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Nucleoside-Derived Antagonists to A₃ Adenosine Receptors Lower Mouse Intraocular Pressure and Act across Species

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Abstract

The purpose of the study was to determine whether novel, selective antagonists of human A₃ adenosine receptors (ARs) derived from the A₃-selective agonist CI-IB-MECA lower intraocular pressure (IOP) and act across species. IOP was measured invasively with a micropipette by the Servo-Null Micropipette System (SNMS) and by non-invasive pneumotonometry during topical drug application. Antagonist efficacy was also assayed by measuring inhibition of adenosine-triggered shrinkage of native bovine nonpigmented ciliary epithelial (NPE) cells. Five agonist-based A₃AR antagonists lowered mouse IOP measured with SNMS tonometry by 3–5 mm Hg within minutes of topical application. Of the five agonist derivatives, LJ 1251 was the only antagonist to lower IOP measured by pneumotonometry. No effect was detected pneumotonometrically over 30 min following application of the other four compounds, consonant with slower, smaller responses previously measured non-invasively following topical application of A₃AR agonists and the dihydropyridine A₃AR antagonist MRS 1191. Latanoprost similarly lowered SNMS-measured IOP, but not IOP measured non-invasively over 30 minutes. Like MRS 1191, agonist-based A₃AR antagonists applied to native bovine NPE cells inhibited adenosine-triggered shrinkage. In summary, the results indicate that antagonists of human A₃ARs derived from the potent, selective A₃ agonist CI-IB-MECA display efficacy in mouse and bovine cells, as well. When intraocular delivery was enhanced by measuring mouse IOP invasively, five derivatives of the A₃AR agonist CI-IB-MECA lowered IOP but only one rapidly reduced IOP measured non-invasively after topical application. We conclude that derivatives of the highly selective A₃AR agonist CI-IB-MECA can reduce IOP upon reaching their intraocular target, and that nucleoside-based derivatives are promising A₃ antagonists for study in multiple animal models.

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Keywords

Aqueous humor; Servo-Null Micropipette System; pneumotonometry; nucleoside-based antagonists; bovine nonpigmented ciliary epithelial cells

1. Introduction

Intraocular pressure (IOP) is commonly elevated in glaucoma, leading to death of retinal ganglion cells and optic nerve atrophy. Reducing IOP is the only intervention known to delay the onset and slow progression of blindness, even in patients with normotensive disease (Collaborative Normal-Tension Glaucoma Study Group, 1998a; Collaborative Normal-Tension Glaucoma Study Group, 1998b; Kass et al., 2002; The AGIS investigators, 2000). IOP can be reduced by lowering either the rate of inflow or the resistance to outflow of aqueous humor.

Among novel strategies for lowering IOP, focus on adenosine receptors (ARs) has seemed promising because knockout of A₃-subtype ARs reduces IOP in the living mouse (Avila et al., 2002a), likely through a reduction in inflow. Several observations obtained with isolated cells have suggested that A₃ARs physiologically increase inflow of aqueous humor by activating Cl⁻ channels of the nonpigmented ciliary epithelial (NPE) cells at the aqueous surface of the ciliary epithelium (Carré et al., 1997; Carré et al., 2000; Mitchell et al., 1999). In contrast to the robust effects of A₃AR agonists on isolated cells from the inflow pathway, A₃AR agonists have been found to exert relatively modest actions on whole-cell currents of cells cultured from the trabecular meshwork (Fleischhauer et al., 2003) and from Schlemm's canal inner wall (Karl et al., 2005).

Based on the results obtained with isolated NPE cells, adenosine and selective A₃AR agonists would be expected to increase inflow and IOP, and A₃AR antagonists would be expected to exert opposite effects. The predicted changes in IOP triggered by A₃AR agonists and antagonists have been confirmed in the living mouse (Avila et al., 2001b; Avila et al., 2002b; Yang et al., 2005). While these responses in mice suggest a potential relevance of A₃AR-selective antagonists to humans, the binding affinities of these antagonists display substantial species variation (Jacobson et al., 1997; Linden, 2001). For instance, the binding inhibition constants (K_i) of some antagonists can vary by more than 30,000-times between rat and human A₃ARs (Yang et al., 2005). Interestingly, the responses of A₃ARs to selective agonists are much more highly conserved across species (Yang et al., 2005). We previously tested one A₃AR-selective antagonist, MRS 1292 (Yang et al., 2005), generated by modifying the A₃AR agonist IB-MECA (Gao et al., 2002), and observed antagonist activity in both the living mouse and immortalized human NPE cells (Yang et al., 2005).

Compared to the parent agonist previously modified (IB-MECA), the A₃AR agonist Cl-IB-MECA displays 3–4-fold greater potency and a 50-fold greater selectivity for A₃ receptors than for A₁ and A_{2A} receptors in rat brain (Kim et al., 1994). In the present work, we have examined the effects of five new A₃AR-selective antagonists constructed (Gao et al., 2006; Jeong et al., 2007) by modifying the far more selective agonist Cl-IB-MECA (Figure 1), with the hope that this strategy might lead to the generation of even more selective A₃AR antagonists that are active across species. Measurements of mouse IOP have been conducted with both invasive SNMS (Avila et al., 2001a) and non-invasive pneumotonometric techniques (Avila et al., 2005). Similar baseline IOP values are obtained by applying the two techniques to the same eyes (Avila et al., 2005). However, under experimental conditions, the measurements can be complementary, rather than identical, because the intact mouse eye can present a substantial barrier to drug penetration (Wang et al., 2007). The fine-tipped micropipettes used

for SNMS tonometry enhance entry of topically-applied drugs into the mouse eye (Wang et al., 2007), so that drug efficacy can be detected even if topical drug permeation is too slow to alter IOP measured non-invasively over similar periods.

2. Methods

2.1. Mice and anesthesia

Black Swiss outbred mice of mixed sex, 25–30 g in weight and 7–9 weeks old (Taconic Inc., Germantown, NY, USA) were maintained under a 12:12-h light/dark illumination cycle with unrestricted access to food and water. Animals were anesthetized with intraperitoneal ketamine (250 mg kg⁻¹), complemented with 0.5% topical proparacaine HCl (Allergan, Bausch & Lomb). Anesthetized mice were secured in a surgical stereotaxic device (David Kopf Instruments, Tujunga, CA), and body temperature was maintained with a heating pad at 37°C (Delta Phase Isothermal Pad, Braintree Scientific, Braintree, MA) (Avila et al., 2001a). As previously described (Wang et al., 2007), IOP was measured invasively with the Servo-Null Micropipette System (SNMS) and non-invasively by pneumotonometry in separate animals. The rate of cardiac contraction was monitored with a pressure transducer wrapped around the mouse tail (MLT1010, Adinstruments, USA). All procedures were performed in adherence with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Measurement of IOP invasively by SNMS tonometry

With this electrophysiologic approach (Avila et al., 2001a), an exploring micropipette, 5–10 µm in outer diameter, is filled with a highly conducting solution and advanced through the cornea into the anterior chamber (Fig. 2A). To enhance stability and reduce background noise, our current micropipettes are filled with 2M NaCl and display resistances of 0.1–0.3 MΩ (Wang et al., 2006; Wang et al., 2007). Upon advancing the micropipette into the anterior chamber of the eye, the IOP forces aqueous humor into the tip. The aqueous humor has a much lower conductance than the original filling solution, increasing the micropipette resistance. The IOP is measured as the hydrostatic pressure necessary to restore the position of the column of filling solution and returning the resistance to its initial value. Measurements are conducted at 1 kHz for tens of minutes during the course of drug applications.

2.3. Measurement of IOP non-invasively by pneumotonometry

Non-invasive measurements were conducted with a pneumotonometric technique adapted and validated for measuring mouse IOP (Avila et al., 2005; Wang et al., 2006; Wang et al., 2007) (Fig. 2B). The technique incorporates a commercially available tip of the ocular blood tomography pneumotonometer (Blood Flow Analyzer [BFA] probe tip; Paradigm Medical Industries Inc.) that is fit to a custom-built mount.

A constant pressure source provides air flow to the diaphragm at the end of the BFA tip. The diaphragm is distended by the air flow, enabling air to escape through holes in the probe-tip wall. Pressure at the base of the BFA tip is monitored through a T-connection to a transducer. The probe is advanced to the cornea with a micromanipulator until a shift in the baseline output reading indicates that contact is made. The tip is then visually displaced from the tear film and the output readjusted to zero before advancing the probe again. Contacting the cornea depresses the diaphragm of the BFA tip, reducing access of the air flow to the escape holes, and thereby increasing the pressure at the tip base. The probe is then advanced in ~10 steps of ~50 µm each at intervals of ~10 s. Elevation in pressure with probe advance displays a relative plateau or inflection region, taken to be the endpoint for the IOP (Wang et al., 2006; Wang et al., 2007). The endpoint is considered technically acceptable if the pressure recording also displays pressure oscillations synchronous with heart rate (Avila et al., 2005; Wang et al., 2006; Wang et al., 2007). The position along the axis of advance is recorded, and the probe is withdrawn

to avoid possible artifacts arising from prolonged corneal pressure. Measurements are then conducted at 10-minute intervals by advancing the probe to the same position (Wang et al., 2007). As illustrated by the control trace of Figs. 4A, measurements with this protocol were stable for more than 30 min.

2.4. Reduction of measurements with living mouse

As previously described (Wang et al., 2006), IOP and heart rate signals were band-pass filtered (1–100 Hz), amplified using a signal conditioner (CyberAmp 380, Axon Instruments, Inc., USA) and digitized at 1 kHz with an analog-to-digital converter (MiniDigi 1A two-channel acquisition system, Axon Instruments, Inc., USA) in the gap-free mode. The resulting digital files were analyzed offline using Clampfit 9 (Axon Instruments).

2.5. Preparation of native bovine NPE cells

Fresh bovine eyes were obtained from a local abattoir. Native bovine NPE cells were dissociated by enzymatic digestion (Do et al., 2006). Ciliary processes were excised and washed with Dulbecco's phosphate-buffered saline (PBS) (GIBCO, Grand Island, NY). Cells were dissociated by incubating the preparation with 0.25% trypsin in a shaker for 30–35 min (250 rpm) at 37°C. Cells were subsequently rinsed twice with PBS and plated on coverslips in medium 199 for ≥ 2 h in 5% CO₂ at 37°C. The medium included 10% fetal bovine serum and 0.1% gentamicin (GIBCO-BRL). The cells were prepared after a post-mortem time of about 1.5 h.

2.6. Cell volume measurements

Cell volume was monitored continually by measuring both calcein fluorescence quenching (Do et al., 2006; Karl et al., 2007) and cell area (Do et al., 2004; Mitchell et al., 2002). Coverslips containing the cells were placed in a chamber connected to a Nikon Diaphot microscope. Cells were loaded with 2 μ M calcein-AM and 0.02% Pluronic for 30–40 min, and then perfused with isotonic Tyrode's solution for 30 min at room temperature. Bleaching was minimized by exciting calcein at intervals of 20 s at 488 nm; light emitted at 520 nm was collected with an IC-200 charge-coupled device (CCD) camera (Photon Technology International, Princeton, NJ). Data were analyzed with Imagemaster software (Photon Technology International).

The cell volume was measured specifically of NPE cells. Although pigmented ciliary epithelial (PE) cells were also present in the dissociated cell preparations, those cells were readily distinguished by their prominent cell granules. The presence of the granules has permitted measurement and differentiation of the electrophysiologic and volume-regulatory properties of the NPE and PE cells, even in mixed harvested preparations (Do et al., 2005; Do et al., 2004; Zhang & Jacob, 1997). Regions for analysis were chosen to include nonconfluent cells with similar diameter and loading, and usually included one to four cells. In measuring volume through fluorescence quenching, total calcein fluorescence was monitored (Do et al., 2006). Calcein fluorescence quenching is increased by shrinkage and lowered by swelling. In monitoring volume by cell area, the number of pixels above threshold within the region of interest was determined using Imagemaster software (Photon Technology International) (Mitchell et al., 2002).

2.7. Solutions and pharmacologic agents

LJ 979 and LJ 1251 were synthesized at Ewha Womans University (Jeong et al., 2007) and MRS 3771 (Gao et al., 2006), as well as MRS 3827 and MRS 3826 (Besada et al., 2006), were prepared at the National Institute of Diabetes and Digestive and Kidney Diseases. Figure 1 provides the structures of the purinergic drugs used in the study, and Table 1 presents the

pharmacologic potencies. Ketamine HCl was purchased from Phoenix Pharmaceutical, Inc. (St. Joseph, MO), and other drugs were obtained from Sigma Chemical (St. Louis, MO).

Drugs were dissolved in DMSO and applied topically. The droplets contained $\leq 2\%$ DMSO at an osmolality of 295–300 mOsm. This vehicle solution did not significantly affect mouse IOP (N=6, Table 2), consistent with previous measurements (Avila et al., 2002a). Drug actions on IOP were evaluated with invasive measurements 10 min after topical application (Table 2) since the continued presence of the micropipette can be associated with a downward drift of the IOP after 10 min. However, smaller droplets (5 μ l rather than 10 μ l) were topically applied more frequently during the course of the non-invasive measurements to prevent drying the eye with the airflow from the pneumotonometer.

2.8. Statistical analysis

Results are provided as means $\pm$ SEM. Significance was evaluated between two sets of data with Student's t-test (paired or unpaired). Groups of three or more measurements were evaluated with one- or two-way analysis of variance (ANOVA), as appropriate, followed by the Bonferroni post-test. Differences were considered significant if the probability of the null hypothesis (P) was <0.05 .

3. Results

3.1. Measurements of IOP conducted by SNMS tonometry

At a concentration of 250 μ M in a 10- μ L droplet (1.44 μ g dose), topical application of the nucleoside-derived antagonist LJ 979 reduced IOP within minutes, when measured by invasive SNMS tonometry (Fig. 3). The relationship between drug concentration in the applied droplet and aqueous humor is considered in the Discussion. The maximal fall in IOP was 4.2 ± 1.2 mm Hg (mean $\pm$ SEM, N=10, $P<0.01$). Subsequent application of 10 mM adenosine (26.4 μ g) in a 10- μ L droplet did not elicit the increase in IOP (Fig. 3) characteristically observed after adding adenosine without drug pretreatment (Avila et al., 2001b; Avila et al., 2002b). Each of the other four nucleoside-derived antagonists described in Table 1 and Fig. 1 also reduced invasive-measured IOP. The reductions ranged from 3.0 ± 1.1 mm Hg [MRS 3771, droplet concentration (dose) of 250 μ M (1.4 μ g, N=9, $P<0.05$)] to 4.9 ± 1.7 mm Hg [LJ 1251, droplet concentration (dose) of 25 μ M (0.13 μ g), N=6, $P<0.05$] (Table 2, Fig. 5). For purposes of comparison, mouse IOP was also monitored by SNMS tonometry after topical application of the prostaglandin analogue latanoprost (Table 2). Consistent with our previous findings (Avila et al., 2001a), latanoprost lowered SNMS-measured IOP by 3.8 ± 0.8 (N=6, $P<0.01$).

3.2. Measurements of IOP by pneumotonometry

The single nucleoside derivative that lowered IOP by pneumotonometry as well as by SNMS tonometry was LJ 1251 (Fig. 4B). At a droplet concentration of 250 μ M and droplet dose of 0.630 μ g, LJ 1251 lowered IOP measured non-invasively by 4.2 ± 0.7 mmHg ($P<0.002$, Table 2). As a positive control, 0.1 mL water g^{-1} was introduced intraperitoneally at the conclusion of the measurements (Figs. 4A–F). The increase in IOP characteristically triggered by systemic hypotonicity was uniformly observed by non-invasive pneumotonometry.

In contrast to LJ 1251, neither the vehicle alone (2% DMSO, Fig. 4A) nor the other four nucleoside derivatives significantly affected IOP measured with pneumotonometry. The four other derivatives were LJ 979 (Fig. 4C), MRS 3826 (Fig. 4D), MRS 3827 (Fig. 4E) and MRS 3771 (Fig. 4F). The mean measured changes, droplet concentrations, numbers of experiments and probabilities are specified in Table 2, with summary results after 30 min shown in Fig. 5.

The IOP was also monitored non-invasively after topical application of the prostaglandin analogue latanoprost (Table 2). In agreement with our earlier pneumotonometric observations (Wang et al., 2007), latanoprost had no significant effect within 30 min ($N=6$, $P>0.9$).

3.3. Effects of nucleoside derivatives on bovine ciliary epithelial cell volume

The potencies of the antagonists studied have previously been quantified against human adenosine receptors (Table 1), and the whole-animal work of the present study was conducted with mice. We also tested the effects of CI-IB-MECA-derived antagonists, three of the putative cross-species nucleoside derivatives, by studying cells from an additional species, the cow. The cell volume of native bovine nonpigmented ciliary epithelial (NPE) cells was monitored by measuring both cell area (Do et al., 2004) and calcein-fluorescence quenching (Do et al., 2006).

After an initial baseline period, cells were sequentially and briefly perfused with 25% hypotonic, isotonic, 25% hypertonic and isotonic solutions (Fig. 6). These initial perfusions served as controls to test whether the measured isotonic cell volume responded appropriately by swelling and shrinking during exposure to the hypo- and hypertonic challenges, respectively. Cells were perfused at $\sim 2 \text{ mL min}^{-1}$, leading to application of 24–48 nmol of derivative in each experiment. Sufficient material was synthesized to test the first two derivatives developed, LJ 979 and MRS 3771, and also LJ 1251, the derivative that reduced mouse IOP measured either invasively or non-invasively (Table 2, Figs. 4B and 5). For purposes of comparison, the previously studied dihydropyridine A_3 antagonist, MRS 1191, was also tested. The values displayed in Fig. 6 were normalized to the area or fluorescence measured just before perfusing with the test solution, at $t=35 \text{ min}$.

After the initial control perfusions with isosmotic and anisosmotic solutions, adenosine with or without an antagonist was perfused for 40 min, 35 min after beginning the experiment. Perfusion with adenosine, the physiologic A_3 agonist, triggered a concentration-dependent shrinkage (light blue symbols, Fig. 6) in comparison to controls perfused with 0.1% DMSO alone ($N=10$, yellow symbols, Fig. 6). We previously found that the physiologic A_3 agonist, adenosine, is less potent in activating Cl^- channels of native bovine nonpigmented ciliary epithelial (NPE) cells than of immortalized human NPE cell lines (Carré et al., 1997). Consistent with the previous finding, we found that the EC_{50} for adenosine-triggered shrinkage was $\sim 250 \mu\text{M}$, based on perfusion with adenosine concentrations ranging from $10 \mu\text{M}$ to 1 mM ($N=30$, data not shown). This observation guided our choice of studying the effects of antagonists on cell shrinkage elicited by $300 \mu\text{M}$ adenosine. At 100 nM , the selective A_3 agonist CI-IB-MECA (Table 1) produced shrinkage ($N=19$, data not shown) insignificantly different from that elicited by high concentrations of adenosine, tested either by area ($P>0.7$) or fluorescence quenching ($P>0.05$, one-way ANOVA with Bonferroni post-test).

As illustrated by Fig. 6, the A_3 antagonists significantly inhibited adenosine-triggered cell shrinkage (two-way ANOVA with Bonferroni post-test). MRS 3771 ($N=5$, $P<0.001$) significantly inhibited the action of adenosine ($N=14$), monitored as cell area. LJ 979 ($N=5$), LJ 1251 ($N=8$) and MRS 1191 ($N=5$) significantly inhibited adenosine-produced cell shrinkage, monitored both by cell area and by calcein fluorescence quenching; significance was attained at the 0.001 level, with the exception of the inhibition of the adenosine-triggered reduction in area by LJ 1251 ($P=0.006$). Ranking the antagonists according to the mean cell area at the conclusion of the test period ($t=75 \text{ min}$), the ranking of antagonist efficacy was: LJ 979 > MRS 1191 $\sim$ MRS 3771 > LJ 1251. Monitored as calcein fluorescence quenching, the ranking was similar, with a single inversion: LJ 979 > MRS 1191 > LJ 1251 > MRS 3771. The effect of LJ 979 in stabilizing cell volume was particularly striking (red symbols, Fig. 6), significantly reducing even the baseline rate of shrinkage noted under control conditions. This stabilizing action may have reflected inhibition of baseline A_3AR activity since the LJ 979 had

no effect on the time course or magnitude of swelling or shrinkage produced by anisotonic solutions in the control period (ending at $t=35$ min, Fig. 6).

4. Discussion

The salient results of the present study are that: (1) five derivatives of the selective A_3AR agonist CI-IB-MECA lowered IOP, measured by SNMS tonometry; (2) of these derivatives, only LJ 1251 rapidly reduced IOP, measured non-invasively after topical application; and (3) the three nucleoside derivatives applied to native bovine nonpigmented ciliary epithelial cells inhibited adenosine-triggered shrinkage.

4.1. Nucleoside-derived A_3 antagonists

The five antagonists of the human A_3AR were modifications of the parent nucleoside agonist CI-IB-MECA (Fig. 1). The chemical strategy was two-fold. Choosing an A_3 agonist as the parent compound enhanced the probability that the derived antagonist would be effective against many mammalian species [Table 1, (Yang et al., 2005)]. Second, the specific modifications were aimed at reducing the H-bond-donating capability associated with activation of the 5'-uronamide of the full agonist, CI-IB-MECA. This was achieved by adding an additional methyl group to the 5'-uronamide, with (LJ 979) or without (MRS 3771) concomitant 4'-thio substitution (Gao et al., 2006). The derivatives MRS 3826 and MRS 3827 are esters of MRS 3771 and LJ 979, respectively. The fifth derivative LJ 1251 was developed by removing the 5'-uronamide, entirely, in order to eliminate H-bonding at that position, accompanied by the 4'-thio substitution (Jeong et al., 2007).

4.2. Effects of nucleoside-derived A_3 antagonists on IOP measured by SNMS

All five of the derivative compounds reduced invasively-measured IOP by 4–5 mm Hg within minutes after topical addition (Figs. 3 and 5, Table 2). We have previously observed that corneal impalement of mice with the exploring micropipette used in SNMS tonometry, enhances delivery of drugs to their intraocular targets (Wang et al., 2007). This increased delivery likely reflects enhanced permeation of the cornea around the tips of the small micropipettes, ~5–10 μ m in diameter. We do not know the drug concentration actually delivered to the intraocular aqueous humor following topical application. However, comparison of the droplet concentrations with known potencies of 13 ocultensive drugs or drug combinations led us to estimate that the concentration of drug topically delivered to the aqueous humor during SNMS tonometry is no more than 1%, and for some drugs may be closer to 0.1–0.2%, of the concentration within a 10- μ L droplet (Avila et al., 2001b). This approximation is consonant with the commonly-applied assumption of ~1% penetration of drugs topically applied to rabbits (Honjo et al., 2001) and primates (Asseff et al., 1973).

Comparison of the delivered concentrations, calculated as 1% of the effective droplet concentrations (Table 2), with the known potencies at A_3AR s (Table 1) is consistent with the view that the derivatives lowered IOP by acting selectively on A_3AR s.

4.3. Effects of nucleoside-derived A_3 antagonists on non-invasively measured IOP

The measurements by SNMS tonometry indicate that all five agonist-based A_3AR antagonists can reduce IOP upon reaching their intraocular target. LJ 1251 was the only one of the derivatives that lowered non-invasively-measured IOP at any of the droplet concentrations during the durations of the current studies. The other four derivatives had no effect on non-invasively-measured IOP at any of the droplet concentrations during the durations of the current studies (Figs. 4–5, Table 2). Similarly, topical application of latanoprost in the current study and CI-IB-MECA in a prior work (Wang et al., 2007) did not significantly alter IOP measured by pneumotonometer over ~30 min.

The foregoing observations indicate that delivery of multiple drugs was substantially reduced by the ocular coats of the mouse eye. Corneal impalement by even fine-tipped micropipettes can significantly improve the entry of topically-applied drugs into the mouse aqueous humor. We speculate that this enhancement is mediated either by direct diffusion around the tip or by a more complex impalement-triggered change in ocular barrier properties. SNMS tonometry and noninvasive pneumotonometry can be complementary in facilitating study of ocular hypotensive drugs. The SNMS provides a tool for measuring IOP with accuracy, prolonged stability and excellent time resolution, while the noninvasive pneumotonometry gives the opportunity to estimate the efficiency of topical drug delivery.

It is unclear why the apparent permeability of LJ 1251 was different from the other four agonist-based A₃AR antagonists. Relative hydrophobicity of the derivatives can in general play a role. However, we have previously observed that both the agonist adenosine and the dihydropyridine antagonist MRS 1191 also alter IOP measured non-invasively, although the responses to topical application of these drugs was slower and smaller than those measured with SNMS tonometry (Wang et al., 2007). Table 3 presents calculated values of calculated log concentration ratios (cLogP) of drugs in octanol:water (a measure of relative hydrophobicity) (Lipinski et al., 2001). The most hydrophilic (adenosine), most hydrophobic (MRS 1191) and the moderately hydrophobic derivative (LJ 1251) were all apparently able to permeate the eye's ocular coats. Thus, relative hydrophobicity does not provide a ready explanation for the differential drug effects.

4.4. Effects on Isolated Bovine Ciliary Epithelial Cells

The strategy of developing antagonists by modifying nucleoside agonists addressed the need to obtain drugs inhibiting A₃ARs of many mammalian species with high selectivity, potency and efficacy (Gao et al., 2002). The nucleoside derivatives of the present study have been found to be highly potent and selective in studies of human ARs (Table 1). The current measurements of IOP by SNMS tonometry demonstrate the functional efficacy of these antagonists in the mouse.

The effects of three of the derivatives were also studied on the adenosine-triggered shrinkage of native bovine nonpigmented ciliary epithelial cells. This shrinkage is thought to reflect A₃AR-mediated activation of Cl⁻ channels, leading to release of cellular Cl⁻, accompanied by K⁺ and water (Carré et al., 1997; Carré et al., 2000; Mitchell et al., 1999). The drugs displayed differing, but significant inhibitions of the shrinkage, suggesting that these novel antagonists are efficacious against bovine, as well as mouse and human A₃AR receptors.

4.5. Summary

Agonists of A₃ARs stimulate Cl⁻ channels in the basolateral membranes of nonpigmented ciliary epithelial cells, accelerating the rate of aqueous humor formation and increasing IOP. Antagonists of A₃ARs inhibit these effects, thereby reducing IOP. Investigation of A₃AR antagonists has been complicated by their highly variable binding affinities in different mammalian species. This issue has been addressed by modifying the highly-selective full agonist Cl-IB-MECA to form A₃AR antagonists designed to act across species. The five derivatives studied all lower IOP in the living mouse, and the three derivatives applied to isolated bovine cells inhibit A₃AR-mediated effects. One of the derivatives, LJ 1251, appeared to permeate the cornea particularly rapidly, but identification of the underlying mechanism will require further study.

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Figure 1.

Structures of the physiologic agonist adenosine, the A₃-selective agonist CI-IB-MECA and the A₃-antagonist nucleoside derivatives, MRS 3771, LJ 979, MRS 3826, MRS 3827 and LJ 1251.

Figure 2. Measurement of mouse IOP by (A) invasive Servo-Null Micropipette System (SNMS) and by (B) non-invasive pneumotonometry.

Figure 3. Effect of LJ 979 on IOP measured invasively IOP by SNMS tonometry. The SNMS-measured IOP began to fall almost immediately upon applying a droplet concentration of 250 μ M. Following LJ 979, the agonist adenosine exerted a negligible effect on IOP, at a droplet concentration of 10 mM.

Figure 4.

Effects of the five A₃-antagonist nucleoside derivatives measured non-invasively. At the conclusion of the experiments, water was introduced intraperitoneally to verify that expected changes in IOP could be detected. As indicated by the asterisks, Measured by pneumotonometry, LJ 1251 did reduce IOP (B). Analyzed by one-way ANOVA with the Bonferroni post-test, the IOP was significantly reduced at times 20 min ($P < 0.05$) and 30 min ($P = 0.01$) at times 20 and 30 min. However, no significant changes in IOP were noted following: topical addition of vehicle alone (2% DMSO, A), LJ 979 (C), MRS 3826 (D), MRS 3827 (E) and MRS 3771 (F). Further details are provided in Table 2.

Figure 5.

Comparison of the effects of the five A₃-antagonist nucleoside derivatives on mouse IOP measured non-invasively (Pneumotonometry) and invasively (SNMS). Each of the drugs significantly lowered IOP measured by SNMS tonometry, but only LJ 1251 significantly reduced IOP measure by pneumotonometry. The probabilities that the differences were random between the two techniques were estimated by the unpaired t-test: (LJ 979) $P < 0.02$, (MRS 3771) $P < 0.02$, (MRS 3826) $P < 0.02$, (MRS 3827) $P = 0.056$, and (LJ 1251) $P > 0.7$. Asterisks indicate differences with a null probability of < 0.05 . Further details are presented in Table 2.

Figure 6.

Effects of A_3 antagonists on cell volume of native bovine nonpigmented ciliary epithelial cells. Volume was simultaneously monitored as area (A) and calcein fluorescence quenching (B). For purposes of clarity, the standard errors have been omitted and the data points are averages of the three measurements obtained each minute. Results for each series of measurements are presented as means normalized to the average of the three points measured just before application of adenosine at 35 min. Brief initial perfusions with 25% hypotonic and 25% hypertonic solutions produced swelling and shrinkage, respectively. Adenosine (300 μ M, Aden) produced cell shrinkage that was partially/completely prevented by perfusion with 300

nM LJ 979, 300 nM MRS 3771, 300 nM MRS 1191 or 150 nM LJ 1251. Data were analyzed by two-way ANOVA with the Bonferroni post-test.

Table 1

Potency of a series of agonists and agonist-derived antagonists at the four subtypes of human adenosine receptors.

Action	Drug	Potency, nM				Source (Ref.)
		A ₁	A _{2A}	A _{2B}	A ₃	
Nonselective agonist	Adenosine	310	700	24,000	290	(Fredholm et al., 2001)
A ₃ agonist	Cl-IB-MECA	222±22	5360 ± 2470	>10,000	1.4 ± 0.3	(Gao et al., 2002; Tchifibon et al., 2005)
A ₃ antagonist	MRS 3771	6,220±640	>10,000	>10,000	29.0±4.9	(Gao et al., 2006)
A ₃ antagonist	LJ 979	>10,000	>10,000	>10,000	15.5±3.1	(Gao et al., 2006)
A ₃ antagonist	MRS 3826	>10,000	>10,000	ND	910±330	(Besada et al., 2006)
A ₃ antagonist	MRS 3827	ND	ND	ND	ND	-
A ₃ antagonist	LJ 1251	2,490±940	341±75	>10,000	4.16±0.50	(Jeong et al., 2007)

ND: not determined because MRS 3827 is not a pure compound, but a mixture of two isomers (in a 3:2 ratio) of MRS 3771. In MRS 3827, the two free OH groups in MRS3771 were incompletely acetylated. The completely acetylated form is MRS3826.

Table 2

Drug	Class	Concentration	Pneumotonometry			SNMS		
			N	$\Delta(IOP)$	P	N	$\Delta(IOP)$	P
Control								
DMSO								
A ₃ antagonists								
MRS 3771	Vehicle	2%	9	-1.4±1.5	>0.3	6	-0.5±0.6	>0.4
	A ₃ antagonist	2.5mM (2% DMSO)	4	+0.3±2.6	>0.9			
		250μM (2% DMSO)	9	+0.4±0.6	>0.5	9	-3.0±1.1	<0.05
LJ 979	A ₃ antagonist	250μM (2% DMSO)	6	+0.2±1.1	>0.8	10	-4.2±1.2	<0.01
MRS 3826	di-acetyl ester of LJ 979	250μM (2% DMSO)	6	-0.5±0.8	>0.5	4	-4.0±0.8	<0.05
MRS 3827	mono-acetyl ester of LJ 979	250μM (2% DMSO)	9	-0.8±1.1	>0.4	6	-4.4±1.3	<0.05
LJ 1251	modified from LJ 979 (rat/human)	250μM (2% DMSO)	6	-4.2±0.7	<0.002	7	-1.5±0.6	<0.05
	A ₃ antagonist	75μM (0.6% DMSO)	6	-0.3±0.8	>0.7	6	-3.1±1.4	<0.08
		25μM (0.2% DMSO)	6	-0.6±1.5	>0.7	6	-4.9±1.7	<0.05
		5μM (0.2% DMSO)				6	-2.2±0.7	<0.03
		500mM (0.2% DMSO)				6	-0.09±3.4	>0.8
Outflow Enhancer								
atanoprost	prostaglandin analogue	116μM	6	-0.1±1.6	>0.9	6	-3.8±0.8	<0.01

Concentrations provided are for the drugs in topically-applied droplets. Values are means $\pm$ SE. N is the number of mice in each protocol; only one eye was studied in each mouse. P is the probability of the null hypothesis.

Table 3

Calculated log concentration ratios (cLogP) of drugs in octanol:water to estimate relative hydrophobicity.

COMPOUND	cLog P
Adenosine	-2.16
Cl-IB-MECA	1.20
MRS 3771	1.69 (Gao et al., 2006)
LJ 979 (MRS 3642)	1.73 (Gao et al., 2006)
LJ 1251 (MRS 3820)	2.25
MRS 3826	3.40
MRS 1191	6.86 (Gao et al., 2006)