This in vitro effect of L-cysteine plus PLA suggests the possibility of producing melanoma growth inhibition in vitro by these means.

MATERIALS AND METHODS

Tissue Culture. As previously described, S-91 mouse melanoma tissue culture explants were prepared, incubated, scored, and the % inhibition calculated (6). Explants were prepared and scored in a similar manner, using hearts of newborn albino mice. Culture pH was adjusted by addition of dilute HCl or NaHCO3 in sterile water in small amounts, and by the use of air or CO2 or O2 gas. Whenever oxygen gas was used, all tube medias were oxygen gased shortly before use.

Solubilities. The solubilities of PLA, its ethyl ester PLAEE,
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dL-mandelic acid, its ethyl ester MAEE, protocatechuic acid, tyrosine, and DOPA were determined in water and absolute ethyl alcohol as follows: saturated water and alcohol solutions of the fine solids were prepared at room temperature (approximately 25°C). Clear 2-ml portions of supernatant were evaporated, employing gentle boiling and a nitrogen jet. The residues were dried over concentrated sulfuric acid and weighed. The results, multiplied by 50 and rounded off to two significant figures, are given in Table 2.

Reagents. The PLA selected for use was Grade B (purest commercially available) obtained from the California Corporation for Biochemical Research. The PLA was purified by recrystallization from acetone-H2O mixture three times, with a 10% final yield. The mandelic acid used was Eastman Kodak white label, protocatechuic acid was obtained from the California Corporation for Biochemical Research, and N-APA was obtained from Mann Research Laboratories, Inc. MeDOPA-MeE was obtained in 5-ml ampules (50 mg/ml) from Merck, Sharp and Dohme (Aldomet).

PLAAE and MAEE were prepared by direct esterification, using standard methods (16). Purification was effected by repeated washing with aqueous, chemically pure NaHCO3 and distilled water. After storage over concentrated sulfuric acid, the PLAAE and MAEE were obtained as clear liquids. The results of combustion analyses (average of duplicate determinations) are as follows: Analysis calculated for PLAEE (C12H16O5): C, 68.06%; H, 7.27%; O, 24.7%. Found: C, 67.84%; H, 7.16%; O, 25.0% (by difference). Analysis calculated for MAEE (C9H12O3): C, 66.6%; H, 6.71%; O, 26.7%. Found: C, 66.3%; H, 6.64%; O (by difference), 27.1%. No ash was noted after combustion of both substances.

RESULTS

Tissue culture results are given in Table 1. Individual values refer to separate experiments, and all the trials with each agent are included. Solubility data are presented in Table 2. Inhibitory effectiveness is in the order PLA > mandelate > PLAAE > protocatechuate > MAEE > N-APA. L-Cysteine at 2.0 mg/ml produces 46-50% inhibition of S-91 mouse melanoma growth, as previously reported (6). The combination of L-cysteine and PLA produces 95–100% growth inhibition of S-91 mouse melanoma explants in vitro. L-Cysteine at 2.0 mg/ml plus PLA produces about 26% inhibition of newborn mouse heart, as previously reported for L-cysteine alone (6). Therefore, PLA alone and in the presence of L-cysteine produces essentially no growth inhibition of newborn mouse heart.

MeDOPA-MeE showed a nonspecific strong toxic effect in tissue culture alone, with production of a black product. These effects are very similar to that produced by DOPA itself in tissue culture.

Solubility data results show that solubilities in absolute ethanol decrease in the order PLAAE and MAEE > PLA > mandelate > protocatechuate > tyrosine or DOPA. Solubilities in water decrease in the order mandelate > PLA > DOPA or protocatechuate > MAEE > N-APA. L-Cysteine at 2.0 mg/ml produces about 26% inhibition of newborn mouse heart, as previously reported for L-cysteine alone (6). Therefore, PLA alone and in the presence of L-cysteine produces essentially no growth inhibition of newborn mouse heart.

DISCUSSION

Structural formulas for tyrosinase substrates and the agents tested are presented in Chart 1. L-ß-2-Thiophenylalanine is not a specific tyrosinase inhibitor but it is a phenylalanine antimetabolite (5, 13). It has been shown that PLA at 2 mmole/liter has a selective inhibitory effect on the respiration and growth of pigmented melanoma but not on amelanotic melanoma (4, 5) and regimens employing phenylalanine and tyrosine restriction plus

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**TABLE 1**

In Vitro Inhibition of Growth

<table>
<thead>
<tr>
<th>Agent*</th>
<th>% inhibition of S-91 mouse melanoma</th>
<th>% inhibition of newborn mouse heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>dL-ß-Phenyllactate</td>
<td>62-64</td>
<td>0-5</td>
</tr>
<tr>
<td>dL-Mandelate</td>
<td>33-45</td>
<td></td>
</tr>
<tr>
<td>dL-ß-Phenylactic acid ethyl ester</td>
<td>21-41</td>
<td></td>
</tr>
<tr>
<td>Mandelic acid ethyl ester</td>
<td>3-12</td>
<td></td>
</tr>
<tr>
<td>Protocatechuate</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-L-phenylalanine</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Methyl ester of L-2-methyl-dihydroxyphenylalanine</td>
<td>100-100</td>
<td>100-100</td>
</tr>
<tr>
<td>Dihydroxyphenylalanine</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>dL-ß-Phenyllactate + L-cysteine</td>
<td>95-100</td>
<td>25-27</td>
</tr>
<tr>
<td>Mandelate + L-cysteine</td>
<td>46-50</td>
<td></td>
</tr>
<tr>
<td>dL-ß-Phenylactic acid ethyl ester + L-cysteine</td>
<td>46-53</td>
<td></td>
</tr>
<tr>
<td>Mandelic acid ethyl ester + L-cysteine</td>
<td>43-48</td>
<td></td>
</tr>
<tr>
<td>Protocatechuate + L-cysteine</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl L-phenylalanine + L-cysteine</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Methyl ester of L-2-methyl-dihydroxyphenylalanine + L-cysteine</td>
<td>100-100</td>
<td></td>
</tr>
<tr>
<td>Dihydroxyphenylalanine + L-cysteine</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>47-50-46</td>
<td></td>
</tr>
</tbody>
</table>

* dL-ß-Phenyllactate, dL-ß-phenylactic acid ethyl ester, mandelate, mandelic acid ethyl ester, protocatechuate, N-acetyl-L-phenylalanine, dihydroxyphenylalanine at 2 mmoles/liter (approximately 0.3 mg/ml); L-cysteine at 17 mmoles/liter (2 mg/ml); L-2-methyl-dihydroxyphenylalanine (0.5 mg/ml). All trials are listed.

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**TABLE 2**

Solubility Parameters of Tyrosinase Substrates and Analogs

<table>
<thead>
<tr>
<th>Substance</th>
<th>Solubility (gm/100 ml) in saturated solution</th>
<th>Solubility ratio (absolute alcohol/water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Dihydroxyphenylalanine</td>
<td>1.2</td>
<td>0.0</td>
</tr>
<tr>
<td>dL-ß-Phenyllactic acid</td>
<td>5.3</td>
<td>53</td>
</tr>
<tr>
<td>dL-Mandelic acid</td>
<td>14</td>
<td>52</td>
</tr>
<tr>
<td>Phenylactic acid ethyl ester</td>
<td>0.5</td>
<td>Miscible</td>
</tr>
<tr>
<td>dL-Mandelic acid ethyl ester</td>
<td>1.0</td>
<td>Miscible</td>
</tr>
<tr>
<td>Protocatechuate</td>
<td>1.2</td>
<td>34</td>
</tr>
</tbody>
</table>

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Melanoma Inhibition by Analogs

**Chart 1.** Structural formulas of tyrosinase substrates, analogs, and L-cysteine.

Penicillamine considerably retard the growth of S-91 mouse melanoma in vivo and may produce regression of metastatic malignant melanoma in man (2, 3). These findings are the major bases for stating that melanoma growth inhibition by PLA and analogs is due to a selective antityrosinase action. S-91 mouse melanoma ultrastructural changes due to phenylalanine and tyrosine restriction support the concept that tyrosinase is a vital melanoma tissue respiratory enzyme (11).

PLA was found to be the best inhibitor tested, alone and with L-cysteine. PLA did not inhibit the growth in vitro of hearts of newborn albino mice. Results are reported as % of controls to reduce experimental variation. It is of interest that PLA, lacking any ring hydroxyls, is not really a close structural analog of the natural tyrosinase substrates tyrosine and DOPA, which both possess ring hydroxyls.

For many substances, the rate of cellular penetration is proportional to lipid solubility or the lipid-water partition coefficient (1). On this basis, the powerful inhibitory action of PLA is probably not explainable by the concept that PLA penetrates the cell membrane more readily than the other substances tested.

PLA might inhibit tyrosinase by chelation of copper, an essential tyrosinase cofactor. A PLA-copper chelation constant datum was not found in the literature, but a value for lactic acid-copper chelate was, and by analogy it suggests that PLA forms a relatively weak copper chelate. The log chelation constant for lactic acid with Cu(II) is 2.70 at 18–25°C (9). Benzene ring substitution...
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\[(A) \quad 4 \text{RSH} + \text{O}_2 = \text{RSSR} + 2\text{H}_2\text{O} \]
\[(B) \quad 2 \text{RS}^- + 2 \text{E-Cu(II)} = 2\text{E-Cu(I)} + \text{RSSR} \]
\[2 \text{RS}^- + 2 \text{E-Cu(I)} = 2 \text{RS-Cu(I)} + 2\text{E} \]
\[4 \text{RS}^- + 2 \text{E-Cu(II)} = 2 \text{RS-Cu(I)} + \text{RSSR} + 2\text{E} \quad \text{(inactivated)} \]
\[\text{NH}_3^+ \]
\[\text{R} = \text{CH}_2 - \overset{\text{C}}{\text{C}} - \overset{\text{\text{O}}}{\text{\text{O}}} \]
\[\text{H} \]
\[\text{E} = \text{Tyrosinase} \]
\[(C) \quad \text{RSH} + \text{E-Cu(I)} = \text{RS-Cu(I)} + \text{H} + \text{E} \quad \text{(inactivated)} \]
\[(D) \quad 2 \text{RSH} + 2 \text{R'} = 2\text{RS} + 2\text{R'H} \]
\[2 \text{RS}^- = \text{RSSR} \]
\[2 \text{RSH} + 2 \text{R'} = 2 \text{R'H} + \text{RSSR} \]
\[\text{R'} = \text{Free Radicals scavenged by L-cysteine} \]

\[\begin{align*}
\text{O} & \quad \text{R} \\
\text{O} & \quad \text{SR} \\
\end{align*} \]
\[\text{HO} \quad \text{HO} \quad \text{R} \quad \text{SR} \]

\[\begin{align*}
\text{O} & \quad \text{R} \\
\text{O} & \quad \text{SR} \\
\end{align*} \]

\[\text{HO} \quad \text{HO} \quad \text{R} \quad \text{SR} \]

\[\begin{align*}
\text{(a)} & \quad \text{Reaction of oxygen with L-cysteine.} \\
\text{(b)} & \quad \text{Conversion of melanoma related Cu(II) to Cu(I) by L-cysteine.} \\
\text{(c)} & \quad \text{Chelation of melanoma related Cu(I) by L-cysteine.} \\
\text{(d)} & \quad \text{Scavenging of melanoma related free radicals by L-cysteine.} \\
\text{(e)} & \quad \text{1,4 addition to dihydroxyphenylalanine quinone by L-cysteine.} \\
\end{align*} \]

Chart 2. Possible mechanisms of action of L-cysteine upon melanoma-related copper, free radicals, and quinones.

of a lactic acid β-hydrogen to form PLA would not be expected to produce a powerful copper chelate (9). Therefore, it is not likely that PLA would inhibit tyrosinase in vitro by copper chelation.

A specific growth-inhibitory effect of PLA and L-cysteine on S-91 mouse melanoma is shown by the findings that the growth inhibition of hearts of newborn mice caused by PLA plus L-cysteine is only 25-27%, as previously reported for L-cysteine alone (6), whereas for PLA alone a growth inhibition of only 0-5% was found for hearts of newborn albino mice explants.

The nonspecific inhibitory effect of MeDOPA-MeE and DOPA, along with their formation of a black material in the media, suggests the formation of a toxic product in both cases.

Concerning the mechanism by which L-cysteine facilitates the inhibitory action of PLA, several possibilities exist: (a) the similarity of results obtained by transient tissue chilling or anoxia suggests that L-cysteine, a strong reducing agent, may be producing anoxia due to formation of cystine, with attendant oxygen consumption; (b) L-cysteine readily reduces Cu(II) to Cu(I); and (c) L-cysteine also forms a strong chelate with Cu(I), which has a chelation constant \( \log K \) of 19.2 at 25°C (14). Therefore, the effect of transient chilling and anoxia as well as L-cysteine may be to cause the reduction of Cu(II) to Cu(I) in tyrosinase; (d) free radical semiquinone intermediates exist in the S-91 mouse melanoma in low-speed-cleared sucrose homogenates, as previously reported in an EPR study, and these intermediates are scavenged by cysteine, which reduces the EPR signal (6, 14); (e) reaction of L-cysteine with DOPA quinone by 1,4 addition is also a possibility (10). It is suggested that the mechanisms of in vitro facilitating action of L-cysteine are most probably related to its ability to convert Cu(II) to Cu(I) by reduction and chelation, while its ability to produce inhibition in vitro when used alone is possibly related to all the proposed mechanisms diagrammed in Chart 2.

The inhibition of S-91 mouse melanoma growth in vitro by PLA plus L-cysteine is 95–100% effective. PLA alone does not produce growth inhibition of S-91 mouse melanoma carried in DBA/2 mice once explant growth has begun (H. B. Demopoulos, personal communication). The finding that L-cysteine, a relatively nontoxic chemical, facilitates inhibition of melanoma growth by PLA in the same manner as tissue chilling and transient anoxia suggests that such powerful specific facilitated inhibition by PLA in vivo is potentially possible. Experimentation is in progress to evaluate this possibility.
ADDENDUM

Concerning the action of PLA plus l-cysteine on S-91 mouse melanomas in vivo, a short communication has been published (P. S. Duke and H. B. Demopoulos, In Vivo Necrosis of Large S-91 Mouse Melanomas by dl-β-Phenyllactic Acid Plus l-cysteine. Life Sci., 6: 951-957, 1967). A paper by the same authors entitled "Increased Host Life-span and in Vivo Growth Inhibition of Large S-91 Mouse Melanomas by PLA Plus l-Cysteine" has been submitted for publication.

REFERENCES

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