Affinity of Ocular Acid-Insoluble Melanin
For Drugs In Vitro

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The authors examined spectrophotometrically the ability of drugs to bind with ocular acid-insoluble melanin. Each drug, at 5 × 10⁻⁵ M, was incubated at 37°C for 8 hr with 4 mg of melanin in 20 mM potassium phosphate buffer (pH 7.4 and 8.0) or in 20 mM acetate buffer (pH 4.8). The authors’ findings showed that chloroquine, thiordiazine, befunolol, pindolol, daunomycin, and 5-fluorouracil bound to melanin (pH 4.8, 7.4, and 8.0). Methotrexate bound to melanin (pH 4.8 but not at 7.4 and 8.0). Pilocarpine, epinephrine, acyclovir, vincristine, and colchicine did not bind to ocular acid-insoluble melanin. Invest Ophthalmol Vis Sci 28:822-825, 1987

Some drugs have an affinity for melanin and accumulate in the pigmented tissues of the eye.⁴⁻⁵ Among them, chloroquine and thiordiazine induce a retinopathy.⁴⁻⁵ The pathogenesis of the retinopathy⁴⁻⁵ involves the drug’s affinity for melanin. By spectrophotometric means, the authors examined drugs used currently and those planned for future clinical employment to assess their affinity for ocular acid-insoluble melanin.

Materials and Methods

Drugs and Solution

The following drugs were used in this study (numbers given in parentheses indicate their maximum light absorption wavelength). Chloroquine (255 nm), pilocarpine (214 nm), epinephrine (280 nm), and colchicine (352 nm) were purchased from Sigma Chemical Co. (St. Louis, MO). Pindolol (264 nm) and vincristine (297 nm) were generous gifts from Shionogi Pharmaceutical Co. (Tokyo, Japan). The authors also purchased thioridazine (315 nm) from Sandoz Co. (Basel, Switzerland); befunolol (295 nm) from Kakenyaku-Kako Co. (Tokyo, Japan); daunomycin (480 nm) from Meiji-Seika Co. (Tokyo, Japan); 5-fluorouracil (266 nm) from Kyowa-Hakko Co. (Tokyo, Japan); methotrexate (370 nm) from Takeda Pharmaceutical Co. (Tokyo, Japan); and acyclovir (254 nm) from Sumitomo-Kogyo Co. (Tokyo, Japan).

Each drug, at 5 × 10⁻⁵ M, was dissolved in 20 mM potassium phosphate buffer (pH 7.4 and 8.0) and in 20 mM acetate buffer (pH 4.8). The absorption was read with a Hitachi Spectrophotometer type U-3200.

Preparation of Ocular Acid-Insoluble Melanin

Deproteinized acid-insoluble melanin was isolated from bovine ciliary body, iris, choroid, and retinal pigment epithelial cells by the method of Nicolaus et al.⁶ The uvea and retinal pigment epithelial cells of 240 bovine eyes were dissected into 240 ml of 20 mM potassium phosphate buffer (pH 7.0) and homogenized in a Waring blender. After centrifugation of the homogenate, the pellet was suspended in 500 ml of 11 N HCl, and the suspension was gently stirred at room temperature for 2 weeks. The suspension was centrifuged at 15,000 g for 20 min to obtain the crude pigment. The crude pigment was washed 10 times by suspension in 500 ml of distilled water and centrifugation at 15,000 g for 20 min. The crude pigment was resuspended in 500 ml of 11 N HCl, and the mixture was gently stirred at room temperature for 2 weeks. The pigment was washed 10 times with water and 2 times with acetone. Thereafter, 500 ml of fresh 11 N HCl was added to the washed pigment, and the mixture was heated at 75°C for 2 min. The heated, black pigment in 11 N HCl was left at room temperature for 2 weeks and was washed 20 times by suspension in 500 ml of water and centrifugation at 15,000 g for 10 min. The final suspension was determined to be neutral when measured by a pH meter. The supernatant after centrifugation of the final suspension showed no absorption from 200-800 nm, and was ascertained to be protein free when determined by the method of Lowry et al.⁷ The final black pigment was dried in vacuo. The total mean yield of the dry pigment calculated for 100 eyes was 2.7 g.

Binding of Drugs to Melanin In Vitro

Each drug, at 5 × 10⁻⁵ M, was incubated at 37°C at various pH ranges with ocular acid-insoluble melanin in a total volume of 2.0 ml. Buffers used were 20
Fig. 1. Binding of chloroquine to ocular acid-insoluble melanin. Ocular acid-insoluble melanin, 4 mg (O) or 1.3 mg (●), was incubated at 37°C with $5 \times 10^{-3} \text{M}$ chloroquine at pH 4.8 (a), 7.4 (b), and 8.0 (c). Means and SD of three experiments are shown.

mM potassium phosphate (pH 7.4 and 8.0) and 20 mM acetic acid–sodium acetate (pH 4.8). Incubation was performed with continuous shaking. After incubation, the mixture was centrifuged at 15,000 g for 30 min to pellet the drug bound to melanin. The absorbance of free drug in the supernatant was determined with a Hitachi spectrophotometer. As control, the drug or melanin only was incubated in the buffer. Bindings of the drugs to melanin were usually run in triplicate. The percent of bound drug was calculated from the amount of free drug and the initial concentration.

**Results**

There was no breakdown of any of the drugs during the incubation for 8 hr at 37°C in the absence of melanin. The effect of incubation time on chloroquine binding to ocular acid-insoluble melanin is shown in Figure 1. Chloroquine bound to melanin (pH 4.8, 7.4, and 8.0). Sixty percent of the drug bound (pH 4.8) to 4 mg of ocular acid-insoluble melanin without incubation. The percent binding increased to 84% for up to 4 hr incubation.

Percent binding for each drug with 4 mg of ocular acid-insoluble melanin is shown in Table 1. Chloroquine, thioridazine, befunolol, pindolol, daunomycin, and 5-fluorouracil bound to melanin (pH 4.8, 7.4, and 8.0). Methotrexate bound to melanin (pH 4.8 but not at 7.4 or 8.0). Pilocarpine, epinephrine, acyclovir, vincristine, and colchicine did not bind to ocular acid-insoluble melanin.

**Discussion**

We demonstrated spectrophotometrically that some drugs have an affinity for ocular acid-insoluble melanin. Although the chemical structure of this form of melanin is unclear at present, Nicolaus and co-workers and Binns et al have greatly contributed to our current knowledge of its chemistry. Potts demonstrated that polycyclic aromatic compounds have a high affinity for melanin. Atlasik and collaborators indicated that compounds with one aromatic ring also may have this affinity. The authors found, however, that vincristine, which has an aromatic ring, showed no affinity for melanin. The nature of the binding between melanin and drugs has not been established.

The present findings showed chloroquine to have an affinity for ocular acid-insoluble melanin, which correlates closely to previous reports by Potts, Bernstein and associates, and Ings. Thioridazine also bound well with melanin in the present study and in previous work described by Potts.

**Table 1. Percent binding of drugs to ocular acid-insoluble melanin**

<table>
<thead>
<tr>
<th>Drug*</th>
<th>pH 4.8</th>
<th>pH 7.4</th>
<th>pH 8.0</th>
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<tbody>
<tr>
<td>Chloroquine</td>
<td>85 ± 4</td>
<td>72 ± 3</td>
<td>65 ± 2</td>
</tr>
<tr>
<td>Thioridazine</td>
<td>63 ± 5</td>
<td>62 ± 2</td>
<td>51 ± 3</td>
</tr>
<tr>
<td>Befunolol</td>
<td>60 ± 3</td>
<td>52 ± 4</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>Pindolol</td>
<td>60 ± 4</td>
<td>60 ± 2</td>
<td>52 ± 4</td>
</tr>
<tr>
<td>Daunomycin</td>
<td>38 ± 4</td>
<td>55 ± 4</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>21 ± 3</td>
<td>15 ± 1</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>35 ± 2</td>
<td>0 ± 4</td>
<td>0 ± 4</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>0 ± 1</td>
<td>0 ± 1</td>
<td>0 ± 1</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>0 ± 2</td>
<td>0 ± 3</td>
<td>0 ± 2</td>
</tr>
<tr>
<td>Acyclovir</td>
<td>0 ± 1</td>
<td>0 ± 2</td>
<td>0 ± 2</td>
</tr>
<tr>
<td>Vincristine</td>
<td>0 ± 2</td>
<td>0 ± 4</td>
<td>0 ± 2</td>
</tr>
<tr>
<td>Colchicine</td>
<td>0 ± 4</td>
<td>0 ± 1</td>
<td>0 ± 1</td>
</tr>
</tbody>
</table>

* Each drug, at $5 \times 10^{-3} \text{M}$, was incubated at 37°C for 8 hr with 4 mg of ocular acid-insoluble melanin in a total volume of 2.0 ml. Buffers used were 20 mM potassium phosphate (pH 7.4 and 8.0) and 20 mM acetic acid–sodium acetate (pH 4.8). Incubation was performed with continuous shaking. Means and SD of three experiments are shown.
Yamabayashi and co-workers demonstrated radioautographically that \(^{3}H\)-befunolol is incorporated into the pigment granules of the iris, ciliary body, choroid, and retina of the rabbit. Araie and associates suggested pharmacokinetically that befunolol and timolol are bound to melanin-containing ocular tissues in pigmented rabbits. The present study also showed the binding of befunolol to ocular melanin, suggesting that this drug accumulates in the uvea and retina. Matsuda and associates, however, have indicated that befunolol produces retinal toxicity in cultured retinal pigment epithelial cells of chick embryo. The authors noted that pindolol had an affinity to acid-insoluble melanin, similar to other \(\beta\)-adrenergic antagonists such as timolol, labetalol, and practolol. Although the retinal toxicity of befunolol has been suggested, the ocular side effects of \(\beta\)-adrenergic blocking agents may be minimal or related to deficient tear secretion.

Intravitreal daunomycin, which has been tried therapeutically in experimental proliferative vitreoretinopathy, may produce retinal toxicity. Because it also shows an affinity for melanin, daunomycin should be evaluated carefully for clinical use. Similar care should be taken with 5-fluorouracil, which has been used intravitreally for the treatment of massive preretinal proliferation. Electrophysiologic findings have demonstrated the possible retinal toxicity of this drug. Because 5-fluorouracil demonstrates an affinity for ocular acid-insoluble melanin, it should be evaluated carefully for clinical use. Methotrexate has been assessed in the treatment of meningeval leukemia and retinoblastoma. Although intravitreal, subconjunctival, and intravenous doses of methotrexate have failed to produce retinal toxicity in albino rabbits, re-evaluation in pigmented animals should be done, because the drug has an affinity for melanin at an acidic pH.

Lyon and Krohn demonstrated that pigmented irides and ciliary bodies took up two to three times more pilocarpine than did albino tissues. However, pilocarpine did not bind to ocular melanin in our present study. Patil and Jacobowitz showed that twice as much epinephrine accumulated in pigmented iris as in nonpigmented iris. Lindquist demonstrated the uptake of epinephrine by uveal melanin granules in vitro, the binding of \(^{14}C\)-epinephrine to melanin structures in pigmented mice in vitro, and the high uptake of \(^{125}I\)-epinephrine in the uveal tract of adult pigmented mice. However, epinephrine did not bind to ocular melanin in the present study. These discrepancies between the previous investigations and the authors’ data may occur because of differences in assay conditions.

Lyon and Krohn and Patil and Jacobowitz used excised iris and ciliary body as pigmented tissue. Lindquist employed melanin granules and autoradiographic techniques, and the authors used ocular acid-insoluble melanin and spectrophotometry. It is possible that acid-insoluble melanin has been modified and reflects in-vivo binding capacity partially.

Acyclovir has been used to treat systemic and ocular infections from herpes virus organisms. The toxicity of intravitreal antiviral drugs has been tested by other investigators. Acyclovir did not bind to ocular acid-insoluble melanin in the assay conditions that the authors employed. Vincristine, which has been tried for the treatment of proliferative vitreoretinopathy, also had no affinity for melanin in this study. Colchicine, which inhibits mitosis, and has been used to treat Behçet’s disease, showed no affinity for ocular melanin. Although some drugs have an affinity for ocular melanin, they do not always produce retinal toxicity. However, the drugs that do bind with melanin should be evaluated carefully for clinical use.

**Key words:** melanin, affinity, drugs, ocular

**References**