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The Cross-Species A₃ Adenosine-Receptor Antagonist MRS 1292 Inhibits Adenosine-Triggered Human Nonpigmented Ciliary Epithelial Cell Fluid Release and Reduces Mouse Intraocular Pressure

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Abstract

Purpose—Antagonists to A₃ adenosine receptors (ARs) lower mouse intraocular pressure (IOP), but extension to humans is limited by species variability. We tested whether the specific A₃AR antagonist MRS 1292, designed to cross species, mimicks the effects of other A₃AR antagonists on cultured human nonpigmented ciliary epithelial (NPE) cells and mouse IOP.

Methods—NPE cell volume was monitored by electronic cell sorting. Mouse IOP was measured with the Servo-Null Micropipette System.

Results—Adenosine triggered A₃AR-mediated shrinkage of human NPE cells. Shrinkage was blocked by MRS 1292 (IC₅₀ = 42 ± 11 nM, p < 0.01) and by another A₃AR antagonist effective in this system, MRS 1191. Topical application of the A₃AR agonist IB-MECA increased mouse IOP. MRS 1292 reduced IOP by 4.0 ± 0.8 mmHg at 25-μM droplet concentration (n = 10, p < 0.005).

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Disclosure of interest: R.A.S., K.A.J., and M.M.C. are co-inventors on patent applications and issued patents assigned to the University of Pennsylvania and NIH dealing with the use of A₃ adenosine-receptor antagonists as agents in treating glaucoma. K.A.J. is a co-inventor of MRS 1292 on a patent assigned to the National Institutes of Health. The other authors have no commercial conflicts of interest.

Conclusions—MRS 1292 inhibits A₃AR-mediated shrinkage of human NPE cells and reduces mouse IOP, consistent with its putative action as a cross-species A₃ antagonist.

Keywords

A₃ adenosine receptors; aqueous humor inflow; Cl[−] channels; intraocular pressure; MRS 1292

INTRODUCTION

Intraocular pressure (IOP) is determined by the rate of inflow of aqueous humor across the ciliary epithelium and the resistance to outflow from the anterior chamber. At fixed outflow resistance, an increase in inflow will increase IOP until the sum of the pressure-dependent and pressure-independent outflows matches inflow. Many transport components underlying inflow are known (Fig. 1), but their regulation is poorly understood. For example, the normal 50–60% fall in ciliary epithelial secretion of aqueous humor during sleep remains unexplained.¹ Clarifying the mechanisms and regulation of aqueous humor formation can lead to novel strategies for lowering IOP, the only intervention known to retard the onset and progression of blindness in glaucoma.^{2–4}

Many hormones, drugs, and signaling cascades have been reported to modify inflow.⁵ Purinergic modulation, especially by A₃ adenosine receptors (A₃ARs), is of particular interest because of its likely physiologic^{6–8} and pathophysiologic importance.^{9,10} A₃ adenosine receptors upregulate Cl[−] channels of the nonpigmented ciliary epithelial (NPE) cell layer abutting the aqueous humor *in vitro*, an action expected to increase inflow and IOP *in vivo*. As predicted, A₃AR agonists increase, and A₃AR antagonists decrease, IOP in mice.⁶

Although the results obtained with A₃AR antagonists are promising, striking species differences have been noted in adenosine-receptor pharmacology, especially for antagonists to A₃ARs.^{11,12} Highly selective and potent A₃AR antagonists have been unavailable for non-primate species.^{13–15} In contrast to the species diversity of responses to antagonists, adenosine agonists characteristically display high affinity across species.¹⁶ Recently, structural modification of the highly specific, cross-species agonist IB-MECA^{17–20} has been found to retain high affinity to A₃ARs, but with zero efficacy.¹³ This new A₃AR antagonist, a cyclized 4',5'-uronamide derivative (MRS 1292; Fig. 2) displays K_i values at both human A₁ and A_{2A} receptors of >10,000 nM (Jacobson *et al.*, unpublished observations). Because the K_i value at the human A₃ receptor is 29 nM (compound 14),¹³ this compound is highly selective for the A₃ receptor. However, the efficacy across species remains to be tested.

In the current study, we have tested the effects of MRS 1292 on human nonpigmented ciliary epithelial (NPE) cells, a potential therapeutic target, and the mouse, the most promising species available for transgenic studies of the molecular pharmacology and genetics of glaucoma.

MATERIALS AND METHODS

Experimental Strategies

We chose to test the effects of MRS 1292 on cultured human NPE cells for two reasons. First, human cells *in vitro* are expected to mimic the functional properties of human NPE cells *in vivo* more closely than other species. Second, A₃ receptors are the only ARs that regulate Cl[−] channels and cell volume in these cells,²¹ simplifying the experimental protocol. Under baseline conditions, there is little tonic activity of A₃ receptors in these cells.^{21,22} We can, however, stimulate all ARs nonselectively with the physiologic agonist

adenosine and determine the effects of MRS 1292 on the changes in cell volume uniquely triggered by A₃ARs.

We chose to test MRS 1292 on the mouse, again for two reasons. Transgenic mice provide a convenient opportunity for studying the molecular physiology and pharmacology in the living animal (e.g., Avila *et al.*⁷). Second, measurement of IOP permitted us to assess the effects of MRS 1292 on the target parameter. However, the adenosine-stimulation protocol was not used in the mouse because it not only stimulates A₃ receptors but also activates other ARs that have independent effects on IOP. Here, we directly assessed the effect of MRS 1292 on the living mouse by monitoring IOP before and after drug application.

Cultured NPE Cells

The NPE cells studied were from clone-4, derived from a primary culture of human nonpigmented ciliary epithelium.²³ Confluent human NPE cells²¹ were harvested from a T-75 flask by trypsinization 3–10 days after passage.^{24,25} An 0.5-ml aliquot of the suspended cells was added to 20 ml of each test solution which included (in mM): 110.0 NaCl, 15.0 HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 2.5 CaCl₂, 1.2 MgCl₂, 4.7 KCl, 1.2 KH₂PO₄, 30.0 NaHCO₃, and 10.0 glucose, at a pH of 7.4 and osmolality of 298–305 mOsm. Gramicidin D (5 μM) was present in all solutions to provide a K⁺-release pathway so that release of KCl, and secondarily water, was limited solely by Cl[−] release.²⁴ One aliquot of cells in suspension was untreated and the other three aliquots were exposed in parallel to different experimental solutions on the same day. The same amount of solvent vehicle (DMSO) was added to the control and experimental aliquots, and the order of studying the suspensions was varied to preclude time-dependent artifacts. Cell volume was monitored with a Coulter counter (model ZBI-Channelyzer II, Beckmann Coulter, Inc., Fullerton, CA) with an aperture of 100 μm. The cell volume was measured as the peak of the distribution function.

Cell shrinkage was fit as a function of time (t) to the exponential function:

$$v_c = (100 - \Delta v_{\infty}) + (\Delta v_{\infty}) \cdot e^{-t/\tau} \quad (1)$$

where Δv_{∞} (in %) is the steady-state shrinkage and τ (in min) is the time constant of the shrinkage. Fits were generated by nonlinear least-squares regression analysis, allowing both Δv_{∞} and τ to be variables.²⁶

Mouse Intraocular Pressure

Black Swiss outbred mice of mixed sex, 7–9 weeks old and ~30 g in weight (Taconic, Inc., Germantown, NY, USA) were maintained under 12-hr light-dark illumination cycle and allowed unrestricted access to food and water. All procedures conformed to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. Mice were anesthetized with intraperitoneal ketamine (250 mg/kg) supplemented by topical proparacaine HCl 0.5% (Allergan, Hormigueros, Puerto Rico) for the IOP measurements.

IOP was monitored with the Servo-Null-Micropipette System (SNMS), a highly reliable electro physiologic approach that has been extensively validated and used in previous studies.^{6,7,27-29} Data reported were obtained from one eye of each mouse studied. Mean values of IOP were calculated by averaging 3–5 min of data acquired at 3 Hz before and after drug application. Thus, each mean was obtained from ~540 to 900 measurements.

Drugs were applied topically in 10-μl drops with an Eppendorf pipette at the stated concentrations. The agents were initially dissolved in DMSO and then added to a saline solution containing benzalkonium chloride to enhance corneal permeability. The final

droplet solution contained the drugs at the stated concentrations together with 0.5% DMSO and 0.0005% benzalkonium chloride at an osmolality of 295–300 mOsm. Control saline solutions (0.9% NaCl) did not contain DMSO and benzalkonium. We have previously found that the DMSO-benzalkonium solution itself does not affect mouse IOP at DMSO concentrations as high as 10% and a benzalkonium concentration of 0.003%.²⁷

Drugs

Adenosine, *N*⁶-(3-iodobenzyl)-adenosine-5'-*N*-methyluronamide (IB-MECA), MRS 1191, and benzalkonium chloride were purchased from RBI Sigma (St. Louis, MO, USA). MRS 1292 was synthesized as previously described.¹³

RESULTS

Cultured Human NPE Cells

Cultured human NPE cells suspended in control solution containing gramicidin D displayed slight shrinkage over the 60 min of study ($\Delta v_{\infty} = 1.2 \pm 0.1\%$; Fig. 3). Adenosine ($10 \mu\text{M}$) increased the degree of shrinkage several-fold. MRS 1292 significantly reduced the magnitude ($\Delta v_{\infty} = 1.9 \pm 0.2\%$, $p < 0.001$ by Student's *t* test; Fig. 3) and slowed the rate of the adenosine-triggered shrinkage. In the presence of MRS 1292, the time constant (τ) of the shrinkage was prolonged from 3.8 ± 0.6 to 11.7 ± 2.6 min ($p < 0.02$). In the presence of the 1,4-dihydropyridine A_3 antagonist MRS 1191,¹¹ adenosine-treated cells displayed no exponential shrinkage. MRS 1191 has been previously used successfully to antagonize human,^{11,21,22} rat,¹¹ and mouse⁶ ARs. Further details are provided in the legend to Figure 3.

The concentration-response relationship for the action of MRS 1292 on adenosine-triggered shrinkage is presented in Figure 4 ($n = 19$, including the experiments of Fig. 3). The IC_{50} estimated from the exponential fit was 41 ± 11 nM.

INTRAOCULAR PRESSURE OF LIVING MICE

Topical addition of droplets containing $25 \mu\text{M}$ of the putative antagonist MRS 1292 produced a maximum reduction in IOP by 8–19 min (mean 15 ± 1 min) of 4.4 ± 0.8 mm Hg ($n = 10$, $p < 0.005$, Student's *t* test; Fig. 5). In comparison, addition of the same volume of saline at the same osmolality produced no significant change in IOP (-0.3 ± 1.2 mmHg, $n = 6$, $p > 0.8$) 14 min later.

In agreement with our previous observations,^{6,7} the A_3 AR agonist IB-MECA produced a rapid increase in IOP of 4.6 ± 1.6 mmHg ($n = 6$, $p < 0.05$ by Student's *t* test; Fig. 5) at 140 nM. At a 10-fold lower concentration, IB-MECA increased IOP by 2.2 ± 0.5 mm Hg ($p < 0.02$; Fig. 5). In contrast, at a very high droplet concentration (1400 nM), IB-MECA exerted no significant effect (-1.2 ± 1.9 mmHg; Fig. 5), presumably because of cross-reaction with A_2A ARs.^{6,30-32}

DISCUSSION

Purine receptors modulate cell activity in the outflow and inflow pathways of the aqueous humor. Regulation of inflow reflects yin-yang modulation of ciliary epithelial secretion.³³ Activating PE-cell ATP receptors can reduce,^{25,33,34} and activating NPE-cell ARs can increase,^{21,22,26} net secretion, leading to corresponding changes in IOP. The current work focuses on the development of cross-species A_3 antagonists potentially useful in glaucoma by reducing inflow and IOP.

The strategy of exploiting A₃ antagonists was initially suggested by *in vitro* evidence that adenosine stimulates A₃AR-mediated Cl⁻ channels in freshly dissociated bovine and cultured human NPE cells and in NPE cells of rabbit ciliary epithelium.^{21,22,26} The *in vitro* results led to the prediction that agonists of A₃ARs would raise, and antagonists of A₃ARs would lower, IOP. This prediction was subsequently confirmed by IOP measurements in living mice.^{6,7}

Modulation of IOP by A₃ARs is likely of both physiologic and pathophysiologic importance. Knockout of A₃ARs reduces baseline levels of IOP,⁷ and topical application of A₃AR antagonists to nonhuman primates lowers IOP.⁸ As a potential link to clinical glaucoma, A₃ARs of NPE cells are upregulated by an order of magnitude in the pseudoexfoliation syndrome, a major cause of secondary open-angle glaucoma.⁹ The aqueous humor concentration of adenosine is also elevated in glaucomatous patients,¹⁰ but whether the association represents a cause or an effect is unknown.

In previous studies, A₃ antagonists were found both to block adenosine-triggered shrinkage of cultured human nonpigmented ciliary epithelial cells²¹ and to lower intraocular pressure.⁶⁻⁸ In those studies, non-purine antagonists of the receptor, such as the dihydropyridine MRS 1191 and the pyridine MRS 1523, were used. Most of these heterocyclic derivatives suffer from a lack of generality of the A₃ affinity across species. For example, note the high ratio of the binding affinities of rat to human A₃ receptors in Table 1. This nongenerality of antagonist selectivity has limited the ability to evaluate the potential clinical relevance of the known A₃ antagonists in animal models, in which the A₃ selectivity of the compounds would be greatly diminished. However, the current antagonist MRS 1292 is a nucleoside derivative¹³ structurally related to the agonist IB-MECA, whose high affinity of IB-MECA at A₃ receptors extends across species. MRS 1292 is A₃-receptor-selective in both the human and the rat.¹³ Note that the ratio of rat-to-human affinities for A₃ receptors is similar for MRS 1292 and selective A₃ agonists (Table 1). In this study, we have found that MRS 1292 acts as an A₃ adenosine-receptor antagonist in mimicking effects of nonpurine A₃ antagonists on cultured human NPE cells and mouse IOP.

The precise concentration of MRS 1292 in the aqueous humor resulting from our topical applications is unknown: The cornea is a significant barrier to topical delivery of nucleosides into the aqueous humor,³⁵ but the mouse anterior chamber volume is only ~2 μ l,²⁷ so that we cannot readily measure drug concentration in the aqueous humor. To estimate an approximate range, we have calculated a “penetrance,” defined as the ratio of the published K_i value at receptors *in vitro* to the minimally effective droplet concentration, for a number of adenosine agonists and antagonists. This penetrance ranges from 1:100 to 1:1000 for purinergic drugs we have tested in the mouse and is not very different from the drug penetrance of 1:100 for agents applied topically to rabbits and primates, as well.⁶ This rule of thumb also applies to acylguanidine blockers and bumetanide, whose topical effects we have also studied in the mouse eye.²⁸ Thus, the approximately 1:1000 penetrance we have noted with MRS 1292 in the present experiments is consistent with our past studies of mouse intraocular pressure.

The structural basis for the conversion of a full agonist, IB-MECA, into an antagonist is in the addition of a spiro lactam ring, which rigidifies the ribose moiety.¹³ This has been proposed to preclude a required conformational change of the ribose moiety in order for the receptor-bound nucleoside to activate the A₃ receptor.³⁶ A conformational change within the receptor involving transmembrane helical domain 6 and a conserved tryptophan residue in that helix has already been proposed, based on mutagenesis and molecular modeling studies. Thus, this conformationally constrained ligand (i.e., the nucleoside MRS 1292) behaves as an A₃ receptor antagonist in the mouse eye, as in studies of the heterologously expressed

recombinant human A₃ receptor. With the current evidence that MRS1292 works in the mouse, we suggest that this compound may be the antagonist of choice for additional animal testing, both for more fully understanding the role of A₃ ARs in regulating IOP and for potentially using A₃ antagonists in glaucoma treatment.

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FIGURE 1.

Purinergic regulation of inflow. NaCl is thought to be taken up from the stroma by Na⁺-K⁺-2Cl⁻ symports and Na⁺/H⁺ and Cl⁻/HCO₃⁻ antiports of the PE cells, to permeate gap junctions into the NPE cells and to be released into the aqueous humor by Na⁺,K⁺-activated ATPase and Cl⁻ channels of the NPE cells.⁴⁴ ATP released by PE cells can trigger production of cAMP, which acts directly on maxi-Cl⁻ channels to enhance Cl⁻ return to the stroma, thereby reducing net secretion.^{5,34} ATP-stimulated recycling is enhanced by the anti-estrogen tamoxifen.³³ At the opposite tissue surface, ATP released by NPE cells can be metabolized to adenosine that, by stimulating A₃-regulated Cl⁻ channels, can increase net secretion.^{21,22,26}

FIGURE 2.

Chemical structures of the physiologic agonist adenosine, the full agonist IB-MECA, and the current nucleoside derivative MRS 1292, an antagonist of A_3ARs .

FIGURE 3.

A_3AR antagonists inhibit adenosine-triggered shrinkage of cultured human NPE cells. Cells were suspended in isotonic (Iso) solution containing gramicidin (Gram). Adenosine ($10 \mu M$) triggered shrinkage. Both the novel A_3AR antagonist MRS 1292 (100 nM) and the established antagonist MRS 1191²¹ (100 nM) prevented that shrinkage ($N = 10$, passages 10–14). Cells were pretreated with the antagonists for 2 min before initiating the measurements. Solid curves are nonlinear least-square fits to Eq. (1): control ($\Delta v_{\infty} = 1.2 \pm 0.1\%$; $\tau = 4.5 \pm 1.7$ min), adenosine ($\Delta v_{\infty} = 4.2 \pm 0.2\%$; $\tau = 3.8 \pm 0.6$ min), and adenosine + MRS 1292 ($\Delta v_{\infty} = 1.9 \pm 0.2\%$; $\tau = 11.7 \pm 2.6$ min). The symbols Δv_{∞} and τ symbolize the steady-state shrinkage and the time constant of that shrinkage, respectively. Cells exposed to adenosine and MRS 1191 did not display exponential shrinkage and their volumes could not be fit to Eq. (1) so that the data points are connected by interrupted lines.

FIGURE 4.

Concentration-response relationship of MRS 1292. The cell shrinkages were measured after 60 min of exposure, as in the experiments of Figure 3, and corrected for the shrinkage displayed by the parallel control aliquots of cells. The curve was generated from an exponential two-parameter fit of the results.

FIGURE 5.

Effects of topical control physiologic saline ($n = 6$), the antagonist MRS 1292 (at a droplet concentration of 25 μ M, $n = 10$), and of the agonist IB-MECA at droplet concentrations of 14 nM ($n = 5$), 140 nM ($n = 6$), and 1,400 nM ($n = 4$) on IOP of living mice. Values are means $\pm$ SEs.

TABLE 1

Comparison of the Binding Affinities (K_i Values in nM) of Various A_3 Adenosine Receptor Ligands in Rat (r) and Human (h) Species. (The Compounds are Selective in Binding to the Human A_3 Adenosine Receptor, Unless Noted.)

Compound	Ratio (r/h)	K_i h A_3	K_i r A_3	Ref.
Antagonists (non-nucleoside)				
MRS 1067	10	561	5,500	37
MRS 1097	39	108	4,160	38
MRS 1191	45	31.4	1,420	39,40
MRS 1220	>1000	0.65	>1000	39,40
MRS 1523	6.0	18.9	113	40
MRE 3008F20	>30,000	0.29	>10,000	41
XAC ^a	760	38	29,000	41,42
OT-7999	>16,000	0.61	>10,000	8
Agonists (nucleoside)				
IB-MECA	0.61	1.8	1.1	39,43
Cl-IB-MECA	0.72	1.8	1.3	13,39
DBXRM	0.28	816	229	13,39
Antagonist (nucleoside)				
MRS1292	1.7	29.3	49	13

^aNonselective.