Variable Effects of Previously Untested Muscarinic Receptor Antagonists on Experimental Myopia

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PURPOSE. Atropine, pirenzepine, and himbacine prevent form-deprivation myopia (FDM) when administered intravitreally. The mechanisms and sites of action of these drugs against myopia are not clear. To shed further light on whether this mechanism is muscarinic, several other muscarinic antagonists were tested.

METHODS. Various concentrations of atropine, pirenzepine, dextetimide, scopalamine, tropicamide, benzatropine, dicyclomine, gallamine, meperazolote, oxyphenonium, propantheline, procyclidine, 4-diphenylacetoxy-N-methylpiperidine (4-DAMP), hexahydro-sila-difenidol (HHSiD), p-fluorohexahydro-sila-difenidol (pF-HHSiD), methoctramine, AFDX-116, and quinuclidinyl benzilate (QNB) were injected into goggled eyes of Leghorn cockerels three times at 48-hour intervals. Fellow control eyes received saline. Control animals received saline in both eyes. Twenty-four hours after final injections, refraction, eye weight, and axial length were measured, and eyes were prepared for microscopy.

RESULTS. Other than atropine and pirenzepine, only oxyphenonium caused full rescue from FDM (goggled versus control; mean ± SD; refraction differences: −9.50 ± 0.22 D vs. 0.83 ± 0.31 D, P < 0.001; wet weight differences: 75.67 ± 3.84 mg vs. 2.33 ± 6.14 mg, P < 0.001; axial length differences: 0.80 ± 0.05 mm vs. 0.03 ± 0.04 mm, P < 0.001). Oxyphenonium-treated retinas showed no damage. Of the other compounds, several elicited partial rescue and/or damaged the retina, whereas others had no effect.

CONCLUSIONS. Oxyphenonium prevents FDM in chicks. The ineffectiveness or partial effectiveness of other compounds, coupled with the high concentrations of effective compounds required to prevent FDM, suggests that muscarinic antagonists act to prevent FDM, either at sites distant from the retina, or through a nonmuscarinic mechanism, on which only some of these drugs act. (Invest Ophthalmol Vis Sci. 2003;44:1330–1338) DOI:10.1167/iovs.02-04796

Ocular growth and refraction are influenced by visual image quality and focus in the developing eye. Poor image quality or inability to compensate for induced or acquired defocus may lead to either insufficient or excessive axial elongation, causing hyperopia or myopia, respectively. It is of both theoretic and therapeutic interest to understand how these changes come about.

A muscarinic cholinergic mechanism has long been implicated in the visual control of ocular growth, especially in experimentally induced form-deprivation myopia (FDM), whereby an image-degrading goggle induces axial elongation and myopic refraction in the growing eye. The muscarinic antagonists atropine and pirenzepine prevent or decrease the development of FDM in a number of species, including humans,1 macaque monkeys,2–4 chicks,5–7 and tree shrews,8 as does another muscarinic antagonist, himbacine, in chicks.9 This myopia-preventing activity has been taken to indicate that a muscarinic mechanism participates in the control of eye growth, and that acetylcholine may be a key transmitter in the transduction pathway linking visual image quality to changes in scleral growth. Further support for this hypothesis comes from the finding that in chicks, treatment of open eyes with muscarinic agonists (carbachol, pilocarpine, and McN-A-343) causes excessive axial elongation.10

In contrast to the myopia-preventing actions of atropine and pirenzepine, two other muscarinic antagonists, methoctramine and 4-diphenylacetoxy-N-methylpiperidine (4-DAMP), were reported not to prevent myopia when injected into the subconjunctival space of lid-sutured chick eyes.5 However, in a study of pirenzepine distribution in ocular tissues after intravitreous versus subconjunctival delivery, Cottriaul et al.11 found that the vitreous, retina, choroid, and sclera each contained a much greater concentration of drug after intravitreous injection than after subconjunctival administration. Therefore, the failure of methoctramine and 4-DAMP to prevent myopia could have been due to ineffective delivery of drug to intraocular targets through the subconjunctival route. In any case, assuming that the drugs reached appropriate targets in effective concentrations, the inactivity of methoctramine and 4-DAMP could indicate either that growth regulation is mediated by specific M1-like muscarinic acetylcholine receptors (mAChRs), responsive only to certain antagonists, as suggested previously,5 or that muscarinic receptors are not involved in growth regulation.

Further evidence against a muscarinic mechanism for growth control is that the concentrations of atropine, pirenzepine, and himbacine required to prevent FDM are high compared with the effective doses in other tissues, or even in retinal studies. For example, Yamashita et al.12 were able to block completely the release of intracellular Ca2+ in embryonic chick retinal cells in vitro with 1 μM atropine (in the presence of 100 μM acetylcholine), whereas a short-term concentration 1010 times higher than this is required to prevent FDM. This raises the possibility that at high concentrations, these muscarinic antagonists prevent excessive eye growth by binding to noncholinergic receptors in the retina, or that their action is mediated by muscarinic receptors located far from the site of application inside the eye. Indeed, the data of Fischer et al.13 suggest that neither retinal sources of acetylcholine nor mAChRs in the retina are necessary for atropine-mediated prevention of FDM. In their study, most sources of retinal acetylcholine were ablated by treatment with quisqualic acid (QA), with the exception of a few type II cholinergic cells that had...
damaged dendritic arbor or were located in the periphery, where they are assumed not to be able to influence growth in the posterior pole of the eye. QA treatment also severely depleted immunolabeling for most muscarinic acetylcholine receptors (mAChRs). If normal levels of retinal acetylcholine and/or mAChRs are essential for induction of excessive ocular growth, then atropine should not have the basis of deprivation-induced myopia in QA-treated retinas. However, this was not the case: Eye growth and the response to atropine were normal in QA-treated eyes. These results suggest that if a muscarinic mechanism is involved in the visual control of ocular growth, it is extraretinal, or at least that it is extraordinary in being able to function perfectly after approximately 90% destruction. In another study of the location of muscarinic mechanisms relevant to FDM, McBrien et al.6 demonstrated that atropine does not prevent FDM through an accommodative mechanism, because accommodation and light-induced pupillary constriction are not mediated by muscarinic mechanisms in chicks. However, myopia-preventing actions of muscarinic antagonists through other muscarinic systems, such as the cholinergic innervation of choroidal perivascular tissues, have not been ruled out.

Therefore, it remains uncertain whether the FDM-preventing actions of atropine, pirenzepine, and himbacine are mediated by mAChRs, or by nonmuscarinic processes. Our purpose in this study was to characterize the responses of visually regulated eye growth in the chick to a variety of muscarinic antagonists that have not been tested previously by intravitreal application, because if FDM is indeed prevented by a muscarinic mechanism, then a wide range of other muscarinic antagonists should also be able to prevent it. We also retested 4-DAMP and methoctramine, but by intravitreal rather than subconjunctival delivery. Two drugs that were previously found to be ineffective, gallamine and p-fluorophenylalanidine (pFH),14 were included in this study. The results presented herein show that only one of the previously untested muscarinic antagonists, oxyphenonium, was as effective at preventing FDM as atropine, pirenzepine, and himbacine.

**Materials and Methods**

**Animals**

White Leghorn cockerels were obtained from Lilydale Hatchery (Calgary, Alberta, Canada) on the day of hatching. Chicks were maintained in a 24-h light cycle (light on 7:00 AM) in a brooder with free access to food and water for 8 days, to allow them time to gain size and physical strength. For the experiments, chicks were transferred to clear plastic cages with steel mesh lids.

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and protocols were approved by the University of Calgary Animal Care Committee according to guidelines of the Canadian Council on Animal Care. A total of 250 chicks was used.

**Goggles**

Goggles of approximately 1 cm in diameter and 0.5 cm in depth were cut from clear plastic pharmaceutical bubble packaging. A 1-mm circumferential flange of plastic was left around the base of the goggle for periorbital adhesion, and a small triangular cutout was made in the posterodorsal quadrant to allow the needle to pass for injection of drugs without removing the goggle. The outside surfaces of the plastic goggles were roughened multiple times with fine sandpaper to create the form-depriving lenses. Goggles were affixed to the feathers surrounding the eyes with contact cement (Pres-tite; LePage, Brampton, Ontario, Canada).

**Injections**

On the eighth day after hatching, injections of muscarinic antagonists were started. All injections were administered with chicks under anesthesia with 1.5% halothane in 50% oxygen and 50% nitrous oxide. Drug solution (20 μL) was injected through the posterodorsal side of the left eye into the vitreous chamber using a 25-μL syringe (Hamilton, Reno, NV) with a 26-gauge needle. Immediately after the first drug injection, the left eye of each chick was fitted with a form-depriving goggle. Fellow control eyes were injected similarly with 20 μL of saline. Because one of the drugs, quinclidinyl benzilate (QNB), is not water soluble, it was dissolved in saline plus 12.5% dimethylsulfoxide (DMSO), and therefore saline plus 12.5% DMSO was used as vehicle for the control as well as treated eyes in the QNB group. Ethanol (70%) was used to disinfect feathers and skin surrounding the injection site and to sterilize needles between injections. Injections were given on days 1, 3, and 5, at 48-hour intervals. For each experiment, a group of control chicks received only saline vehicle (drug dose = 0) in both goggled and open eyes; therefore, a substantial number of control groups was run, and the ratio of experimental to control groups could vary from one experiment to another.

**Measurements**

Myopia is characterized by a negative refraction, accompanied by increased eye size and weight. On the day after the last injection, refraction without cycloplegia was performed in treatment and control eyes, with a slit retinoscope and test lenses. The chicks were then killed by chloroform inhalation; the eyes enucleated, trimmed clean, and weighed; and the axial length measured with digital calipers. The resolution of the calipers is ±200 μm.

**A-scan Ultrasonography**

Axial dimensions were measured by high-resolution A-scan ultrasonography, using a modification of a custom-designed computer-based system described in Wildsoet and Wallman15 and Nickla et al.16 Pulses were generated by a pulser/receiver (Model 5072PR; Panametrics, Waltham, MA) with a 30-MHz piezoelectric probe (Model PZ25-0.25), and reflections along the visual axis were sampled at a rate of 100 MHz (DAQ Board STR8100D; Sonix, Springfield, VA), scaled and displayed with programs written by Kevin Rada (University of North Dakota, Grand Forks, ND) in commercial software (LabView ver. 4.01; National Instruments, Austin, TX) on a generic computer. The distance between peaks marking the back of the retina and the front of the sclera was considered to indicate choroidal thickness. The physical limit of resolution on-axis is estimated as ±21 μm: resolution = (sound velocity)/2 × (bandwidth), with velocity = 1536 m/sec and bandwidth = 37 MHz,17 although with repeated measurements and averaging, the practical resolution may be better than ±10 μm.16

The A-scan ultrasonograph was acquired after most of the experiments in this study were completed. Therefore, only a portion of experimental groups was measured with this technique.

**Statistical Analysis**

Statistical analysis of raw data was performed on computer (InStat ver. 2.01, or Prism 3; GraphPad, San Diego, CA; for Macintosh, Apple Computer, Cupertino, CA). For intergroup comparisons, we used an ANOVA followed by the Dunnett post-test for comparisons of each treatment group with the control group.

**Drugs**

Each one of 18 muscarinic antagonists was injected into the form-deprived eyes of chicks in various concentrations (Table 1). Drug concentrations shown are those of stock solutions in the syringe. Because the volume of the vitreous body in the eye in 1- to 2-week-old White Leghorn chicks is approximately 180 μL (Luft WA, unpublished data, 2001), the effective dilutions of drugs in the eye were one tenth of the stock concentration. All drugs were purchased from Sigma-
Because high concentrations of drugs that are nonspecific for mACHR subtypes, such as atropine and pirenzipine, are necessary (20 μL of 100 mM stock solution, or 10 mM in the vitreous, every other day) to prevent FDM, it was logical to assume that similar concentrations of other muscarinic antagonists would be needed to elicit rescue effects. We were surprised to find that 9 of the 16 previously untested muscarinic antagonists were toxic at 100 mM (stock concentration). At this concentration, eight of these (benztropine, dicyclomine, mepenzolol, oxyphenonium, propantheline, procyclidine, 4-DAMP, and dextrometorphan) induced inflammatory responses and clouding of the vitreous accompanied by varying degrees of damage to the retina in the affected eyes. In this study, the terms “inflammation” and “inflammatory response” refer to pus formation in the vitreous cavity and infiltration of the retina and retinal pigmented epithelium by macrophages. Although it was impossible to measure refraction in the cloudy eyes, we noted that the affected eyes were considerably smaller in size and weight than saline-injected, form-deprived eyes. This is surprising, because the form deprivation due to clouding by the vitreous infiltrates would be expected to make eyes myopic and larger than normal. The ninth drug, gallamine, caused a strong systemic effect that resulted in death a few minutes after the intraocular injection of 100 mM stock. To avoid such toxic reactions, experiments using drugs described in this section were repeated at lower concentrations.

### Prevention of FDM

Open control eyes were approximately emmetropic, after correction for the +5.0 D artifact in refracting such small eyes. As expected, both atropine and pirenzipine caused an almost complete rescue of form-deprived eyes from myopia. Atropine and pirenzipine greatly reduced the excessive myopic refractive error, wet weight, and axial length differences normally induced by form deprivation, as shown in Table 2. We noted that there were significant differences in some of the axial length measurements depending on whether they were obtained with digital calipers or by A-scan. We explored a number of explanations for these differences, none of which was compelling. The discrepancy has no bearing on the conclusions to be drawn from this study, however, because both methods of axial length measurement showed a similar magnitude and the same direction of change, and both support the results obtained by the other measured parameters. Ultrasound measurements revealed no significant differences in choroidal thickness between treated and control eyes.

Only one other muscarinic antagonist tested, oxyphenonium bromide (10 mM), completely prevented FDM (Table 2, Fig. 1). Histologic analysis and immunocytochemical labeling showed no detectable morphologic damage or immune reaction in these retinas (Fig. 2). Figures 2A and 2B show retinas stained with toluidine blue, an acidophilic dye used to examine the overall appearance of tissue. Histologic examination revealed no difference in retinal structure or integrity between eyes injected with saline or 10 mM oxyphenonium. Figures 2C

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**Table 1. Drugs and Concentrations Injected in 20-μL Doses Three Times at 48-hour Intervals**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
<th>Drug</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Atropine</td>
<td>100 mM</td>
<td>Oxyphenonium</td>
<td>100, 10 mM</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>100 mM</td>
<td>Propantheline</td>
<td>100, 10 mM</td>
</tr>
<tr>
<td>Dextrometorphan</td>
<td>100, 10 mM</td>
<td>Procyclidine</td>
<td>100, 10 mM</td>
</tr>
<tr>
<td>Scopolamine</td>
<td>200, 100, 10, 0.1 mM</td>
<td>4-DAMP</td>
<td>100, 10 mM</td>
</tr>
<tr>
<td>Tropicamide</td>
<td>100 mM</td>
<td>HHSID</td>
<td>100, 10 mM</td>
</tr>
<tr>
<td>Benztpropine</td>
<td>100, 10, 1 mM</td>
<td>pfHHSID</td>
<td>100, 10 mM</td>
</tr>
<tr>
<td>Dicyclomine</td>
<td>100, 10 mM</td>
<td>Methocramine</td>
<td>5 μM</td>
</tr>
<tr>
<td>Gallamine</td>
<td>100, 1 mM</td>
<td>AF-DX-116</td>
<td>11.5 mM</td>
</tr>
<tr>
<td>Mepenzolone</td>
<td>100, 10 mM</td>
<td>QNB</td>
<td>7.4 mM</td>
</tr>
</tbody>
</table>

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**Results**

### Toxic Effects of Previously Untested Muscarinic Antagonists on FDM

After they were weighed and measured, eyes were hemisected equatorially, the vitreous removed, and the eyecups placed into chilled paraformaldehyde fixative solution (4% paraformaldehyde, and 3% sucrose in 0.1 M phosphate buffer) overnight. After fixation, eyes were washed in phosphate-buffered saline (PBS: 14.2 g Na2HPO4, 16 g NaCl in 2 L distilled water, pH adjusted to 7.4) for a minimum of three 20-minute washes. The eyecups were then cryoprotected overnight in PBS and 30% sucrose, for storage or sectioning. Three eyecups from each treatment group were preserved for immunohistochemistry.

**Histologic Staining**

Prepared slides were washed in PBS to remove mounting medium. Slides were incubated with 0.1% toluidine blue in dH2O for 5 minutes, and then washed a second time (three times for 20 minutes each). Slides were viewed by bright-field microscopy.

**Immunocytochemistry**

Prepared slides were washed in PBS (three times for 20 minutes each) to remove mounting medium and then incubated with 150 μL primary antibody solution at room temperature in a humidified chamber overnight. Slides were washed again in PBS (three times for 20 minutes each) to remove unbound primary antibody, incubated for 1 to 2 hours with 150 μL secondary antibody solutions, and washed in PBS. The sections were then covered in a 4:1 solution of glycerol-distilled water and glass coverslips and viewed by fluorescence microscopy. Images were digitally captured using a cooled charge-coupled device (CCD) black-and-white digital camera (Spot RT; Diagnostic Instruments, Inc., Sterling Heights, MI).

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**Fixation**

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**Sectioning**

Cryoprotected eyecups were embedded in optimal cutting temperature (OCT) compound (Tissue Tek; Sakura Finetek Inc., Torrance, CA), snap frozen in liquid nitrogen and mounted onto sectioning blocks. Sections approximately 15 μm in thickness were cut using a cryotome and mounted onto coated slides. The sections were then ringed with rubber cement and allowed to dry for approximately 2 hours before being used for histologic staining and immunocytochemistry.
TABLE 2. Refraction, Weight, Axial Length, and Choroidal Thickness Differences between Form-Deprived and Fellow Saline-Treated Control Eyes in Atropine, Pirenzepine, or Oxyphenonium-Treated Chicks

<table>
<thead>
<tr>
<th></th>
<th>Refraction Difference (D)</th>
<th>Weight Difference (mg)</th>
<th>Axial Length Difference (mm)</th>
<th>Axial Length Difference by A Scan (mm)</th>
<th>Choroid Thickness Difference (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine (100 mM) n = 5</td>
<td>-1.00 ± 0.45</td>
<td>27.40 ± 10.64</td>
<td>0.06 ± 0.07</td>
<td>0.08 ± 0.18</td>
<td>-0.02 ± 0.04</td>
</tr>
<tr>
<td>Saline n = 6</td>
<td>-8.83 ± 0.30</td>
<td>92.50 ± 8.97</td>
<td>0.67 ± 0.10</td>
<td>0.35 ± 0.13</td>
<td>0.07 ± 0.04</td>
</tr>
<tr>
<td>Pirenzepine (100 mM) n = 5</td>
<td>-4.2 ± 0.34</td>
<td>42.90 ± 7.56</td>
<td>0.41 ± 0.05</td>
<td>0.18 ± 0.12</td>
<td>-0.01 ± 0.07</td>
</tr>
<tr>
<td>Saline n = 5</td>
<td>-9.50 ± 0.22</td>
<td>75.67 ± 3.84</td>
<td>0.80 ± 0.05</td>
<td>0.39 ± 0.07</td>
<td>0.05 ± 0.05</td>
</tr>
<tr>
<td>Oxyphenonium (10 mM) n = 6</td>
<td>-0.83 ± 0.31</td>
<td>2.33 ± 6.14</td>
<td>0.03 ± 0.04</td>
<td>-0.14 ± 0.20</td>
<td>-0.05 ± 0.06</td>
</tr>
<tr>
<td>Saline n = 6</td>
<td>-9.5 ± 0.22</td>
<td>75.67 ± 3.84</td>
<td>0.80 ± 0.05</td>
<td>0.39 ± 0.07</td>
<td>0.03 ± 0.05</td>
</tr>
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</table>

Data are expressed as the mean ± SD.
Axial length was measured using both calipers and A-scan ultrasonography. The concentration is that of stock solution in the syringe; dilution in vitreous after injection is one tenth of this.

and 2D show retinas labeled with LEP100, an antibody for lysosomal membrane, which appears after infiltration of a tissue by phagocytic cells, such as macrophages. There was very little labeling in either of these images, demonstrating that 10 mM oxyphenonium injections do not induce any more macrophage invasion than in a saline-injected eye.

Most of the other drugs that were tested for effect on FDM in this study significantly but only partially counteracted the effects of form-deprivation on refraction and eye size (Table 3). Ultrasound measurements revealed no significant differences in choroidal thickness between drug-treated and control eyes. On refraction, wet weight, and axial length measurements, 4-DAMP had the greatest effect, followed (in order of decreasing effectiveness) by QNB (7.4 mM), p-fl-HHSiD, hexahydro-siladifenidol (HHSiD), scopolamine, AF-DX 116 (11.5 mM), propantheline, dextemidime, and tropicamide (all injected at 10 mM, unless stated otherwise).

As mentioned, 100 mM propantheline, benzotropine, dextemidime, and 4-DAMP each caused inflammation and severe retinal damage. However, when the concentrations of these compounds were reduced tenfold to 10 mM in the syringe, eyes appeared undamaged, but the weight and dimensions of the drug-treated form-deprived eyes were less than those of the saline-treated, open control group. These results raise the possibility that the effective concentrations of propantheline, benzotropine, dextemidime, and 4-DAMP may also cause covert retinal damage or inflammation, rather than act through mAChRs, because inflammation causes an absolute reduction in eye growth, whereas atropine and pirenzepine only prevent the form-deprivation-induced enlargement.2,18

Microscopic analysis of retinas treated with 10 mM 4-DAMP, propantheline, benztropine, and dextemidime revealed obvious retinal damage only with the former two. In propantheline-treated retinas, labeling of photoreceptors with 4D2, an antibody to rhodopsin, showed complete disruption of the rods (Fig. 3B) compared with those in saline-treated retinas (Fig. 3A). LEP100 labeling for lysosomal membranes was very strong.

![Figure 1](image1.png)  
**Figure 1.** Intercular differences (treated [T] minus control [C]) in (A) refraction, (B) wet weight, (C) axial length, and (D) axial length measured by A-scan ultrasonography in the eyes of chicks treated with 10 mM (in the syringe) oxyphenonium compared with the saline-treated control. All the data for the drug-treated group were found to be significantly different from those for the control (**P < 0.0001), by unpaired Student’s t-test. Error bars, SD (n = 6).

![Figure 2](image2.png)  
**Figure 2.** (A) Control retina and (B) retina treated with 10 mM oxyphenonium and stained with toluidine blue. (C) Control retina and (D) retina treated with 10 mM oxyphenonium and labeled for lysosomal membrane with a 1:50 solution of the LEP 100 antibody. Scale bar, 50 μm.
Throughout the propantheline-treated retina, indicating highly active phagocytosis. Immunolabeling of propantheline-treated retinas with antibodies to calretinin and calbindin also revealed disorder in the inner and outer nuclear and plexiform layers (data not shown). In retinas treated with 10 mM 4-DAMP, toluidine blue staining (Fig. 4B) showed that all layers were in disarray, with extensive damage to the photoreceptors. LEP100 labeling for lysosomal membrane (Fig. 4D) was strong throughout the retina, especially in the photoreceptor and ganglion cell layers. Large gaps in the retinal pigmented epithelium that were visible to the unaided eye were also apparent in the unprepared eyecups from 4-DAMP-treated eyes (data not shown).

Retinas treated with partially effective drugs that limited FDM, but did not cause obvious damage or inflammation, were also examined microscopically (data not shown). Retinas treated with scopolamine, tropicamide, HHSiD, or pfHHSiD did not show any signs of damage or inflammation. Retinas treated with QNB showed very slight damage to the photoreceptor outer segments, but no evidence of inflammation.

**Zero Effects of Previously Untested Muscarinic Antagonists**

The following drugs had no significant effect on eye growth in form-deprived chick eyes (data not shown): dicyclomine (10 mM), gallamine (10, 1 mM), procyclidine (10 mM), mepenzolate (10 mM), and methochromine (5 μM). Methochromine was administered at a much lower dose than the other drugs because of its known inhibitory action on the guanosine triphosphatase (GTPase) activity of G-proteins at micromolar (and higher) concentrations (according to the manufacturer), and therefore it is still unknown whether a higher concentration of this drug would elicit any rescue, damage, or inflammation.

**DISCUSSION**

Atropine, pirenzepine, and himbacine have been reported to prevent FDM. The present study was undertaken because of the uncertainty regarding whether mAChRs mediate this activity. It was expected that if blockade of muscarinic receptors mediates prevention of eye growth, then many if not all antagonists to the relevant muscarinic receptors should also be effective. It was also expected that intraocular delivery should make all drugs effective, including 4-DAMP and methochromine, even if they have not been shown to be effective subconjunctivally. Use of high doses should circumvent problems due to subtype specificity, which should disappear at sufficiently high drug concentrations.

The results of this study demonstrate that most muscarinic antagonists prevent FDM to some extent, but not as effectively as atropine, pirenzepine, and himbacine, even at comparatively high doses. Oxyphenonium (10 mM) is the only previ-
to cm2 and cm4 in chicken), himbacine is selective for particular muscarinic receptor subtype (Table 4). For example, among the drugs that elicit a full rescue, atropine is nonselective, pirenzepine is selective for M1 receptors (corresponding to cm2 and cm4 in chicken), himbacine is selective for mammalian M2 and M4 receptors (binding not characterized in chicken), and oxyphenonium is considered nonselective, like atropine. Another surprising finding is that scopalamine, the structure of which differs from that of atropine by only a single cyclic ether located in a region that is not supposed to have much effect on mAChR binding, did not cause full rescue from FDM, even at 200 mM in the syringe (20 mM in the eye), equivalent to the concentration of atropine in 100 mM atropine sulfate (Atr2SO4). As well, there are structural similarities between pirenzepine and AF-DX 116, and between oxyphenonium and mepenzolate, that suggest a greater degree of functional similarity than was observed.

We have also considered the possibility that one or more of the tested drugs were unstable in the intraocular environment. According to the manufacturers' information, however, all the compounds are stable when not in the presence of reactive chemicals, such as strong oxidizing agents or strong bases. It is therefore unlikely that any of the compounds tested was degraded spontaneously in the eye and lost its pharmacologic activity.

Subliminal Damage and Inflammation
The effect of retinal damage on eye growth in chicks has been seen previously in our laboratory in experiments with anti-sense oligodeoxynucleotide molecules with phosphorothioate backbones (Lencses KA, Luft WA, Stell WK, unpublished data, 2000), mercuric ions (Ramal-Shah A, Stell WK, unpublished data, 2001), the cholinotoxin, ECMA,13,29 nitric oxide donors (Gudgeon et al., manuscript submitted; Baird KJ, Stell WK, manuscript submitted), and experimental uveitis caused by platelet-activating factor (PAF).30 Each of these treatments causes various degrees of retinal damage through either an immune response or direct toxicity to retina and/or retinal pigment epithelium. The reduction in eye growth in these cases is not likely to be related to a specific pharmacologic action of these molecules, but rather to the destruction of cells and signaling mechanisms that are crucial for regulation of ocular growth. Given that propantheline, dexetimide, benztropine, AFDX-116, QNB, and 4-DAMP elicited partial rescue effects that were accompanied by signs of toxicity or inflammation, it is impossible to determine whether growth prevention
by these drugs was due to muscarinic blockade, toxicity, or inflammation. We assume that the reduction in eye size caused by some antagonists at doses that caused damage was not due to specific muscarinic antagonism but rather to disruption of cellular signaling pathways. It is possible that testing lower concentrations of these drugs would be more useful in assessing their FDM rescue abilities, because nonspecific actions would thus be minimized. It is unlikely that subtype-specific antagonists would have a significant effect at lower doses, however, because even a nonselective antagonist such as atropine had to be administered at millimolar concentrations to be effective.

That retinal damage prevents eye growth raises the possibility that the partially effective drugs (scopolamine, tropicamide, pirenzepine, himbacine, and oxyphenonium) may prevent FDM by subliminal retinal damage or inflammation, even though eyes treated with these drugs show no physical or immunocytochemical evidence that could be detected by our methods. For example, an undetected inflammatory response to the partially and fully effective drugs may prevent FDM by subliminal retinal damage or inflammation, because nonspecific actions would thus be minimized. It is unlikely that subtype-specific antagonists would have a significant effect at lower doses, however, because even a nonselective antagonist such as atropine had to be administered at millimolar concentrations to be effective.

Having considered the possibility that an undetectable immune response may be responsible for inhibiting deprivation-induced growth and myopia, we must also state the obvious alternative. The effective and partially effective drugs that do not cause retinal damage may be exerting their activity by binding to muscarinic receptor subtypes, or to other types of receptors.

### Nonretinal Receptors and Receptor Accessibility

A likely explanation for the discrepancy in activity among antagonists is that the muscarinic activity controlling eye growth is located, not in the retina, but rather in its surrounding tissues (ciliary body, choroid, or sclera). This is supported by the evidence in Fischer et al. which indicated that the actions of atropine and pirenzepine are not affected by ablation of most cholinergic activity and cholinergic neurons in the chick retina, including complete ablation in the fundus, where deprivation-induced growth takes place. In addition to the mAChRs located in the retina, immunoreactive muscarinic receptor subtypes cm2, cm3, and cm4 were identified in both the choroid and ciliary body. Lind et al. inferred that mAChRs are present in the chick sclera, because atropine, pirenzepine, and 4-DAMP inhibited scleral chondrocyte proliferation and glycosaminoglycan synthesis in vitro. The effective concentrations of these agents were high (1 mM), however, and because these experiments were performed on isolated chondrocytes in culture, tissue barriers cannot be a factor. If the muscarinic growth-controlling action is mediated by these extraretinal receptors, then the intravitreal delivery method may not be optimal, because any compound that is presented this way must diffuse from the vitreous, through the retina and across the blood-retina barrier of the pigmented epithelium to reach its target. Atropine, pirenzepine, himbacine, and oxyphenonium may have properties that allow them to pass through these layers and reach mAChRs in the choroid, ciliary body, or sclera in concentrations high enough to block the action of acetylcholine, and thus prevent the generation or transmission of growth-enhancing signals. Conversely, the diffusive properties of the other partially active compounds may slow their passage through the various tissue layers. In the case of the ineffective compounds, their chemistry may restrict diffusion so much that they remain bound in the vitreous, or may cause them to be transported or to diffuse out of the eye before reaching their targets, resulting in ineffective concentrations at the target. This is unlikely, however, because oxyphenonium (one of the four completely effective compounds) is a quaternary amine, known to cross tissue barriers poorly, and if poor diffusion were a problem, then it is expected that this drug would not have been effective.

### Nonmuscarnic Receptors

Although we identified another muscarinic antagonist that prevents FDM, it was surprising that so many others were rather ineffective. The binding affinities and potencies of many of the antagonists that were found to be ineffective against FDM in the present study cover a wide range and overlap with those of atropine, pirenzepine, himbacine, and oxyphenonium (Table

<table>
<thead>
<tr>
<th>Agent</th>
<th>Pharmacologic Selectivity</th>
<th>Relative Affinity</th>
<th>Vitreous Concentration</th>
<th>Effect on FDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine</td>
<td>Nonselective</td>
<td>m1, m3, m4 &gt; m5 &gt; m2</td>
<td>10 mM</td>
<td>Full rescue</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>M1</td>
<td>M1 &gt; m3, m4, m5 &gt; m2</td>
<td>10 mM</td>
<td>Full rescue</td>
</tr>
<tr>
<td>Oxypenydronium</td>
<td>Nonselective</td>
<td>m2 &gt; m5 &gt; m3 &gt; m4</td>
<td>1.0 mM</td>
<td>Partial rescue; damaged retina</td>
</tr>
<tr>
<td>Propantheline</td>
<td>Nonselective</td>
<td>m2 &gt; m5 &gt; m3 &gt; m1</td>
<td>0.01 mM</td>
<td>Partial rescue</td>
</tr>
<tr>
<td>Scopolamine</td>
<td>None Selective; &gt; pirenzepine</td>
<td>Similar to that of atropine, M1 &gt; M2</td>
<td>20, 10, 1, 0.1,</td>
<td>10 mM Partial rescue</td>
</tr>
<tr>
<td>Tropicamide</td>
<td>Small M4 selectivity</td>
<td>m2 &gt; m5 &gt; m3</td>
<td>10 mM</td>
<td>Partial rescue</td>
</tr>
<tr>
<td>Dexetimide</td>
<td>Nonselective</td>
<td>m1, m3, m4 &gt; m5 &gt; m2</td>
<td>10, 1, 0.1 mM</td>
<td>Partial rescue</td>
</tr>
<tr>
<td>Benztropine</td>
<td>M1 &gt; M2</td>
<td>m1 &gt; M2</td>
<td>10, 1, 0.1 mM</td>
<td>Partial rescue</td>
</tr>
<tr>
<td>Dicyclomine</td>
<td>M1</td>
<td>m1 &gt; m3 &gt; m4 &gt; m5</td>
<td>10, 1 mM</td>
<td>No rescue</td>
</tr>
<tr>
<td>Gallamine</td>
<td>Cardioselective (i.e. m2)</td>
<td>m2 &gt; m4 &gt; m1 &gt; m3</td>
<td>1.0 mM</td>
<td>Toxic, no rescue</td>
</tr>
<tr>
<td>Methochromain</td>
<td>Cardioselective, (m2)</td>
<td>m2 &gt; m4 &gt; m3 &gt; m1</td>
<td>10, 1 mM</td>
<td>No rescue</td>
</tr>
<tr>
<td>Procyclidine</td>
<td></td>
<td>m2 &gt; m4 &gt; m3 &gt; m1</td>
<td>10, 1 mM</td>
<td>No rescue</td>
</tr>
<tr>
<td>h-DAMP</td>
<td>M3, M5, M4 selective &gt; M2</td>
<td>m3, m1 &gt; m5 &gt; m4 &gt; m2</td>
<td>10, 1, 0.1 mM</td>
<td>Partial rescue</td>
</tr>
<tr>
<td>HHSID</td>
<td>M5, M4 &gt; M2</td>
<td>m3 &gt; m1 &gt; m5 &gt; m4 &gt; m2</td>
<td>10, 1, 0.1 mM</td>
<td>Partial rescue</td>
</tr>
<tr>
<td>pHHSID</td>
<td>M5</td>
<td>m3 &gt; m1 &gt; m4 &gt; m5 &gt; m2</td>
<td>10, 1, 0.1 mM</td>
<td>Partial rescue</td>
</tr>
<tr>
<td>AF-DX 116</td>
<td>M2</td>
<td>m2 &gt; m4 &gt; m3 &gt; m1</td>
<td>1.15 mM</td>
<td>Partial rescue</td>
</tr>
<tr>
<td>QNB</td>
<td>M2</td>
<td>m2 &gt; m4 &gt; m3 &gt; m1</td>
<td>0.5 μM</td>
<td>No rescue</td>
</tr>
</tbody>
</table>

### Table 4. Pharmacological Selectivities and Binding Affinities of Muscarinic Antagonists Tested in Form-Deprived Chick Eyes

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4). As well, we found no consistent correlation between effectiveness against FDM and chemical properties thought to be predictive of muscarinic antagonism.33 Thus, the question remains whether rescue by atropine, pirenzepine, himbacine, and oxyphenonium could be mediated by a nonmuscarinic mechanism. Evidence against a specific action of these drugs can be inferred from the study by Lind et al.32 cited earlier. The action of muscarinic antagonists in the absence of any known source of acetylcholine in scleral cell culture may argue for a non-mACHr-mediated action.

The ability of atropine and pirenzepine to prevent FDM even when cholinergic cells and receptors are absent from the retina could be explained by action through some noncholinergic, nonmuscarinic receptor or cellular signal mechanism, even one located in the retina, that is necessary for control of eye growth. The phenomenon of binding and action of drugs from one class on receptors of another class has been observed in previous studies. For example, many opiate agonists and antagonists are known to bind to and block N-methyl-D-aspartate (NMDA) receptors.34,35 As well, one of the partially effective muscarinic antagonists, benztpine, is known to block dopamine transporters36 and to increase dopamine efflux from neurons of the substantia nigra.37 Benztpine did not elicit full rescue in the present study, however. Still, it is conceivable that oxyphenonium, pirenzepine, and atropine could exert some benztpine-like activity through the dopaminergic system, although atropine is not an effective dopamine reuptake blocker.38 Some of these muscarinic compounds may also be acting through nicotinic receptors in the eye, as has occurred in studies of atropine on rat nACHRs.38 Stone et al.39 reported that four nicotinic antagonists have myopia- and growth-preventing effects in form-deprived chicks and that nACHRs may therefore be involved in eye growth.

In view of the evidence cited herein, it is possible that some receptor types other than mAChRs mediate the FDM rescue activity of atropine, pirenzepine, himbacine, and oxyphenonium. If the binding affinities of muscarinic antagonists for nonmuscarinic receptors were significantly lower than for mAChRs, then higher concentrations of the drugs would be required to stimulate or inhibit the signal mechanism, even if the target receptors were located in the retina. Therefore, this hypothesis is consistent with our observations.

In summary, given the evidence from this study and from Fischer et al.,13 it remains a strong possibility that cholinergic receptors in the retina are not the route by which atropine and other muscarinic antagonists prevent FDM. Whether the mechanism is through retinal nonmuscarinic mechanisms, or muscarinic receptors in extraretinal tissues, is still not clear. However, if a muscarinic mechanism outside the retina was the functional route, then it would be expected that several of the antagonists tested—especially those with relatively nonselective binding profiles, such as scopolamine, dexetimide, QNB and 4-DAMP—would have elicited a full rescue effect. Because these drugs were not fully effective and effectiveness was not strongly correlated with structural indicators of specific anti- muscarinic activity, we suggest that the few antagonists that fully prevent FDM probably do so through nonmuscarinic mechanisms.

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References


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