Dry-eye disease (DED) or keratoconjunctivitis sicca is one of the most common ophthalmic conditions encountered in clinical practice, and significantly lowers the quality of life of affected individuals.

Currently, two prescription drugs have been approved for the treatment of DED in the United States: 0.05% cyclosporine ophthalmic emulsion (formulated as Restasis®) and 5% lifitegrast ophthalmic solution (formulated as Xiidra®). However, both drugs are associated with a significant incidence of adverse effects affecting up to 25% of patients that include mostly instillation irritation, but also ocular pain, dysgeusia, and decreased visual acuity. Thus, despite recent advances in the management of DED, novel safe and well-tolerated therapeutic approaches that can improve the ocular surface microenvironment are urgently needed.

DED is considered a multifactorial disease associated with tears and the ocular surface that causes discomfort, visual disturbance, and possibly damage to the ocular surface due to inadequate tear production and/or instability. Symptoms of DED are common and often pose a chronic problem causing patients to experience irritation, scratchy, gritty, or burning sensations. Additionally, patients experience foreign body sensation, excess watering, and/or blurred vision. Many factors are associated with the development of dry eye, yet the pathogenesis of DED remains elusive. However, the role of inflammatory-mediated mechanisms are becoming well-established in both the development and prolonged amplification of signs and symptoms associated with DED.

Adenosine is a naturally occurring purine nucleoside and an extracellular signaling molecule with diverse, yet essential, functions in human physiology. Best known for its role as a local modulator in response to cellular stress and injury, adenosine exerts its biological effects via binding to distinct, but conserved, metabotropic receptors (A1R, A2AR, A2BR, A3R) located throughout the body.

One of the local effects that adenosine has been shown to possess is anti-inflammatory via reducing leukocyte recruitment as well as blocking neutrophil adhesion to vascular endothelium. Furthermore, adenosine mediates inhibition of phagocytosis and inflammatory cytokines by M1 macrophages, and stimulates M2 macrophage differentiation (for review, see...
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Hasko and Cronstein\textsuperscript{14}). The current consensus is that most of these effects are mediated by agonism of the A\textsubscript{2A}R.\textsuperscript{14}

The rationale of the present study is based on intriguing evidence suggesting that preconditioning by an adenosine receptor type 1 (A\textsubscript{1}R) agonist can promote the resolution of inflammation via the subsequent upregulation of A\textsubscript{2A}R.\textsuperscript{15} Specifically, agonism of the A\textsubscript{1}R has been shown to exert potent anti-inflammatory and cytoprotective effects in other organ systems (e.g., the kidney).\textsuperscript{16} Furthermore, A\textsubscript{1}R deletion increased the clinical severity score in a mouse model of experimental allergic encephalomyelitis, and caffeine-induced A\textsubscript{1}R expression improved disease severity in wild-type mice.\textsuperscript{17} However, despite strong expression throughout all ocular tissues, there is only limited knowledge regarding the role of A\textsubscript{1}R in ocular inflammation.\textsuperscript{18}

We hypothesized that targeting A\textsubscript{1}R may reduce the inflammatory pathology associated with experimental DED. Therefore, we tested the efficacy of the A\textsubscript{1}R agonist, trabodenoson, in a preclinical mouse model for DED. We chose trabodenoson (INO-8875) given its established safety and pharmacokinetic profile that was tested in several clinical trials, including a randomized Phase I dose-escalation study in healthy volunteers\textsuperscript{19} as well as a Phase II study in adults with primary open-angle glaucoma and ocular hypertension.\textsuperscript{20}

**METHODS**

**Animals**

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the European Commission Directive 86/609/EEC for animal experiments, using protocols approved and monitored by the Animal Experiment Board of Finland. C57BL/6j mice were originally purchased from The Jackson Laboratories (Bar Harbor, ME, USA) and bred at the Animal Center of the University of Eastern Finland. Mice were housed at a constant temperature (22 ± 1°C) and in a light-controlled environment (lights on from 7 AM to 7 PM) with ad libitum access to food and water. Male mice (6–10 weeks of age) were used for experiments.

**Induction of Chronic Experimental DED and Drug Administration**

DED was induced using a combination of desiccating environment and scopolamine administration, which is routinely used to induce dry-eye disease in mice.\textsuperscript{21–25} Our method is based on previous reports by others that have for example used a blower hood combined with scopolamine administration,\textsuperscript{21} or a controlled-environment chamber with desiccating conditions.\textsuperscript{26} Briefly, scopolamine was administered by placing a 3 × 3-mm piece of a scopolamine patch (ScopoDerm; Novartis, Basel, Switzerland) into each ear. The presence of patches was checked twice daily at the time of drug administration. Patches were replaced twice weekly. Concomitantly, mice were placed in a controlled desiccating environment of 5% to 15% humidity and 15 L/min airflow (SiccaSystem; K&P Scientific LLC, Oak Park, IL, USA) for 21 days. Air supply for the SiccaSystem cages is driven by a low-noise, oil-free linear air pump with 120 L/min airflow (Alita AL-120; N-ECO, spol. s r.o., Ružomberok, Slovak Republic). Air was subsequently dried using an in-line water separator (SMC Pneumatics, Yorba Linda, CA, USA) and two in-line custom-built 4-L-capacity desiccating columns filled with orange silicagel desiccant (Acros Organics, Immuno Diagnostic Oy, Hämeenlinna, Finland). Pressure gauges with release valves (McMaster-Carr, Elmhurst, IL, USA) were placed before and after the desiccating column to maintain a steady airflow of 4 psi. Dried air was distributed via a four-channel manifold into four individual flow meters (Dwyer Instruments, Michigan City, IN, USA), which were used to regulate airflow to 15 L/min into each of four cages (Super Mouse 750Micro-Isolator cage; Lab Products, Inc., Seaford, DE, USA) per system. Air was pumped into each cage through two access points with barbed fittings (5/32-inch ID). The access points were placed 15 cm apart, 4 cm above floor level, and have been chosen to correspond to the height of the mouse’s eyes, as originally described in Barabino et al.\textsuperscript{26}

**Experimental Compounds**

Both vehicle and 6% trabodenoson (INO-8875) were provided by Inotek Pharmaceuticals Corp. (Lexington, ME, USA) as preservative-free ophthalmic suspensions. Cyclosporine (0.05%) ophthalmic emulsion was Restasis (Allergan Inc., Irvine, CA, USA).

All test compounds and vehicle were administered to both eyes twice daily (8 AM and 5 PM) by pipetting 10 μL into the conjunctival sac using a P20 micropipettor. Treatments were started 3 days before induction of chronic DED and continued throughout the duration of the 3-week experimental DED-induction period (Fig. 1A).

**Quantification of Tear Volume**

Tear volume quantification was performed using a sterile phenol red-soaked cotton thread (ZoneQuick; FCI Ophthalmics, Pembroke, MA, USA) that was applied in the lateral canthus for a duration of 30 seconds, using forceps. The wetting length of the thread was read by an examiner blinded to treatment designation under a microscope and estimated using a ruler. Resolution of the measurements was 0.5 mm. Tear volume was measured in all groups, in both eyes, at baseline and at the end of the 3-week follow-up time.

**Quantification of Ocular Surface Inflammation**

To determine ocular surface inflammation, we quantified corneal fluorescein staining, essentially as described previously, with some modifications.\textsuperscript{27} Briefly, 2 μL 0.2% liquid sodium fluorescein were applied into the conjunctival sac of both eyes. After 2 minutes, corneal epithelial damage was assessed by acquiring an image using a fluorescence microscope (Leica Microsystems, Buffalo Grove, IL, USA). The total corneal fluorescein score was determined and scored by an investigator blinded to treatment designation by assessing fluorescein puncta and patches per a previously published scoring system\textsuperscript{27}: absent, 0; slightly punctate staining, 1; strong punctate staining but not diffuse, 2; small positive plaque areas, 3; large area fluorescein plaque, 4.

**Tissue Collection and Histology**

At the end of the 3-week induction period, mice were killed by transcardial perfusion with 0.9% NaCl solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffer solution, pH 7.4. The eyes, including the lids and lacrimal glands, were dissected and postfixed overnight in 4% paraformaldehyde. Intraorbital lacrimal glands were embedded in paraffin and sectioned in 10-μm-thick sections for histological analysis. The eyeball was cryoprotected, embedded in optimum cutting temperature compound, and cryosectioned in 10-μm-thick sections for immunocytochemical analysis. Detailed histological analysis, using hematoxylin-cosin (H&E) staining was performed as...
Effects on Tear Production

Using a desiccating chamber in combination with transdermal scopolamine administration, induction of DED in mice that received twice-daily instillations of vehicle (n = 20 eyes) had significantly reduced tear volumes (2.0 ± 0.4 mm) as compared with naïve animals maintained in a normal humidified environment (4.0 ± 0.6 mm; n = 12 eyes; P < 0.05; Fig. 1B). By contrast, DED-induced animals receiving twice-daily treatments of 6% trabodenoson (n = 18 eyes) had higher tear volumes (3.0 ± 0.4 mm) as compared with vehicle-treated animals on day 21, and did not differ significantly from tear volumes in naïve mice (P = 0.48). DED-induced mice treated twice daily with cyclosporine (n = 20 eyes) had similar tear volume levels to the trabodenoson-treated animals after 21 days (3.1 ± 0.4 mm; P > 0.99).

Statistics

All data are presented as median ± interquartile range for nonparametric data or mean ± SEM for parametric data. Data were analyzed using either Kruskal-Wallis ANOVA (for nonparametric data) or 1-way ANOVA (for parametric data). Differences between groups were subsequently determined using either Dunn’s, Dunnett’s, or Tukey’s multiple comparisons tests as appropriate. Differences were considered statistically significant at the P < 0.05 level.

RESULTS

Effects on Tear Production

Corneal surface damage was assessed using fluorescein staining and scored as described above. Representative examples of fluorescein staining are shown in Figure 2A. Naïve animals did not exhibit any detectable fluorescein staining (data not shown). In contrast, significant levels of staining representative of inflammation of the ocular surface were observed after 21 days in the desiccating chamber in eyes from untreated mice (median score: 2.5; n = 20 eyes; Fig. 2B); in vehicle-treated eyes, the median fluorescein score reached 3 (n = 20 eyes; Fig. 2B). The presence and distribution of staining in vehicle-treated eyes was not different from eyes that were left untreated but subjected to the same desiccating conditions for 21 days indicating no vehicle-related effects (Kruskal-Wallis ANOVA, P < 0.01; Dunn’s multiple comparisons test P = 0.99; Fig. 2B). By contrast, in mice treated with 6% trabodenoson (n = 18 eyes), corneal surface changes were much less severe and yielded a significant attenuation of fluorescein staining patterns as compared with vehicle-treated eyes (median score: 1; Kruskal-Wallis ANOVA, P < 0.01; Dunn’s multiple comparisons test P < 0.05; Fig. 2B). These changes in trabodenoson-treated eyes were similar to the reductions on ocular surface staining as observed in cyclosporine-treated eyes (n = 18 eyes; P < 0.05 versus vehicle; Fig. 2B).

Effects on Corneal Surface Staining

Infiltration of immune cells into the lacrimal gland was assessed and scored using a modification of a previously reported scoring method.25 In these experiments, the 21-day induction of DED caused significant infiltration of immune cells into the lacrimal gland, reaching a median score of 3 in untreated eyes (Figs. 3A, 3B), indicating greater than five foci of infiltration and the presence of parenchymal tissue damage. This degree of infiltration was not affected by vehicle treatment (Figs. 3A, 3B). However, twice-daily treatment with 6% trabodenoson reduced lacrimal gland pathology significantly by two scores from a median score of 3 for vehicle- to a median score of 1 for trabodenoson-treated eyes (Kruskal-Wallis test with Dunn’s multiple comparisons test, P < 0.001; Figs. 3A, 3B). The improvement with trabodenoson was similar

Figure 1. Trabodenoson improves preclinical dry-eye pathology. (A) Schematic presentation of the experimental design. Test compounds were administered twice daily at 8 AM and 5 PM by applying 10 μl into the conjunctival sac of both eyes. Tear volume measurements (TVM) and corneal fluorescein staining were performed on study days 0 and 21. (B) Induction of DED resulted in a statistically significant reduction of tear volumes in vehicle-treated mice (n = 20 eyes), whereas tear volumes in trabodenoson-treated animals (n = 18 eyes) were not statistically significantly lower compared with tear volumes in naïve animals (n = 12 eyes), suggesting a positive effect on lacrimal gland function. Tear volumes of cyclosporine-treated eyes (n = 20 eyes) were similar to the trabodenoson group. Data are presented as mean ± SEM and were analyzed with 1-way ANOVA and Tukey’s multiple comparisons test. *P < 0.05.

Effects on Lacrimal Gland Infiltration and Conjunctival Goblet Cells

Infiltration of immune cells into the lacrimal gland was assessed and scored using a modification of a previously reported scoring method.25 In these experiments, the 21-day induction of DED caused significant infiltration of immune cells into the lacrimal gland, reaching a median score of 3 in untreated eyes (Figs. 3A, 3B), indicating greater than five foci of infiltration and the presence of parenchymal tissue damage. This degree of infiltration was not affected by vehicle treatment (Figs. 3A, 3B). However, twice-daily treatment with 6% trabodenoson reduced lacrimal gland pathology significantly by two scores from a median score of 3 for vehicle- to a median score of 1 for trabodenoson-treated eyes (Kruskal-Wallis test with Dunn’s multiple comparisons test, P < 0.001; Figs. 3A, 3B). The improvement with trabodenoson was similar
in magnitude as observed in eyes treated with cyclosporine (Fig. 3B).

Mucin-producing conjunctival goblet cells were identified with PAS staining in the inferior conjunctiva and counted stereologically. Induction of the DED model reduced the number of PAS-stained goblet cells in the inferior conjunctiva by 56.8% in vehicle-treated mice as compared with naïve animals (1-way ANOVA, \( P < 0.05 \); Tukey’s multiple comparisons test, \( P < 0.001 \); Figs. 4A, 4B). Compared with vehicle-treated eyes, trabodenoson-treated eyes had a 35.8% higher goblet cell count as assessed by conjunctival PAS stain histology (\( n = 18 \) eyes; \( P < 0.05 \); Figs. 4A, 4B). In contrast, cyclosporine treatment produced only a 19.1% higher goblet cell count, which was not statistically different (\( n = 18 \) eyes; \( P = 0.37 \); Figs. 4A, 4B) from vehicle-treated eyes.

**DISCUSSION**

In the present study, we tested the efficacy of trabodenoson, an adenosine mimic with selectivity toward the A\(_1\)R,\(^{30}\) in a mouse model of DED. Our results indicate that twice-daily ocular administration of trabodenoson provides therapeutic benefit against the ocular surface changes associated with DED, a benefit that was equal in magnitude to a current therapy approved by the Food and Drug Administration for dry eye: 0.05% ophthalmic cyclosporine emulsion (Restasis). Further, our results show trabodenoson treatment not only decreases fluorescein staining of the ocular surface and exerts anti-inflammatory actions on the lacrimal gland, but preserves mucin-producing goblet cell numbers in the conjunctival layer. Trabodenoson is well-tolerated without instillation pain or irritation associated with currently approved therapies.\(^{19}\) In
A1R is a feasible therapeutic strategy to reduce DED-associated ocular surface damage and inflammation, exerting anti-inflammatory and cytoprotective effects on the lacrimal gland and conjunctival goblet cells.

Notably, trabodenoson resulted in a significant increase in the number of conjunctival goblet cells that was not observed in cyclosporine-treated eyes. Although we can only speculate with regard to the exact molecular mechanism underlying this observation, A1R activation may cause an upregulation of mucin MUC5AC expression in the conjunctival fornix. A similar effect has been observed in the lungs of adenosine deaminase–deficient mice, which show elevated adenosine levels. A concomitant increase in mucin secretion could thus prevent the loss of goblet cells caused by inflammatory processes.

In the present study, we compared trabodenoson against the most commonly used prescription drug for DED, 0.05% ophthalmic cyclosporine emulsion (Restasis). Trabodenoson showed similar or better preclinical efficacy, supporting the development of A1R-targeting pharmaceuticals for DED.

To our knowledge, the data presented herein are the first to demonstrate anti-inflammatory efficacy of an ocular formulation of an A1R agonist. As such, it remains tempting to speculate that trabodenoson has potential therapeutic benefits in and beyond DED.

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**References**


**Figure 4.** Trabodenoson increased the number of conjunctival goblet cells. (A) Representative examples of PAS-stained conjunctivas suggestive of an increased number of conjunctival goblet cells in trabodenoson-treated eyes. Scale bar: 50 μm. (B) Quantification of mucin-producing conjunctival goblet cells showed a 35.8% increase in trabodenoson-treated eyes compared with naive controls (1-way ANOVA with Tukey’s multiple comparisons test, n = 18, *P* < 0.05). Cyclosporine treatment, in contrast, resulted in a nonsignificant increase of 19.1% compared with vehicle treatment (n = 18, P = 0.37).


