The *In Vivo* Effects of the CB₁-Positive Allosteric Modulator GAT229 on Intraocular Pressure in Ocular Normotensive and Hypertensive Mice

Elizabeth A. Cairns,¹ Anna-Maria Szczesniak,¹ Alex J. Straiker,² Pushkar M. Kulkarni,³ Roger G. Pertwee,⁴ Ganesh A. Thakur,³ William H. Baldrige,^{5,6} and Melanie E.M. Kelly^{1,6}

Abstract

Purpose: Orthosteric cannabinoid receptor 1 (CB₁) activation leads to decreases in intraocular pressure (IOP). However, use of orthosteric CB₁ agonists chronically has several disadvantages, limiting their usefulness as clinically relevant drugs. Allosteric modulators interact with topographically distinct sites to orthosteric ligands and may be useful to circumvent some of these disadvantages. The purpose of this study was to investigate the effects of the novel CB₁-positive allosteric modulator (PAM) GAT229 on IOP.

Methods: IOP was measured using rebound tonometry in anesthetized normotensive C57Bl/6 mice and in a genetic model of ocular hypertension [nose, eyes, ears (nee) mice] before drug administration, and at 1, 6, and 12 h thereafter.

Results: In normotensive mice, topical administration of 5 μ L GAT229 alone at either 0.2% or 2% did not reduce IOP. However, a subthreshold dose (0.25%) of the nonselective orthosteric CB₁ agonist WIN 55,212-2, when combined with 0.2% GAT229, significantly reduced IOP compared with vehicle at 6 and 12 h. Similarly, combination of subthreshold Δ^9 -tetrahydrocannabinol (a nonselective orthosteric CB₁ agonist; 1 mg/kg) with topical 0.2% GAT229 produced IOP lowering at 6 h. In nee mice, administration of topical 0.2% GAT229 or 10 mg/kg GAT229 alone was sufficient to lower IOP at 6 and 12 h, and 12 h, respectively.

Conclusions: The CB₁ PAM GAT229 reduces IOP in ocular hypertensive mice and enhanced CB₁-mediated IOP reduction when combined with subthreshold CB₁ orthosteric ligands in normotensive mice. Administration of CB₁ PAMs may provide a novel approach to reduce IOP with fewer of the disadvantages associated with orthosteric CB₁ activation.

Keywords: cannabinoids, cannabinoid receptor 1, allosteric modulator, glaucoma, intraocular pressure

Introduction

GLAUCOMA, THE LEADING CAUSE of irreversible blindness worldwide, is a group of optic neuropathies involving selective retinal ganglion cell (RGC) loss.¹ Current therapeutic strategies for the treatment of glaucoma include management of intraocular pressure (IOP).^{2,3} For every mmHg reduction in IOP, there is a 10% reduced risk of progression of glaucomatous visual field loss.² In the years since the initial reports of cannabis-induced IOP lowering in

humans, the effects of cannabinoids have been studied extensively in the eye.^{4–6} Components of the endocannabinoid system (ECS) are present throughout the eye, including structures associated with both aqueous humor production and outflow, in addition to the retina.^{4,5} Activation of cannabinoid receptor 1 (CB₁), through topical, oral, and inhaled routes of administration of synthetic cannabinoids and phytocannabinoids, decreases IOP in both animal models and humans.^{7–19} The mechanism of these hypotensive effects involves local actions that may affect both aqueous

¹Department of Pharmacology, Dalhousie University, Halifax, Nova Scotia, Canada.

²Department of Psychological and Brain Sciences, Indiana University, Bloomington, Indiana.

³Department of Pharmaceutical Sciences School of Pharmacy, Bouvé College of Health Sciences, Northeastern University, Boston, Massachusetts.

⁴School of Medicine, Medical Sciences, and Nutrition, Institute of Medical Sciences, University of Aberdeen, Aberdeen, Scotland.

⁵Department of Medical Neuroscience, Dalhousie University, Halifax, Nova Scotia, Canada.

⁶Department of Ophthalmology and Visual Sciences, Dalhousie University, Halifax, Nova Scotia, Canada.

humor inflow and outflow,^{20–27} and also appears to involve, at least in part, β adrenergic receptors.²⁸

Although glaucomatous RGC death has been extensively studied,^{29–34} to date, no drug designed specifically as an RGC neuroprotectant has successfully made it to the market.^{35–39} Several studies have reported that, aside from IOP-lowering effects, modulation of the ECS is neuroprotective, including in some models of glaucoma.^{40–44} Therefore, it has been suggested that the ECS, and more specifically CB₁ modulation, may be a strategy that could provide both IOP-lowering properties and direct neuroprotection in glaucomatous RGC death.^{4,34,45}

However, direct activation of CB₁ via orthosteric ligands has several disadvantages, limiting the potential of CB₁ as a clinically relevant druggable target. First, CB₁-mediated hypotensive effects are relatively transient. For example, Hudson et al.²⁸ found that 2 h after administration, the IOP-lowering effect of the nonselective CB₁/CB₂ agonist WIN 55,212-2 (WIN) was no longer present. Second, repeated administration of cannabinoids can lead to desensitization of the receptor, ultimately resulting in a complete loss of effect. This was recently demonstrated by Ghosh et al.,⁴⁶ who showed that repeated high doses of the endocannabinoid enzyme inhibitor JZL184, which increases levels of the endocannabinoid 2-arachidonoyl glycerol (2-AG), caused tolerance in their model of inflammatory nociception. Third, systemic exposure to cannabinoids is known to cause adverse effects, including psychotropic and cardiac effects.^{47,48}

Allosteric modulation of CB₁ may be one means to enhance CB₁ signaling, while minimizing disadvantages associated with direct orthosteric activation.^{49–53} The first CB₁ allosteric modulator was described by Price et al. in 2005,⁵⁴ and since then, several additional allosteric modulators have been synthesized and characterized.^{52,53,55} These compounds have the potential to manipulate orthosteric activation of CB₁, by either changing affinity to the binding site or efficacy of activation; but, unlike agonists, cannot activate the receptor on their own.⁵⁶ Many of the negative side effects associated with CB₁ activation, such as psychoactivity, occur when the receptor is activated at supraphysiological levels, disrupting normally tightly controlled spatial and temporal activation.^{6,57} Use of positive allosteric modulators (PAMs) may enable pharmacological fine-tuning of activation, enhancing receptor activity while maintaining spatial and temporal control.^{51,53,56} This results in an improved safety profile over direct orthosteric activation, similar to that observed with benzodiazepines acting at GABA receptors.⁵⁸

As CB₁ allosteric modulation is yet to be investigated as a therapeutic strategy for the treatment of glaucoma, we aimed to investigate the effects of GAT229, a recently characterized CB₁ PAM,⁵⁹ either alone or in combination with a CB₁ orthosteric ligand, on IOP in normotensive and ocular hypertensive mice.

Methods

Animal husbandry

All experiments were performed in accordance with ethical guidelines from the Canadian Council for Animal Care and the ARVO Statement for the Use of Animals in Ophthalmic and Vision research, and were approved by Dalhousie's University Committee on Laboratory Animals. Male C57Bl/

6J and *Sh3pxd2b* heterozygous mice were obtained from Jackson Laboratory (Bar Harbor, ME). Both male and female nose, eyes, ears (nee) mutant mice were used. Nee mice were identified by mutated phenotypical morphology, as described by Mao et al.,⁶⁰ which included a shortened nose length and domed heads. Mice were kept in a 12 h light/12 h dark cycle (07:00–19:00) and were fed *ad libitum*.

Drugs and reagents

GAT229 was synthesized^{59,61} and provided by Dr. Ganesh Thakur (Northeastern University, Cambridge, MA). Topically delivered drugs [5 μ L, including WIN 55,212-2 (Bio-Techne, Abingdon, OX, United Kingdom)] were dissolved in the standard vehicle containing 2% DMSO (Sigma-Aldrich, Oakville, ON, Canada) and 4% Tween-20 (Sigma-Aldrich) in Tocrisolve (Bio-Techne, Minneapolis, MN). I.p. administered drugs [GAT229 and Δ^9 -tetrahydrocannabinol (Δ^9 -THC); Cayman Chemical, Ann Arbor, MI] were dissolved in a vehicle containing 1:1:18 ethanol, Kolliphor EL (Sigma-Aldrich), and saline at 1 mg/mL.

IOP measurements

To reduce variability due to diurnal changes in IOP and therefore potentially obscuring any drug-mediated IOP change,^{62,63} all measurements were initiated at the same time each day (09:30). Anesthesia was induced in mice using 3% isoflurane at 1 L/min and then maintained at 1.5% throughout the recordings. Once the pedal reflex disappeared ($\sim$ 2 min after induction), 10 recordings were taken in each eye using a handheld rebound tonometer (Tonolab; Icare, Finland) calibrated to mouse. The average of 10 recordings was used for data analysis. Topical and i.p. drugs were administered immediately following the initial baseline IOP measurements, and follow-up IOP recordings were carried out 1, 6, and 12 h after initial administration. All IOPs are reported as the change in pressure (in mmHg) from the baseline recording. Analysis using the change in IOP from baseline, rather than the absolute IOP, controlled for variability in IOP both between eyes and between animals.

Data analysis

Statistical analysis was performed using GraphPad version 6.0 (GraphPad Software, Inc., La Jolla, CA). All data are presented as mean $\pm$ S.E.M. *T*-tests were used to compare between 2 groups. Paired *t*-tests were used to compare between eyes of the same animal. Three or more groups were compared using one-way analysis of variance with a Dunnett's *post hoc* test to compare between groups. A *P* value of <0.05 was considered significant.

Results

CB₁ allosteric modulation of IOP in normotensive mice

To explore the effects of CB₁ PAMs on normotensive mice, male C57Bl/6J mice were administered either topical 0.2% or 2% GAT229 (Fig. 1A) in 1 eye, with the contralateral eye receiving vehicle. While the reduction in IOP caused by CB₁ orthosteric ligands is typically very short in duration (1–2 h),^{11–14,16,28,64–66} the time course of any

FIG. 1. The CB₁ PAM GAT229 alone does not reduce IOP in normotensive C57Bl/6J mice. (A) The structure of the CB₁ PAM GAT229. Given topically at either 0.2% ($n=7$, B) or 2% ($n=4$, C), GAT229 alone does not reduce IOP in normotensive C57Bl/6 mice 1, 6, or 12 h after administration. CB₁, cannabinoid receptor 1; IOP, intraocular pressure; PAM, positive allosteric modulator.

potential CB₁ PAM effect on IOP was unknown. Therefore, we investigated changes in pressure from baseline on an extended timeline; 1, 6, and 12 h after initial administration. As IOP varies diurnally,^{28,62,63} in all mice receiving vehicle, IOP increases were measured at both 6 and 12 h following baseline measurement (2.3 ± 0.5 and 3.6 ± 0.5 mmHg from baseline, respectively, $P < 0.05$). Topical administration of the CB₁ PAM GAT229 alone at either 0.2% or 2% did not appear to have any effect on IOP measured at 1, 6, or 12 h when compared with eyes receiving vehicle (Fig. 1B, C), consistent with expectations of a CB₁ PAM.⁵⁹

To determine whether GAT229 acted as a CB₁ PAM when administered together with a CB₁ orthosteric agonist, a combination of WIN with GAT229 was examined (Fig. 2). The CB₁ orthosteric agonist WIN has been reported to lower IOP in mice at 1 h after topical administration.²⁸ Similar to what was previously reported, 1 h after initial administration, eyes receiving topical 1% WIN had a greater reduction in IOP than eyes receiving vehicle ($P < 0.05$, -1.6 ± 0.7 and 0.4 ± 0.6 mmHg from baseline, respectively, $n=9$). This ef-

fect was no longer present 6 and 12 h later (Fig. 2A), which is also in alignment with previous reports of the transient time course for reported IOP reduction by CB₁ orthosteric agonists.^{13,14,28} However, while topical administration of 0.25% WIN was subthreshold for lowering IOP at all tested time points ($P > 0.05$; Fig. 2B), the combination of 0.2% GAT229 and 0.25% WIN produced a significant reduction in IOP at 6 h, which was sustained at 12 h after initial topical administration ($P < 0.05$, mean difference 3.4 ± 1.1 and 3.2 ± 0.9 mmHg, respectively, $n=5$; Fig. 2C), suggesting a PAM-mediated potentiation of the effect of WIN.

Allosteric modulators may display probe specificity, resulting in differences in downstream signaling.^{67–69} To investigate any potential probe-specific effects, GAT229 was also investigated with Δ^9 -THC, a CB₁ partial agonist (Fig. 3). Δ^9 -THC was chosen due to differences in CB₁ binding compared with WIN;⁷⁰ WIN binds deeper in the CB₁ orthosteric pocket compared with Δ^9 -THC.⁷⁰ Topical delivery of 0.5% or 2% Δ^9 -THC failed to significantly lower IOP at 1, 2, or 4 h after administration compared with

FIG. 2. The IOP-lowering effect of subthreshold WIN is potentiated by the CB₁ PAM GAT229 in normotensive C57Bl/6J mice. (A) One hour after administration, but not at 6 or 12 h, 1% WIN significantly decreased IOP compared with contralateral eye receiving vehicle (Tocrisolve; -1.6 ± 0.7 and 0.4 ± 0.6 mmHg from baseline respectively, $n=9$). However, a lower dose of WIN (0.25%) administered alone did not significantly decrease IOP compared to contralateral vehicle-treated eyes at any of the time points measured ($n=4$), and was therefore considered as a subthreshold dose (B). (C) IOP was significantly lower in eyes receiving 0.2% GAT229 combined with subthreshold WIN (0.25%) at 6 and 12 h after application compared to vehicle (-0.9 ± 0.5 and -0.3 ± 0.9 , 0.8 ± 0.8 and 4.2 ± 0.5 , and 2.4 ± 1.1 and 5.6 ± 1.2 mmHg from baseline, respectively, $n=5$). Paired *t*-tests, $*P < 0.05$. CB₁, cannabinoid receptor 1; IOP, intraocular pressure; PAM, positive allosteric modulator.

contralateral eyes receiving vehicle ($P > 0.05$, $n = 4-9$; data not shown). However, mice receiving i.p. 5 mg/kg Δ^9 -THC had a greater reduction in IOP 1 h after administration compared with a separate group of mice receiving i.p. vehicle ($P < 0.01$, -2.3 ± 0.3 and -0.4 ± 0.4 mmHg from baseline, respectively, $n = 5$ mice; Fig. 3A). This IOP reduction was not seen at 6 and 12 h after administration ($P > 0.05$). A lower i.p. dose of Δ^9 -THC (1 mg/kg; $n = 10$ mice) did not alter IOP significantly at 1, 6, or 12 h, compared with mice receiving i.p. vehicle ($P > 0.05$). The potential PAM effect of GAT229, using Δ^9 -THC as the CB₁ orthosteric partial agonist, was explored by administration of i.p. subthreshold (1 mg/kg) Δ^9 -THC, combined with topical 0.2% GAT229 in one eye and topical vehicle in the fellow eye. At 1 h following i.p. injection of 1 mg/kg Δ^9 -THC, no significant difference between eyes receiving vehicle and those receiving 0.2% GAT229 was observed. However, at 6 h there was a significant decrease in IOP from baseline compared with eyes receiving vehicle ($P < 0.05$, -0.6 ± 0.7 and 1.2 ± 0.4 mmHg from baseline, respectively, $n = 9$; Fig. 3B). Unlike the combination with WIN, the PAM effect of GAT229 plus Δ^9 -THC was not maintained through to 12 h ($P > 0.05$).

CB₁ allosteric modulation of IOP in ocular hypertensive mice

Nee mice have a 1 base pair deletion in *Sh3pxd2b* gene,⁶⁰ causing a loss of function mutation similar to mutations found in the Frank–Ter Haar syndrome, a rare developmental syndrome that frequently involves congenital glaucoma.^{71–73} Nee mice display closed iridocorneal angles, presumably as a result of improper development.⁷¹ While at 16 days postnatal (p16), nee mice of both sexes have IOP similar to their wild-type (WT) littermates ($P > 0.05$; 17.6 ± 1.8 mmHg, $n = 6$, and 12.6 ± 0.4 mmHg, $n = 5$, respectively), and nee mice display a significant progressive increase in IOP, which is sustained over their lifetime (Fig. 4). At 28 days postnatal (p28), IOP in nee mice was, on average, 6.8 ± 2.0 mmHg greater than in WT littermates; and by 37 days postnatal (p37), 11.3 ± 2.3 mmHg. Around 3 months of age [~ 100 days postnatal (p ~ 100)], this difference remained at 10.6 ± 2.2 mmHg. We used both male and female nee mice aged 2–3 months in this study as a model of spontaneous angle-closure glaucoma to examine

FIG. 3. The IOP-lowering effect of subthreshold Δ^9 -THC is also potentiated by the PAM GAT229. (A) 5 mg/kg Δ^9 -THC caused a significant reduction in IOP 1 h after i.p. administration compared to mice receiving i.p. vehicle ($P < 0.01$, mean difference 1.9 ± 0.6 mmHg, $n = 5$ mice per group). Administration of 1 mg/kg Δ^9 -THC ($n = 10$ mice) did not lower IOP compared with mice receiving vehicle at 1, 6, or 12 h ($P > 0.05$). (B) In mice receiving i.p. 1 mg/kg Δ^9 -THC, eyes receiving topical 0.2% GAT229 had significantly larger reduction in baseline IOP at 6 h after administration compared with contralateral eyes receiving topical vehicle ($P < 0.05$, mean difference 1.7 ± 0.6 mmHg, $n = 9$). * $P < 0.05$, ** $P < 0.01$. IOP, intraocular pressure; PAM, positive allosteric modulator; Δ^9 -THC, Δ^9 -tetrahydrocannabinol.

the actions of the PAM GAT229 in a model of ocular hypertension.

Unlike in normotensive mice, topical administration of 0.2% GAT229 alone in nee mice was sufficient to reduce IOP compared with vehicle eyes at both 6 and 12 h after administration ($n = 6$, $P < 0.05$, mean difference 5.8 ± 2.2 and 7.7 ± 3.0 mmHg, respectively; Fig. 5A). To investigate whether the route of administration produced a difference in effect, GAT229 was also administered i.p. at 10 mg/kg. While at 6 h after administration IOP was not significantly different from separate nee mice receiving i.p. vehicle, nee mice had significantly reduced IOP at 12 h ($n = 4$ mice per group, $P < 0.01$, mean difference 8.7 ± 2.5 mmHg; Fig. 5B).

Unlike in normotensive mice (Fig. 2A), topically administered 1% WIN in nee mice failed to produce an IOP-lowering effect at 1, 6, or 12 h ($P > 0.05$, $n = 6$; Fig. 6A). Administration of a topical dose of WIN, which was

FIG. 4. The nee mouse has ocular hypertension. Nee mice (nose, eyes, ears) have a mutation in *Sh3pxd2b*, a podosome adaptor protein, analogous to humans with Frank–Ter Haar syndrome.^{60,71} Compared with WT littermates, nee mice had significantly elevated IOP by p28 (13.9 ± 0.9 and 20.7 ± 2.4 mmHg) that persisted until adulthood (p ~ 100 ; 12.1 ± 0.8 and 22.7 ± 1.6 mmHg). T -tests, * $P < 0.05$, $n = 5-19$. IOP, intraocular pressure; nee, nose, eyes, ears; WT, wild type.

FIG. 5. The CB₁ PAM GAT229 reduces IOP alone in ocular hypertensive nee mice. (A) Six and 12 h following topical administration of 0.2% GAT229, IOP was significantly less compared to baseline than in eyes receiving vehicle (paired *t*-tests, *n*=6, -6.9 ± 2.5 and 0.2 ± 2.5 , and -9.9 ± 3.1 and -2.2 ± 1.2 mmHg from baseline, respectively). (B) Twelve hours following i.p. administration of 10 mg/kg GAT229, IOP was significantly reduced compared with nee mice receiving vehicle (*t*-test, *n*=4 mice per group, -11.1 ± 2.2 and -2.4 ± 1.5 mmHg from baseline, respectively). **P*<0.05, ***P*<0.01. CB₁, cannabinoid receptor 1; IOP, intraocular pressure; PAM, positive allosteric modulator.

subthreshold in normotensive mice (0.25%; Fig. 2B), also did not reduce IOP in nee mice (*P*>0.05, *n*=4; Fig. 6B). Furthermore, IOP was also not significantly different in eyes receiving subthreshold WIN (0.25%) in combination with 0.2% GAT229 compared with vehicle, 1, 6, or 12 h after administration (*P*>0.05, *n*=10, mean difference 0.1 ± 2.0 , 3.0 ± 3.0 , and 4.0 ± 3.3 mmHg, respectively; Fig. 6C). This is in contrast to normotensive mice where 0.2% GAT229 + 0.25% WIN did produce a significant decrease in IOP (*P*<0.05) at the latter time points of 6 and 12 h following administration (Fig. 2C).

Discussion

CB₁ allosteric modulation presents a novel approach to enhance CB₁-mediated actions while minimizing the disadvantages of CB₁ orthosteric activation.^{49–53} We have demonstrated that the novel CB₁ PAM GAT229 not only potentiates the effect of subthreshold WIN and Δ^9 -THC in normotensive mice but can also change the time to reach peak IOP reduction and/or the duration of the effect in normotensive mice, and in a model of ocular hypertension.

The GAT229-induced increase in the duration of the IOP-lowering effect is in contrast to the relatively short-lived actions of acute orthosteric CB₁ activation by can-

nabinoids, typically lasting only about 1–2 h after administration.^{12–14,16,28,65,66} We found that whereas administration of 1% WIN alone reduced IOP for ~1 h, the combination of subthreshold (0.25%) WIN with GAT229 reduced IOP for at least 6 h in normotensive mice. Duration of action is important when considering use as a clinically relevant therapeutic; drugs with short durations of action require frequent administration, which may lead to issues with patient compliance.^{74–77} Therefore, an increase in duration of effect by ~5 h (compared with CB₁ orthosteric activation alone) would significantly reduce the frequency of administration required to achieve therapeutic efficacy.

The exact mechanisms of CB₁-mediated IOP reductions are still obscure, although evidence clearly supports local rather than CNS or systemic actions.^{78,79} CB₁ is expressed by tissues of both the inflow and outflow pathways, and effects of cannabinoids on both aqueous production and outflow facility have been reported.^{4,5} Initially, Colasanti⁹ reported that administration of Δ^9 -THC delivered via osmotic minipump increased aqueous humor outflow facility in cats. Conversely, Chien et al.¹⁴ reported that topical administration of WIN reduced aqueous flow in monkeys, without effect on outflow facility, but this decrease could not be completely attributed to a decrease in aqueous humor production. *In vitro* studies also support multiple sites of

FIG. 6. The IOP-lowering effect of subthreshold WIN is not potentiated by GAT229 in nee mice. Unlike normotensive mice (Fig. 2A), administration of 1% WIN did not reduce IOP in nee mice (*n*=6; A). Neither subthreshold (0.25%) WIN (*n*=4; B) nor the combination of subthreshold WIN and 0.2% GAT229 had a significant effect on IOP (*n*=10, C). Paired *t*-tests. IOP, intraocular pressure.

action that may contribute to the ocular hypotensive actions of CB₁ ligands. For example, stimulation with cannabinoids produces contractile responses in ciliary muscle,^{20,27,80} relaxation of trabecular meshwork cells,^{21,22,24} and enhanced outflow in perfused anterior segment.^{23,25,26} Furthermore, some CB₁ ligands exhibit functional selectivity (bias), due to differential coupling of G proteins.⁸¹ In cultured human trabecular meshwork cells, WIN was more efficacious than CP55,940, a synthetic cannabinoid, at increasing intracellular calcium. In addition, a stable analogue of the endocannabinoid anandamide (AEA), metAEA, did not produce any measurable increase in intracellular calcium.²⁴ This suggests that not only may CB₁-mediated decreases in IOP occur via multiple sites of action but that different CB₁ ligands may produce disparate effects as well.

How exactly GAT229 extends the CB₁-mediated IOP-lowering actions is unclear. However, like other PAMs, it is possible that this effect is due to stabilization of CB₁ in an intermediate state, extended disassociation time, or by decreasing receptor desensitization.^{51,53,56,59}

Selective PAMs cannot activate receptors on their own and are subject to the “ceiling effect”—they require the presence of an orthosteric ligand to exert effects.⁵⁶ Without administration of exogenous cannabinoids, any activity of CB₁ PAMs is therefore dependent on the presence of endocannabinoids. This is true of GAT229, which showed no independent activity at CB₁ in *in vitro* studies, but potentiated the effect of several CB₁ orthosteric agonists, including the endocannabinoids 2-AG and AEA.⁵⁹ In normotensive mice, the lack of an IOP-lowering effect after administration of GAT229 alone suggests insufficient local endocannabinoids at the site of action (eg, sites of aqueous humor production and/or outflow) to potentiate the effect of GAT229 at CB₁. However, the observed IOP-lowering effect of GAT229 alone in *nee* mice, when either administered topically or i.p., could imply increase endocannabinoids at the site of action compared with normotensive WT mice.

The lack of effect of WIN, alone or combined with GAT229, in *nee* mice was unexpected. As *nee* mice represent a model of angle-closure glaucoma,^{71,72} it is possible that IOP modification may not be achievable in models of glaucoma where conventional aqueous humor outflow is restricted. Our results also suggest that the GAT229-potentiated endocannabinoid mechanism of IOP lowering is not the same as the WIN-mediated mechanism.

There is ample evidence of perturbations of the ECS during pathology, including increased endocannabinoids in other models of neurodegeneration such as Alzheimer's disease.^{6,49,82–84} It is unclear if the reasons for this increase in endocannabinoids are a cause, or a result, of pathology.^{83,84} Only 2 studies have attempted to investigate cannabinoid levels in glaucoma. Chen et al.⁸⁵ reported that in human cadaveric eyes, both 2-AG and palmitoylethanolamide (PEA) were decreased in the ciliary body compared with nonglaucomatous eyes (PEA was also decreased in choroid). While AEA levels were reportedly unaffected in the tissues investigated, Nucci et al.⁴² found lowered AEA in the rat retina following an acute model of ocular ischemia/reperfusion injury consisting of 45 min of ischemia and 12 h of reperfusion. However, endocannabinoids are labile; they are degraded rapidly, which poses technical challenges in adequately and consistently capturing true measurements.⁸³ Both these existing studies were limited in that they were

small sample sizes, postmortem tissue (former) or an acute model (latter), and in both cases only a limited number of lipids were measured at a single time point. Additional investigation of endocannabinoids, their metabolites, and related lipid profiles in chronic glaucoma models, including *nee* mice, is therefore warranted in future.

The ability to administer GAT229 alone, like other CB₁ PAMs, is advantageous in that typical adverse effects associated with systemic orthosteric CB₁ activation can be avoided.^{49–51,53} In normal conditions, endocannabinoids are released in a very restricted spatial and temporal pattern.⁶ This is in contrast to systemic administration of orthosteric CB₁ agonists, which results in global CB₁ activation leading to a vast array of on-target, but undesirable, effects (such as changes in heart rate and psychoactivity).⁸⁴ CB₁ PAMs are useful in that if endocannabinoid levels are altered at the site of the pathology (eg, the eye), but not elsewhere (eg, brain regions associated with producing psychotropic effects), PAM administration would cause potentiation of CB₁ activity only at the site of pathology, thereby reducing the potential for off-site activation and adverse effects.^{51,53} We would, therefore, hypothesize that the i.p. administration of GAT229, while still effective at lowering IOP, would not produce tetrad activity (catalepsy, hypothermia, anticonception). In support of this, i.p. administration of a similar CB₁ PAM, ZCZ011, alone produced no tetrad activity when given alone, but significantly reduced both mechanical and cold allodynia in a model of chronic constriction nerve injury.⁸⁶ In addition, administration of the parent compound GAT211 (a racemic mixture of GAT229 and GAT228, a partial allosteric agonist) also did not produce tetrad effects when administered alone in WT mice.⁸⁷

Chronic use of high doses of CB₁ orthosteric ligands may not be a viable clinically relevant approach as it can lead to not only side effects but also receptor desensitization and loss of effect over time.^{88,89} This is specifically relevant in the development of ocular hypotensive drugs, as many patients require treatment over a number of decades.^{1,2,90} For example, chronic activation of CB₁ via monoacylglycerol lipase inhibition (either pharmacologically or genetic) results in not only reduced CB₁ expression in neural tissues but also reduced CB₁ function.^{46,91,92} While we did not perform chronic administration with GAT229, Ignatowska-Jankowska et al.⁸⁶ found that chronic use of their CB₁ PAM (ZCZ011) did not result in any loss of effect over time, nor caused a change in endocannabinoid levels. Therefore, GAT229 may be well suited for long-term use.

Given that CB₁-negative allosteric modulators behaved quite differently *in vivo* compared with what was predicted from *in vitro*, investigation of CB₁ PAMs in animals, and particularly in animal models of disease, is essential.^{93,94} In this study, we have demonstrated for the first time the potential of CB₁ PAMs to modify IOP, either alone in a model of ocular hypertension or in combination with subthreshold CB₁ agonists in normotensive mice. Therefore, administration of CB₁ PAMs may provide a novel approach to reduce IOP without the disadvantages associated with orthosteric CB₁ activation.

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Author Disclosure Statement

M.E.M.K. is the founder and director of Panag Pharma Inc. Panag develops phytotherapeutics for local and regional treatment of pain and inflammation. E.A.C., A.M.S., P.M.K., R.G.P., G.A.T., and W.H.B. have no existing competing financial interests.

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Address correspondence to:

Dr. Melanie E.M. Kelly

Department of Pharmacology

Dalhousie University

5850 College Street

Halifax, Nova Scotia, B3H 4R2

Canada

E-mail: melanie.kelly@dal.ca