Myopia-Inhibiting Concentrations of Muscarinic Receptor Antagonists Block Activation of Alpha2A-Adrenoceptors In Vitro


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Purpose. Myopia is a refractive disorder that degrades vision. It can be treated with atropine, a muscarinic acetylcholine receptor (mACr) antagonist, but the mechanism is unknown. Atropine may block alpha2-adrenoceptors at concentrations ≥0.1 mM, and another potent myopia-inhibiting ligand, mamba toxin-3 (MT3), binds equally well to human mACr M4 and alpha2A- and alpha2C-adrenoceptors. We hypothesized that mACr antagonists could inhibit myopia via alpha2A-adrenoceptors, rather than mACr M4.

Methods. Human mACr M4 (M4), chicken mACr M4 (cM4), or human alpha2A-adrenergic receptor (hADRA2A) clones were cotransfected with CRE/promoter-luciferase (CRE-Luc; agonist-induced luminescence) and Renilla luciferase (RLuc; normalizing control) into human cells. Inhibition of normalized agonist-induced luminescence by antagonists (ATR: atropine; MT3; HIM: himbacine; PRZ: pirenzepine; TRP: tropicamide; OXY: oxyphenonium; QNB: 3quinuclidinyl benzilate; DIC: dicyclomine; MEP: mezenpazolate) was measured using the Dual-Glo Luciferase Assay System.

Results. Relative inhibitory potencies of mACr antagonists at mACr M4/cM4, from most to least potent, were QNB > OXY > ATR > MEP > HIM > DIC > PRZ > TRP > MT3 was 56× less potent at cM4 than at M4. Relative potencies of mACr antagonists at hADRA2A, from most to least potent, were MT3 > HIM > ATR > OXY > PRZ > TRP > QNB > MEP; DIC did not antagonize.

Conclusions. Muscarinic antagonists block hADRA2A signaling at concentrations comparable to those used to inhibit chick myopia (≥0.1 mM) in vivo. Relative potencies at hADRA2A, but not M4/cM4, correlate with reported abilities to inhibit chick form-deprivation myopia. mACr antagonists might inhibit myopia via alpha2-adrenoceptors, instead of through the mACr M4/cM4 receptor subtype.

Keywords: atropine, form-deprivation myopia, nonspecific binding, off-target, muscarinic antagonist, alpha2a-adrenoceptor

Myopia (near- or short-sightedness) is a common eye condition that increases the risk of ocular pathology as it progresses.1,2 As its prevalence continues to rise,3 so does the need for an effective therapy. Topical atropine is effective against myopia in children4; however, treatment requires daily application, and the dosage most commonly prescribed for many years (1%) may induce allergic reactions, as well as muscarinic acetylcholine receptor (mACr) M3-mediated side effects—mydriasis, photophobia, cyclopia—and possibly early presbyopia.4,5 Atropine administered topically at concentrations of 1% loses effectiveness over a period of 1 to 2 years, and upon cessation, “rebound” may occur, wherein myopia returns at a faster rate than in untreated eyes6,7—possibly because of the desensitization of target receptors in response to a high concentration of anticholinergic drug. Recent studies have supported the use of 0.01% atropine to treat childhood myopia.8,9 This comparatively lower dose of atropine appears to retain its effectiveness for inhibiting myopia, and results in a reduced severity of side effects such as allergies, loss of accommodation, and rebound. Some of those treated with 0.01% atropine still complain of muscarinic receptor (mACr) blockade-induced complications, including photophobia and
blurred vision; however, those symptoms are not severe enough to prompt a discontinuation of treatment. Therefore, 0.01% atropine is currently the most favored concentration prescribed by clinicians both in Southeast Asia and North America.9-12 Atropine is a potent nonselective mAChR antagonist. Thus, it has been widely assumed to inhibit myopia via blockade of mAChRs. Another potent mAChR antagonist, mamba toxin-3 (MT3), is strongly selective for mAChR M4 over other mAChR subtypes in mammals,13 and inhibits form-deprivation myopia (FDM) in the chick at far lower concentrations than those required for atropine (17.5–70 pmol versus 20–2000 nmol per injection, respectively)14-16. Therefore, given the ability of MT3 to block myopia development in the chick model, it has been suggested that the mAChR M4 receptor subtype is the target for myopia-controlling action of both atropine and MT3.14,17 It is important to point out, however, that a direct mechanistic link between mAChR systems in the eye and the mAChR antagonist properties of atropine or MT3 for inhibition of myopia has never been demonstrated.

Moreover, some evidence suggests that the ability of atropine to inhibit myopia development may not involve mAChRs at all.18 For example, most mAChR antagonists, even very potent ones such as 3-quinuclidinyl benzilate (QNB) or dicyclomine, do not inhibit myopia in chicks, and those muscarinic antagonists that do so inhibit myopia require concentrations that can be orders of magnitude above their inhibition constants for blocking mAChRs (Table 1).16,19 Furthermore, ablating ≥90% of choline acetyltransferase (ChAT)-containing amacrine cells—the only known source of retinal acetylcholine (ACh; the natural agonist at mAChRs)—in the chick has no effect on the eyes’ ability to achieve emmetropia, nor does elimination of retinal ChAT impair myopia inhibition by atropine.20 Treatment with myopia-inhibiting concentrations of atropine in chick retina-RPE-choroid-sclera preparations causes a massive, nonspecific release of retinal neurotransmitters (including dopamine), and induces spreading depression that affects the entire retina, not just mAChR-related circuits.21 Finally, significant myopia-related changes in eye size in the tree shrew and chick do not result in significant changes in mRNA or protein expression of any mAChR subtype in the retina,22,25 nor does nor-maChR-induced axial elongation result in changes in the concentration of retinal ACh, or its metabolite colchicine.24 Results such as these have led to arguments that favor a nonretinal mechanism of atropine-mediated myopia inhibition, with mAChR binding in the choroid or sclera suggested as the most likely alternative. One way to determine whether the molecular targets of anti-myopia therapy—that are modified directly by treatment with mAChR antagonists—are located in these tissues, is to look for changes in receptor density upon induction of experimental myopia and during atropine treatment. There is no change, however, in mAChR mRNA expression in either the choroid or sclera, upon induction of experimental myopia in intact eyes of tree shrew and chick.22,25 A second method to determine whether the choroid or sclera is directly responsible for atropine-mediated myopia inhibition is to examine the responses to atropine in these tissues specifically, in in vitro preparations of eyecups lacking retina. In such preparations, the action of atropine to inhibit extracellular matrix production and sulfate incorporation into the glycosaminoglycans of chick scleral cartilage26 and the inhibition of carbachol-induced mouse scleral fibroblast proliferation20 were observed only at high concentrations of atropine (0.5–100 μM in the medium), which are 500- to 10,000-fold higher than those that block mAChRs (Kᵢ = 1–10 nM; Table 1). Finally, while pirenzepine treatment has been shown to result in changes in choroidal thickness when applied to in vitro preparations of chick eyecups comprising the RPE-choroid-sclera, it did so only at high concentration (5 mM); other mAChR-targeted antagonists, such as oxyphenonium (1 mM) and dicyclomine (0.6 mM), had no effect on the choroid in these preparations.27 Unfortunately, the effect of atropine on choroidal thickness in chick eyecups lacking retina was not tested.

Of particular interest in the above examples is the requirement of high concentrations of atropine and other mAChR antagonists for myopia-inhibiting effects, relative to the subnanomolar affinities (Kᵢ) of many of these antagonists for the M₄ mAChR (Table 1). Indeed, even if a drug binds selectively to its preferred receptors with high affinity (e.g., Kᵢ ≤ 1 nM), it is expected that at concentrations two to three orders of magnitude higher it will bind to, and affect the activity of, other (“off-target”) receptors.21 Thus, atropine, while receptor-selective at nanomolar concentrations, would very likely have off-target effects at other receptors at the micro- to millimolar concentrations used to mitigate myopia. Additionally, while it is true that MT3 can inhibit myopia at much lower concentrations than atropine,14,15 this result cannot be interpreted to mean that it does so through M₄ or any other mAChR on this basis alone. Although MT3 is selective for the M₄ receptor over other mAChRs,15,28 it has a comparably high inhibitory potency (IC₅₀ = 1–10 nM) at human α₂A, α₂D, and α₂-adrenergic receptors; moderate inhibitory potency (IC₅₀ = 25–50 nM) at α₁B and α₂C-adrenergic receptors; and low inhibitory potency (IC₅₀ = 200 nM) at the mAChR M₁ receptor.29,30 There are also data to indicate that at high concentrations (1–100 μM) atropine can also have antagonist activity at α₁-adrenoceptors,30,31 although the specific adrenoceptor subtypes to which it may bind remain unknown.

Chicks are a popular animal model for myopia drug studies. They have high-quality vision, active accommodation and emmetropization, and large eyes for easy intravitreal drug delivery. Diffusors52-54 and negative lenses55,56 induce large changes in axial length and refractive error (−5 to −15 diopter [D]) in a matter of days, and results from chick experiments can usually be replicated in mammals and nonhuman primates.37 Interpretations of results, using mAChR antagonists to prevent FDM in the chick, have generally presumed that these ligands work the same way at avian receptors as they do at human or other mammalian receptors. That said, the chick retinal structure and G-protein receptor sequences differ significantly from those of mammals. Chickens lack an orthologue for the mammalian mAChR M₄. Instead, chicken M₂ (cM₂), the orthologue for the mammalian M₂ receptor, has some pharmacologic properties of both mammalian M₁ and M₂ receptors, such as relatively high affinity for pirenzepine.38 Thus, in the chick, one can conclude that MT3 will target the M₄ mAChR and not other mAChR subtypes. Furthermore, there are examples of substantial differences in ligand behavior between species, even for mammals, whereby even minor sequence differences can cause receptor-selective ligands to interact differently, with the same G-protein-coupled receptor (GPCR) orthologue.39,40 We therefore considered it likely that MT3, which is highly selective for the mammalian M₄ receptor, might have a significantly altered ability to bind to the chick M₄ receptor.

Given the likelihood that the concentrations of atropine used for treating myopia in humans and chicks can act at receptors other than mAChR M₄, we focused on the molecular pharmacology of the chick cM₂ and human M₄ receptors, and the interactions of these receptors with the mAChR-targeted agents that have been used to evaluate myopia development in the chick model. Furthermore, since both MT3 and atropine can potentially interact with α₁-adrenoceptors, we also explored the interactions of FDM-mitigating agents with the
human α2A-adrenergic receptor ADRA2A (hADRA2A) subtype. To test the hypothesis that non-mAChR mechanisms can mediate the inhibition of myopia development by mAChR antagonists in the chick FDM model, we evaluated (1) whether mAChR antagonists have similar relative inhibitory potencies at chick cM4 compared to human M4, and (2) whether mAChR antagonists can bind to, and inhibit signaling by, hADRA2A. We chose to investigate the binding of mAChR antagonists to α2A-adrenergic receptors because MT3 has a high affinity for these adrenergic receptors, because this adrenoceptor is the most common α-adrenoceptor subtype in the central nervous system,11 and because some α2-adrenergic agonists have been reported to inhibit FDM in the chick (Carr BJ, Stell WK. IOVS 2016;57:ARVO E-Abstract 4738) and guinea pig.42

### Methods and Materials

#### Experimental Strategy

Our strategy to explore the interactions of the FDM-related antagonists with either mAChR M4 or cM4 was to express the recombinant receptors in a mammalian Lenti-X HEK 293T cell line, in which the only endogenously expressed mAChR (M3) was eliminated by a CRISPR/Cas9-mediated genomic editing approach (CRISPR-M3 cells: CR-M3). The HEK cell line is well recognized for its ability to couple the signaling of GPCRs from multiple species efficiently to multiple effectors, including Gs, Gi, Gαq, and G12/13. In this system, the abundance of expressed muscarinic and adrenergic receptors enables coupling to Gs, with a resulting increase in synthesis of cyclic AMP (cAMP).43-44 In turn, the cAMP drives a cAMP response element (CRE)-luciferase reporter construct, such that increased luciferase activity serves as an index of activation of the transfected muscarinic and adrenergic receptors. Carbachol was used to activate the chick and human mAChRs, and clonidine was used as the agonist to activate hADRA2A. The ability of increasing concentrations of antagonists to inhibit the cholinergic and adrenergic responses was used as an index of their relative potencies for blocking the expressed receptors. The relative inhibition constants of the myopia-inhibiting compounds, for blocking either the muscarinic or α2A-adrenergic receptors, were compared directly with the relative ability of the compounds to mitigate myopia in the chick FDM model.

### DNA and Expression Vectors

All receptor clones were expressed in a pcDNA 3.1(+) vector (Invitrogen, Thermofisher Scientific, Waltham, MA, USA). The cM4 genomic clone was obtained as previously described,18 and the M4 (MAR040000) and hADRA2A (AR0A2A000) receptor clones were purchased from the cDNA Resource Center (Bloomburg University Foundation, Bloomburg, PA, USA). The CAMP response element luciferase vector (CRE-Luc; pGL4.29[lu2P/CRE/Hygro]) and the constitutively active Renilla luciferase control vector (RLuc; pRL-RK) were purchased from Promega (Madison, WI, USA).

### CRISPR/Cas9 Knockdown of mAChR M4

CRISPR knockout of mAChR M4 in Lenti-X HEK 293T cells (LX293T; Clontech, Mountain View, CA, USA) was performed to ensure that mAChR ligand-mediated effects on cAMP were the

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### Table 1. Binding Data for Muscarinic Antagonists Tested at mAChR M4 and cM4 in Our Assay

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC_{50} Study</th>
<th>K_{ICP} Study</th>
<th>K_{i} Literature</th>
<th>Inhibits FDM Est. [Vit]</th>
<th>Source</th>
<th>Cat No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATR</td>
<td>*390 nM</td>
<td>*140 pM</td>
<td>*125–250 pM</td>
<td>Yes16, 0.1–10 nM</td>
<td>Sigma</td>
<td>A0257</td>
</tr>
<tr>
<td>MT3</td>
<td>*8 nM</td>
<td>*3 nM</td>
<td>*2 nM</td>
<td>Yes14, 10–350 nM</td>
<td>Peptides International</td>
<td>PMT-4410-s</td>
</tr>
<tr>
<td>HIM</td>
<td>*1 nM</td>
<td>*1 nM</td>
<td>*100–160 pM</td>
<td>Yes15, 3–12 mM</td>
<td>Enzo Life Sciences</td>
<td>ALX-550-061</td>
</tr>
<tr>
<td>QNB</td>
<td>*115 nM</td>
<td>*40 pM</td>
<td>*315 pM</td>
<td>Partial16, 0.74 mM</td>
<td>Sustainable Scientific</td>
<td>BR-143015</td>
</tr>
<tr>
<td>TRP</td>
<td>*155 nM</td>
<td>*55 nM</td>
<td>*166 nM</td>
<td>Partial16, 10 mM</td>
<td>Sigma</td>
<td>T9778</td>
</tr>
<tr>
<td>MEP</td>
<td>*1 nM</td>
<td>*360 pM</td>
<td>*16 nM</td>
<td>No16, 1–10 mM</td>
<td>Sigma</td>
<td>M0651</td>
</tr>
<tr>
<td>DIC</td>
<td>*15 nM</td>
<td>*5 nM</td>
<td>*5–160 nM</td>
<td>No16, 1–10 mM</td>
<td>Sigma</td>
<td>D7909</td>
</tr>
</tbody>
</table>

* Human or mammalian mAChR M4 receptor or tissue.
† Avian mAChR M4 receptor or tissue.

Non-M4/cM4-specific receptors/tissues: rat M4,125; guinea pig atria and ileum,126; chick heart.19,58,127 IC_{50} Study data are the data determined by our binding assay, K_{ICP} Study data are calculated from the IC_{50} values obtained by our assay by the method of Cheng and Prusoff,49 and K_{i} Literature data are data from previously published studies. Maximum inhibition achieved by all antagonists was 100%. ATR, atropine sulfate; OXY, oxyphenonium bromide; PRZ, pirenzepine dihydrochloride; CCh, carbachol; Est. [Vit], estimated vitreal concentrations, assuming uniform dilution in total vitreal volume; required for myopia inhibition in the chick; TRP, tropicamide; N/A, not available.

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**Note:** The text continues with further details on experimental methods, data interpretation, and conclusions related to the study's findings.
result of activity at transfected, and not endogenous, receptors.3,48 Stable CRISPR M3-knockout LX293T cells are referred to as CR-M3 cells. Cell culture medium, serum, transfection materials, culture flasks, and multiwell plates were purchased from Thermofisher Scientific unless specified otherwise. The knockout design and procedures used to derive the CR-M3 cells from the wild-type LX293T cells, using the GeCKO CRISPR protocol (available in the public domain, https://www.addgene.org/crispr/libraries/geckov2/), were as described by Sanjana et al.37 and Shalem et al.48 Three sets of genome-specific single guide RNA sequences to mACHR M3 (F to R) were chosen from the GeCKOv2 Human Library, and both strands of oligonucleotides were synthesized for each target sequence (F: CACCGgcgctttcttaacgggcatcc; R: AAACggatgcccgttgtgacC; F: CACCGgcgctacccgagc; R: AAACgctcgggtgcgcgcttgtgacC; F: CACCGgcgcttacccggcttgtgacC; R: AAACgctcgggtgcgcgcttgtgacC; F: CACCGgcgcttaacgggcatcc; R: AAACgctcgggtgcgcgcttgtgacC; F: CACCGgcgcttcacccgagc; R: AAACgctcgggtgcgcgcttgtgacC; F: CACCGgcgctttcttaacgggcatcc; R: AAACggatgcccgttgtgacC; F: CACCGgcgctttcttaacgggcatcc; R: AAACggatgcccgttgtgacC; F: CACCGgcgctttcttaacgggcatcc; R: AAACggatgcccgttgtgacC). The lower-case sequences are target sequences of the M3 genomic locus, and the uppercase sequences are flanking sequences for cloning. Both strands of the oligonucleotides were annealed and inserted in BonBI restriction enzyme sites under a U6 promoter in a CRISPR/Cas9 vector (lentiCRISPR v2), a gift from Feng Zhang (Addgene plasmid #52961; Cambridge, MA, USA). The lentiCRISPR v2 plasmids containing three M3-targeting sequences were mixed and transfected using Lipofectamine LTX Reagent. The transfected cells were maintained in the presence of 5 μg/ml puromycin to select for knockout cells. Once established, stable-knockout CR-M3 cells were resuspended in high-glucose Dulbecco’s medium (DMEM) + 10% dimethyl sulfoxide and frozen in liquid nitrogen for future use.

Cell Culture and Transfection Protocols

CR-M3 cells were grown in high-glucose DMEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, and 5 μg/ml Plasmocin (InvivoGen, San Diego, CA, USA) in a 5% CO2 environment at 37°C. Cells were maintained at <80% confluency and passaged with phosphate-buffered isotonic saline, pH 7.4, containing enzyme-free EDTA (1 mM), with mild trypsinization (90 μl 0.25% trypsin-EDTA/25 cm² T-flask) to optimize homogeneity during re platting. Transfection was performed in 12-well plates at 30% confluence. For each well to be transfected, 4 μl Lipofectamine LTX was diluted in 50 μl Opti-MEM reduced serum medium; vector cDNAs (CRE-Luc [180 ng], RLuc [160 ng], and receptor genomic clones [160 ng]) were mixed in a separate aliquot of 50 μl Opti-MEM. Equal volumes of the LTX and DNA solutions were then combined (1:1 ratio), and the cells were incubated at room temperature for 5 minutes, after which the LTX-DNA complexes were added (100 μl LTX-DNA complex/well) to wells with gentle mixing. The cells were allowed to grow in 5% CO2 at 37°C, and the cell medium was changed to supplemented DMEM without selection after a minimum exposure of 8 hours to the transfection medium. Twenty-four hours after transfection, cells were seeded (7500 cells/well) into white, clear-bottomed, tissue-treated 96-well plates, fed with supplemented DMEM without selection, and allowed to grow overnight.

CRE-Luc Luminescence Assay and Antagonist IC50 Curves

Forty-eight hours after transfection, the cell medium was aspirated and replaced with various concentrations of antagonist diluted in FluoroBrite DMEM containing a fixed concentration of agonist (50 μM/well); treated cells were incubated for 4 hours at 37°C under 5% CO2 + O2. The fixed agonist concentration (carbachol: 10 μM; or clonidine: 1 μM) was chosen, on the basis of preliminary concentration-response studies, to produce large but submaximal activation of normalized CRE-Luc luminescence (LucN). Submaximal concentrations (78%–90% activation) were used instead of 50% or 100% activation, so as to extend the dynamic range of measurable responses. The choice of antagonists for testing was based on availability and previously published reports of their ability to prevent FDM in the chick (Table 1). All drug stock solutions (10 mM) were made using sterile distilled H2O, with the exception of QNB (5 mM) and himbacine (9 mM), which were dissolved in 100% and 90% methanol, respectively. The maximum concentration of methanol that did not cause cell death, as determined by Robust regression and Outlier removal (ROUT; GraphPad, La Jolla, CA, USA) statistical analysis, was 6%.

Changes in intracellular cAMP were measured indirectly using the Dual-Glo Luciferase Assay System and protocol (Promega). Drug-treated cells and Dual-Glo assay reagents were equilibrated to room temperature, and then 50 μl Dual-Glo Luciferase Reagent was added to each sample well. The plate was incubated for 10 minutes at room temperature, with moderate shaking to ensure complete cell lysis. After incubation, CRE-Luc levels were measured using a Victor X4 Spectrophotometer (PerkinElmer, Waltham, MA, USA). Once CRE-Luc measurements were complete, 50 μl Dual-Glo Stop-and-Go Reagent was added to each of the sample wells, the 10-minute incubation with shaking was repeated, and then the levels of RLuc (normalizing control) in the wells were measured in the same order, using the same spectrophotometer.

Data Analysis

Raw RLuc data were first subjected to outlier detection using ROUT analysis with a 1% false detection rate (GraphPad Prism v 6.07; La Jolla, CA, USA). Flagged data usually represented significant decreases in RLuc expression, due to cell death caused by high-concentration drug or methanol. There were no instances of significant cell death for any of the experiments involving testing at M4 or cM4. At hADRA2A, cell death occurred at dicyclomine hydrochloride (DIC) (>500 μM), QNB (>500 μM, 6% methanol), oxypenonium (OXY) (>3 mM), mepenzolate bromide (MEP) (>10 mM), and himbacine (HIM) (>500 μM). These data were removed along with their corresponding CRE-Luc values in the final analysis. Remaining CRE-Luc data were normalized to RLuc (CRE-Luc/RLuc = LucN), and then either the fold-change LucN (agonist treatment: [treated well − well without agonist] ÷ well without agonist) or the percent maximum response (antagonist treatment: [treated well ÷ well without antagonist] × 100) was calculated. For graphing and IC50 calculations, molar concentrations were graphed on the x-axis, transformed using the algorithm X = Log(X), and then nonlinear regression analysis was performed. Graphical data are represented as curve-fitted linear regressions of the means of the transformed molar concentration values ± SEM (GraphPad Prism, Version 6.07); n = 3 or 4 experiments, performed in duplicate. Inhibitory constants (KIC50) were estimated from these data by the method of Cheng and Prusoff (pKIC50 = IC50 ÷ (1 + ([A]/EC50))),49,50 where [A] = agonist concentration used, and EC50 was the agonist concentration required to elicit a 50% response, as determined by previous experiments using our assay system. Data are reported as either IC50 (assay/system-dependent inhibitory potency), KI (absolute inhibitory potency), or EC50 (assay/system-dependent excitatory potency) values. These values are all concentration-dependent; therefore, the smaller the value, the more effective the ligand.
RESULTS

Human mAChR M4

Carbachol treatment of transiently transfected cells resulted in an increased induction of CRE-Luc-mediated luminescence (maximum 87-fold increase) with an EC₅₀ = 5.6 μM; 10 μM was selected as the fixed agonist concentration, as it resulted in a submaximal induction of CRE-Luc (approximately 80% of maximum response). Relative IC₅₀ values of mAChR antagonists, ranked in order from highest to lowest potency at human mAChR M₄, were QNB (115 pM) > atropine (390 pM) > oxyphenonium (400 pM) > mepenzolate (1 nM) > MT3 (8 nM) > himbacine (10 nM) > dicyclomine (15 nM) > pirenzepine (23 nM) > tropicamide (155 nM) (Fig. 1). Inhibitory constants (Kᵢ), estimated from these data by the method of Cheng and Prusoff, were in good agreement with previously published affinity constant data for these ligands, either at human M₄ receptor or in mammalian tissues (Table 1).

Chicken mAChR M4

Carbachol treatment resulted in an increased induction of CRE-Luc-mediated luminescence (maximum 287-fold increase), similar to results reported previously, with an EC₅₀ = 2 μM; 10 μM was chosen as the fixed concentration, as it gave a large but submaximal activation of CRE-Luc induction (approximately 90% of maximum response). The behavior of antagonists at the cM₄ receptor did not mimic perfectly their behavior at human M₄. The behavior of antagonists at the cM₄ receptor did not mimic perfectly their behavior at human M₄, in order from highest (Kᵢ), estimated from these data by the method of Cheng and Prusoff, were in good agreement with previously published affinity constant data for these ligands, either at human M₄ receptor or in mammalian tissues (Table 1).
to lowest potency, were QNB (310 pM) > oxyphenonium (560 pM) > atropine (710 pM) > mepenzolate (4 nM) > himbacine (6 nM) > dicyclomine (41 nM) > pirenzepine (49 nM) > tropicamide (245 nM) > MT3 (450 nM) (Fig. 2). When converted by the Cheng-Prusoff correction, the IC₅₀ data for atropine, QNB, and pirenzepine are in good agreement with previously published Kᵢ values obtained by utilizing the same receptor clone. Although most antagonists acted with comparable relative inhibitory potencies at the cM₄ receptor and the human M₄ receptor, the Kᵢ for MT3 in blocking cM₄ was 56 times less potent (IC₅₀ = 450 nM; 1 µM; maximum 82% inhibition) compared to that for its interaction with the human M₄ mAChR (IC₅₀ = 8 nM).

**Human α₁A-Adrenoceptor**

Clonidine treatment of cells transiently transfected with hADRA2A resulted in increased CRE-Luc-mediated lucinescence (maximum 26-fold increase), with an IC₅₀ = 430 nM; 1 µM was chosen as the fixed concentration, as it gave a submaximal activation of CRE-Luc (approximately 75% of maximum response). Although increased CAMP synthesis is not the usual response to agonist treatment of Gₛ-coupled receptors, this Gₛ-coupling effect has been reported previously in CHO cells transfected with the hADRA2A receptor (also known as α₂C10). Relative IC₅₀ values for the mAChR antagonists at the hADRA2A receptor, ranked in order from highest to lowest potency, were MT3 (15 nM) > himbacine (17 µM) > atropine (45 µM) > oxyphenonium (463 µM) > pirenzepine (867 µM) > tropicamide (1.5 nM) > QNB (260 µM; maximum 82% inhibition) > mepenzolate (798 µM; maximum 68% inhibition) > dicyclomine (no detectable activity) (Fig. 3). The IC₅₀ for yohimbine, an α₂-adrenergic antagonist, was 5 nM (calculated Kᵢ(CP) = 2 nM). These data are in good agreement with previously published values for the affinity of MT3 (Kᵢ(CP) = 5 nM) and yohimbine (Kᵢ = 400 nM) at the hADRA2A receptor (Table 2). Thus, our data demonstrated unequivocally that myopia-inhibiting mAChR antagonists can bind to hADRA2A, although with affinities much lower than those with which they interact with mAChRs. The noteworthy exception was MT3, which bound to hADRA2A with an inhibitory potency (IC₅₀ = 15 nM) comparable to that for its interaction with the human M₄ mAChR (IC₅₀ = 8 nM).

**Controls**

Three different control experiments were conducted to verify that the changes in CRE-Luc, reflecting increases in intracellular CAMP, were due to activation of the transfected receptor and not stimulation of endogenous receptor signaling pathways. First, cells were transfected with the pcDNA 3.1(+)-plasmid only. There was no significant change in lucinescence in response to carbachol or clonidine treatment (Supplementary Fig. S1). Second, the impacts of elevating intracellular calcium using a calcium ionophore (CI A23187), or activating protein kinase C with phorbol 12-myristate 13-acetate (PMA) were tested. CI A23187 treatment, resulting in an increase in intracellular Ca²⁺, could in principle cause activation of Ca²⁺-dependent cyclase and an increase in cellular CAMP, thereby mimicking the action of Gₛ-coupled GPCRs (Supplementary Fig. S2). Alternatively, the protein kinase C (PKC) activator, PMA, could mimic the potential action of Gₛ-protein–coupled receptors via phospholipase action, and raise both inositol triphosphate and diacylglycerol levels by activating CAMP response element binding protein (CREB)—thus causing increased luminescence without an increase in cellular CAMP (Supplementary Fig. S2). Importantly, we observed no significant change in Lucₜ with treatment of either CI A23187 (1 µM) or PMA (10 µM), especially when compared to the response resulting from agonist treatment of transfected cells (Supplementary Fig. S2).

**Discussion**

The primary finding of our study is that the relative potencies of myopia-inhibiting mAChR antagonists to block the adrenergic hADRA2A receptor are correlated better with their ability to inhibit chick FDM than are the relative inhibitory potencies...
of the same compounds to block the muscarinic M₄/C₄ receptors (Tables 1, 2; Fig. 4). The data correlate the relative abilities of the mAChR antagonists for myopia inhibition versus their relative Kᵢ values for blocking the chick and human mAChRs show MT₃ as an obvious outlier (Figs. 4a, 4b, yellow circles). Yet, the same correlation plot paradigm shows that the relative Kᵢ of MT₃ to block hADRA2A is tightly correlated, as are the Kᵢ values of the other mAChR antagonists, with the ability to attenuate chick FDM (Fig. 4c, yellow circle). These findings support the hypothesis that myopia inhibition by high-concentration mAChR antagonists may be mediated by receptors other than mAChR M₄/C₄, and our data point to members of the α₂-adrenergic receptor family as possible alternatives.

A secondary finding is that, although the relative inhibitory potencies of most myopia-inhibiting mAChR antagonists for blocking the chick C₄ compared with the human M₄ receptors are in good agreement, MT₃ is an anomaly; it has markedly reduced Kᵢ for the chick C₄ receptor compared with the human M₄ receptor. This result is likely due to differences in the ligand-binding sites on the two receptors.

### Distribution of Drugs Within Ocular Tissues After Different Delivery Methods

A question to ask about the use of atropine for myopia inhibition (in humans and animals) is, Does the high concentration of antagonist, as administered, reflect its concentration at the site of action in the eye? Currently, the site of action for atropine-mediated myopia inhibition is unknown, but best guesses identify the retina, choroid, and/or sclera.⁵⁶ In chicks, intravitreal injection is the usual method of delivery, and concentrations of 1 to 100 mM have been used for mAChR antagonist-mediated injection of FDM (Table 1). Unfortunately, no studies exist on the ocular distribution of atropine after intravitreal injection into chick eyes; but one study has investigated the ocular distribution, in chicks, of a single dose of intravitreally injected radioactively labeled pirenzepine.⁵⁹ At 1 hour after injection, reported concentrations (percentage of the original injected amount) were 6% in the retina, 1.5% in the choroid, and 2.5% in the sclera.⁶⁰ Based on these data, and considering that 100 mM was used to achieve full myopia inhibition in the chick,⁶¹ estimated concentrations for ocular tissues would be retina = 6 mM, choroid = 1.5 mM, and sclera = 2.5 mM. These estimated concentrations greatly exceed those that would be expected to have mAChR-only effects, and fall within the range of concentrations that would enable pirenzepine to block the hADRA2A receptor under in vitro conditions, as calculated by our assay (Kᵢ(CP) = 250 μM; Table 2).

In humans, atropine for myopia inhibition is delivered in the form of daily eye drops (0.01%-1%). Under experimental conditions, delivery of a single dose of 2% [³H]atropine to the conjunctival sac of albino rabbits resulted in a distribution of the original concentration of 0.09% in the sclera, 0.01% in the choroid, and 0.008% in the retina, 1 hour after instillation⁶²; similar results (retina: 0.01%; choroid and sclera 0.38%) were seen 30 minutes after topical application of [C]l atropine.⁶³ Therefore, the concentrations of atropine in the eye after a single topical application of 0.01% to 1% (0.13–13 μM in the sclera, 0.07–7 μM in the choroid, and 0.01-1.1 μM in the retina, extrapolated from single-dose studies in albino rabbit⁶⁴) are within the range of what we have found to be the IC₅₀ for atropine’s ability to block the hADRA2A in vitro cell culture preparations (our value = 45 μM; value reported by others = 1–10 μM).⁶⁰,⁶¹ The ocular distribution of atropine after multiple topical doses has not been determined, but studies monitoring the accumulation of atropine in human eyes have shown that atropine buildup can occur. Serum levels of atropine were undetectable after a single inhalation dose of 12 to 25 ng/μL atropine sulfate, but were raised to 1.13 to 5.23 ng/μL when treatment was administered every 4 to 6 hours over a period of 48 hours.⁶³,⁶⁴ Pigmented tissues (RPE, iris, ciliary body, choroid, and retina) are especially apt at retaining atropine, which may lead to an initially weaker but significantly prolonged effect.⁶⁵,⁶⁶ It is possible, then, that topical

### Table 1. Binding Data for α₂-Adrenergic Drugs and mAChR Antagonists at hADRA2A

<table>
<thead>
<tr>
<th>Drug</th>
<th>Ki/ED₅₀ Study</th>
<th>Kᵢ(CP) Study</th>
<th>Kᵢ/Literature</th>
<th>Inhibits Myopia Est. [Vit]</th>
<th>Source</th>
<th>Cat No.</th>
</tr>
</thead>
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<tr>
<td>Alpha₂-adrenergic drugs at hADRA2A</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YOH</td>
<td>5 nM</td>
<td>1 nM</td>
<td>*400 pM–6 nM⁶⁵–⁵⁵</td>
<td>No, 0.01–1 mM</td>
<td>Sigma</td>
<td>Y3125</td>
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<tr>
<td>Clon</td>
<td>425 nM</td>
<td>N/A</td>
<td>*60–160 nM⁵⁹</td>
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<td>Sigma</td>
<td>C7897</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>†630 pM⁵⁸</td>
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<td>†4 nM⁹⁰</td>
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<table>
<thead>
<tr>
<th>Drug</th>
<th>IC₅₀ Study</th>
<th>Kᵢ(CP) Study</th>
<th>Kᵢ/Literature</th>
<th>Inhibits Myopia Est. [Vit]</th>
<th>Source</th>
<th>Cat No.</th>
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<td>Muscarinic antagonists at hADRA2A</td>
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<tr>
<td>ATR</td>
<td>45 μM</td>
<td>14 μM</td>
<td>1–100 μM⁵⁰,⁵¹</td>
<td>Yes⁶⁶, 0.1–10 mM</td>
<td>Sigma</td>
<td>A0257</td>
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<tr>
<td>MT₃</td>
<td>15 nM</td>
<td>5 nM</td>
<td>5 nM⁵⁰,⁵²</td>
<td>Yes⁶⁵–⁵⁷, 10–350 nM</td>
<td>Peptides International</td>
<td>PMT+4410-s</td>
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<tr>
<td>HIM</td>
<td>17 μM</td>
<td>5 μM</td>
<td>N/A</td>
<td>Yes⁶⁸, 5–12 mM</td>
<td>Enzo Life Sciences</td>
<td>ALX-550-061</td>
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<tr>
<td>OXY</td>
<td>470 μM</td>
<td>140 μM</td>
<td>N/A</td>
<td>Yes⁶⁸, 1–10 mM</td>
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<td>PRZ</td>
<td>870 μM</td>
<td>250 μM</td>
<td>N/A</td>
<td>Yes⁶⁸, 10 mM</td>
<td>Sigma</td>
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<tr>
<td>QNB</td>
<td>260 μM (82%)</td>
<td>80 μM</td>
<td>N/A</td>
<td>Partial⁵⁹, 0.74 mM</td>
<td>Sustainable Scientific</td>
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<tr>
<td>TRP</td>
<td>15 μM</td>
<td>440 μM</td>
<td>N/A</td>
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<td>MEP</td>
<td>780 μM (68%)</td>
<td>240 μM</td>
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<td>DIC</td>
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<td>N/A</td>
<td>No⁵⁹, 1–10 mM</td>
<td>Sigma</td>
<td>D7909</td>
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Non-ADRA2A-specific receptors/tissues: chick pineal,⁵² chicken cortex,⁵³ rabbit pulmonary artery.⁵¹ Ki/ED₅₀ Study and IC₅₀ Study data are the data determined by our binding assay; Kᵢ(CP) data are calculated from the IC₅₀ values in our assay by the method of Cheng and Prusoff.⁴⁹ Kᵢ/ED₅₀ Literature and Kᵢ Literature data are from previously published studies. Maximum inhibition achieved was 100%, unless indicated otherwise by percent values in parentheses beside the reported IC₅₀. YOH, yohimbine hydrochloride; Clon, clonidine hydrochloride; ATR, atropine sulfate; OXY, oxyphenonium bromide; PRZ, pirenzepine dihydrochloride; Est. [Vit], estimated vitreal concentrations, assuming uniform dilution in total vitreal volume, required for myopia inhibition in the chick; N/A, not available.

* Human or mammalian mAChR M₄ receptor or tissue.
† Avian mAChR M₄ receptor or tissue.
application of 0.01% atropine daily for long periods (up to 2 years) could result in ocular concentrations much higher than those seen after a single dose, with intracellular organelles such as melanin granules acting as reservoirs for slow release of the drug and possibly providing local concentrations that would be sufficient to interact with non-mACHRs.

Pirenzepine has also been tested for the treatment of childhood myopia, with twice-daily applications of concentrations up to 2%; but the results did not support superiority of clinical efficacy of pirenzepine treatment over atropine treatment,35–38 and further studies have seemingly been abandoned.69 Ocular distribution of a single dose of topically applied [3H]-pirenzepine (2%) has also been tested in the rabbit eye; concentrations in the ocular tissues 1 hour after administration were roughly half those calculated for atropine (retina: 0.004%, choroid: 0.021%, sclera: 0.052%)—possibly because atropine, being more lipophilic, is better at penetrating the ocular tissues and is lost less rapidly than pirenzepine.69 The estimated concentrations of pirenzepine delivered to ocular tissues should be sufficient to activate all mammalian mACHR subtypes fully,70 but are expected to fall below the concentration required to achieve a significant blockade of hADRA2A signaling (retina: 2 μM, choroid: 12 μM, sclera: 30 μM) as calculated by our assay (Ki(cp) = 250 μM, Table 2).

mACHR Antagonists at M4, cM4, and hADRA2A Receptors: Correlations With Myopia Inhibition

The relative inhibitory potencies of myopia-inhibiting mACHR antagonists at the hADRA2A receptor, but not at the chick cM4 and human M4 mACHRs, are well correlated with their reported ability to inhibit chick FDM4,16,19 (Fig. 4). We have confirmed previous reports of high-affinity binding of MT35,52 (Ki(cp) = 5 nM) to hADRA2A, and demonstrated that atropine and himbacine can also bind to this receptor, albeit with potencies much lower than their relative Ki values for blocking the mACHRs. All other mACHR antagonists tested had very low potency for blocking hADRA2A, but 100% inhibition of clonidine-mediated effects was achieved with oxyphenonium, tropicamide, and pirenzepine—drugs that were reported to inhibit myopia fully in the chick when used at high concentrations. The maximum inhibitory effects achieved by QNB (partial myopia inhibition in chick) and mecoponolate (no myopia inhibition in chick) were 82% and 68%, respectively, and diclocynine (no myopia inhibition in chick) had no discernable effect at the concentrations tested. At the human and chick M4/cM4 receptors, atropine, QNB, mecoponolate, and diclocynine are all comparably potent inhibitors, with Ki values in the nanomolar range; yet atropine is the only one of these that can inhibit chick FDM.50 Most interesting is the behavior of MT3 at M4/cM4. It is the most effective drug found to date for mitigating chick FDM,14,15 and we confirmed its high potency for the human M4 receptor. However, we found it to be the least potent of all drugs tested at the chick cM4 receptor. This disconnect, between the potency of MT3 at cM4 and its effectiveness against chick FDM, supports our hypothesis that myopia inhibition is not mediated by the M4/cM4 receptor.

**Ocular α2-Adrenoceptors and Possible Mechanism of Action**

Sympathetic innervation in the vertebrate eye services structures such as the dilator pupillae, the ciliary muscle and epithelium, and ocular blood vessels within and outside the choroid.72 Alpha₂-adrenoceptors are best known as autoreceptors, but they can also act as heteroreceptors.41,73 Alpha₂-adrenoceptors that act as autoreceptors are located on presynaptic adrenergic neurons; they are activated by epinephrine/norepinephrine and inhibit the release of their own neurotransmitters. Alpha₂-adrenoceptors that act as heteroreceptors are located presynaptically on nonadrenergic neurons, and there is evidence that α₂-adrenoceptors can modulate the release of neurotransmitters such as GABA, dopamine, and serotonin in the rat brain and retina.74–79 Interestingly, both GABA and dopamine have been implicated in the regulation of eye growth.80–88 In the rat retina, the epinephrine-synthesizing enzyme phenylethanolamine N-methyltransferase (PNMT) has been localized to amacrine cell populations80 and retinal epinephrine levels have been reported to rise upon light exposure89,91, but there is no further evidence for a role of epinephrine or norepinephrine in retinal visual processing of any vertebrate species examined. In spite of this, α2-adrenoceptors are present on retinal neurons. In rat retina, human, and primate tissue, ADRA2A immunoreactivity has been localized to the nonpigmented ciliary epithelium, cornea and conjunctiva, and retinal ganglion cells and amacrine cells.92 In

**Difference in Binding of MT3 at M4 Versus cM4**

When attempting to assign functional consequences to treatment with a particular ligand, it is important to consider the mechanism of binding of that ligand to its target receptor. Generally, orthosteric binding sites, where the transmitters or primary signaling molecules bind, are highly conserved (100%) between receptors of the same class and between species.71 “Selective” modulatory ligands such as MT3, however, will bind to receptor amino acid residues that are less conserved, usually outside the orthosteric pocket, in the extracellular loops (EL2 and EL3) of the target GPCR.59 In such instances, substitution of even a single amino acid can have a significant impact on ligand-receptor binding characteristics.39,40 Thus, while decreased conservation of receptor sequences in the extracellular loops aids in selectivity of a ligand for a certain receptor subtype, the increasingly stringent requirements for binding can also result in loss of activity at the same receptor subtype in other species. This finding is what we observed in our assay: MT3 bound poorly to cM4 compared to M4. Chicken and human M4 share only a 71.4% to 72.7% sequence identity in EL3 and EL2, respectively (Supplementary Fig. S5). Therefore, it is likely that the decreased activity of MT3 at cM4 is caused by amino acid differences in its EL2 and EL3 sequences, compared to those of M4. All other drugs tested in our assay did not differ significantly in their binding behavior at M4 versus cM4. This result was expected; these ligands are known to be orthosteric inhibitors of ACh at mACHRs, and the reported orthosteric binding site of the mACHR M4/cM4 receptors is 100% conserved between human and chick71 (Supplementary Fig. S5).
Off-Target Binding of Muscarinic Antagonists

4738), in which there is no increase in scleral compliance in myopia,99,104 or a small decrease in IOP before onset of increase in IOP after but not preceding the onset of physiological range) on axial elongation, as hypothesized by attenuation of IOP is responsible for inhibition of myopia in humans with pathologic myopia 109 and mammals with experimental myopia.103,105 Animal studies have found no relationship,100–103 an equivocal result.97,105 A possible reason for this could be measurement bias induced by utilizing indentation or applation tonometry, which calculates IOP from the amount of pressure required to flatten an area of the cornea. The accuracy of tonometry is dependent on factors such as corneal thickness106 and scleral compliance.107,108 and increased scleral compliance (scleral creep) has been observed in humans with pathologic myopia109 and marmals with experimentally induced myopia.110,111 Thus, if the sclera is more compliant than normal, it may lead to a mistaken measurement bias toward lower IOP. In contrast, α2-adrenoceptor antagonists also were reported to effectively inhibit FDM in chicks (Carr BJ, Stell WK. IOVS 2016;57:ARVO E-Abstract 4738), in which there is no increase in scleral compliance in response to myopiogenic stimuli111,112 IOP measurements were not recorded in that study, however, so we cannot exclude a role completely. The relationship between the actions and receptor targets of the compounds we studied and their impact on IOP and myopia remains an interesting topic for future work. Notwithstanding the possible role of IOP in myopia, our work establishes clearly that action of atropine via the mAChR M 1 receptor is highly unlikely to be a factor.

α2-adrenoceptor agonism in the retina can also result in the upregulation of neuroprotective agents and mechanisms, such as an increase in basic fibroblast growth factor (bFGF) in rat photoreceptors112,113 or activation of the extracellular signal-regulated kinase signaling pathway in chick Müller cells.93 Interestingly, both bFGF and extracellular signal-regulated kinase signaling have been implicated in protection against chick FDM114,115 and guinea pig lens-induced myopia.102,116,117 Finally, brimonidine might be involved in the regulation of nitric oxide synthase in retinal arterioles, where it may activate endothelial nitric oxide synthase (eNOS), resulting in vasoconstriction, as reported in pigs.118 It is important to note that although there are no intraretinal blood vessels in the chick retina, it is served by blood vessels in the choroid and an intravitreal structure called the pecten. Whether α2-adrenoceptor ligands can also modulate NOS (neuronal NOS, eNOS, or inducible NOS) activity or expression in retinal neurons is worth investigating further, since synthesis and release of nitric oxide are necessary for myopia inhibition by atropine, and have been associated strongly with reduction of eye growth.57

Caveats and Considerations

There are some discrepancies in the ability of mamba toxins to inhibit myopia in different animal models. Although MT3 inhibits FDM effectively in chicks14 and tree shrews,17 it seems to be ineffective at inhibiting the axial elongation induced by lens-induced myopia (LIM) in the same animals.15,17 Conversely, MT7, a mamba toxin subtype that is highly selective for mAChR M 1 and is not known to act at any other receptor,28–29 inhibits LIM, but not the axial elongation induced by FDM, in the tree shrew.17 MT7 has no effect in the chick,119 which is expected because chicks do not possess the M 1 subtype. Pirenzepine, however, is effective at inhibiting chick FDM, possibly due to binding to the M 1 receptor, which MT5 does not recognize, or to off-target binding at α2-adrenoceptors as evidenced by our work. These differences in treatment effectiveness of mamba toxins are in line with evidence suggesting that the compounds involved in LIM and FDM are in some ways different,20–22 and that—whatever the mechanism affected by atropine (and other myopia-inhibiting mAChR antagonists)—the results of our study may be unique to the circuitry of FDM, and not LIM.

Although we have shown that some mAChR antagonists will bind hADRA2A at high concentrations—comparable to those found to be effective against chick FDM—we have not ruled out other α2-adrenoceptor subtypes, non-M 1 muscarinic subtypes (i.e., mAChR M 1/cM 2), or non-mAChR/non-adrenergic receptor targets. The high concentrations of these drugs required to inhibit FDM in the chick model impede our ability to identify any single receptor as the key target for the impact of any compound on myopia, because of the distinct possibility of many unidentified off-target receptor-mediated effects.21 Furthermore, because of technical issues we could not overcome, we were unable to express a functional chick ADRA2A receptor in our HEK cell expression system, which would have enabled us to compare the “nonspecific” affinities of mAChR antagonists directly for the chick versus human α2A-adrenoceptors. What can be concluded with confidence from our data, however, is that M 1/cM 2 cannot be seen as the unique or even the leading receptor candidate for mediating the inhibition of FDM by atropine (and other mAChR antagonists) in the chick bioassay.

Summary

Our data provide new information about the relative potencies of muscarinic antagonists at the human and chick mAChR M 1/cM 2 receptors and the human α2A-adrenoceptor. This is the first study to characterize the inhibitory action of oxyphephonium, mepenzolate, MT 3, dicyclomine, and tropicamide explicitly at chicken cM 2, and the first to test specifically and report in detail, the potential “off-target” actions of multiple mAChR antagonists at hADRA2A. Consequently, these data provide a concentration range over which these drugs should be expected to act in a mAChR-specific manner, and a concentration range at which they can be predicted to have “off-target” effects at hADRA2A and possibly other receptors. The focus of thinking about the anti-myopia action of atropine as muscarinic, combined with the lack of knowledge of the mechanism of atropine’s therapeutic actions (in the setting of either human or chick myopia), is a deterrent to the discovery of more effective anti-myopia agents with fewer side effects. The data presented here make a compelling case for continued investigation into non-mAChR targets for atropine-mediated
myopia inhibition, with α-adrenoceptors being provocative candidates for novel therapeutic interventions.

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