A Second Generation Prostanoid Receptor Antagonist Acting at Multiple Receptor Subtypes

David F. Woodward,* Jenny W. Wang, Clayton S. Spada, Robert W. Carling, Jose L. Martos, Simon Pettit, Jussi Kangasmetsa, L. David Waterbury, Matthew Lawrence, Wenzheng Hu, and Neil J. Poloso*

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ABSTRACT: It has previously been reported that a prototypical compound (AGN 211377), which blocks pro-inflammatory prostanoid receptors (DP₁, DP₂, EP₁, EP₄, FP, TP) and leaves open IP and EP₂ receptors so that their anti-inflammatory properties could be exerted, produced superior inhibitory effects on cytokine release from human macrophages compared to cyclooxygenase (COX) inhibitors. This favorable activity profile translated into animal studies, with AGN 211377 exceeding the level of inhibition afforded by COX inhibition. AGN 211377 was not, however, a practical drug candidate, having poor bioavailability and cost of goods concerns. Compound 1 (designated AGN 225660) represents a second-generation compound with an entirely different “druggable” core structure. Such a dramatic change in chemical scaffold created uncertainty with respect to matching the effects of AGN 211377. AGN 225660 inhibited RANTES, IL-8, and MCP-1 secretion by at least 50%, from TNF α activated human macrophages. Although AGN 225660 reduced TNF α -evoked MCP-1 release from human monocyte-derived macrophages, it increased LPS-induced MCP-1 secretion (up to 2-fold) from human monocyte-derived dendritic cells. However, AGN 225660 inhibited the release of IL12p 70 and IL-23 from human monocyte-derived dendritic cells stimulated by LPS by more than 70%. This effect of AGN 225660 was reproduced in part by the prototype compound AGN 211377 and a combination of selective DP₁, EP₁, EP₄, FP, and TP antagonists. These findings suggest important effects on T cell skewing and disease modification by this class of therapeutic agents. AGN 225660 exhibited good ocular bioavailability and was active in reducing ocular inflammation associated with phacoemulsification surgery, LPS, and arachidonic acid induced uveitis.

KEYWORDS: prostaglandin, antagonist, inflammation, cytokine, chemokine, interleukin

Prostanoids are established mediators of inflammation, and the therapeutic efficacy of drugs that block their global biosynthesis in conditions such as rheumatoid arthritis is well-known. Detailed investigations of the roles of the various prostanoids and their receptors in mediating inflammatory events reveal an unexpected level of complexity. The actions and interactions of the various prostanoid receptor subtypes in mediating inflammation are not straightforward and modulatory and opposing effects are not uncommon. The receptors mediating the actions of prostanoids are as follows: DP₁ and DP₂ for prostaglandin D₂ (PGD₂), EP_{1–4} for prostaglandin E₂ (PGE₂), FP for prostaglandin F_{2 α} (PGF_{2 α}), IP for prostacyclin (PGI₂), and TP for thromboxane A₂ (TxA₂).^{1,2} Over the years, potent and selective antagonists have been developed for each of these prostanoid receptor subtypes in an effort to devise more targeted and safer therapeutics, but little contemporaneous evidence for clinical success emerged in the public domain.² This situation suggested that blocking a single

receptor with a selective antagonist reflects an oversimplified approach to a complex pharmacological problem.

Whereas most prostanoid receptors are pro-inflammatory, extensive evidence indicates that EP₂ and IP receptors are typically anti-inflammatory. A series of compounds was, therefore, designed in which (a) the IP and EP₂ receptors were left open so that their anti-inflammatory properties could be exerted and (b) pro-inflammatory receptors were blocked, namely DP₁, DP₂, EP₁, EP₄, FP, TP.³ The side effects encountered during treatment by global inhibition of

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Figure 1. Chemical structure of (a) AGN 225660 and (b) AGN 211377.

Table 1. Pharmacological Characterization of AGN 225660 at Human Recombinant Prostanoid Receptors^a

	DP ₁	EP ₁	EP ₂	EP ₃	EP ₄	FP	IP	TP
AGN 225660	220	70	2 900	7100	70	180	1 100	5
AGN 211377	71	370	>10 000	5500	60	180	>10 000	<1

^aComparison of the effects of AGN 225660 and AGN 211377 on intracellular [Ca²⁺] produced by graded concentration (1–10 000 nM) of selective agonists at human recombinant DP₁, EP₁, EP₂, EP₃, EP₄, FP, IP, and TP receptor stable transfectants. Values are mean IC₅₀ (nM), *n* = 3 of triplicate determinations.

prostanoid biosynthesis with cyclo-oxygenase-2 (COX-2) inhibitors would also be avoided by leaving IP and EP₃ receptors open. IP receptors inhibit platelet aggregation, and inhibition of prostacyclin biosynthesis is the underlying consequence of the cardiovascular liability in some patients treated with cyclo-oxygenase-2 (COX-2) inhibitors.² EP₃ receptors inhibit gastric acid secretion, and blocking them could ultimately lead to gastric ulceration.² The prototypical compound that embodied this pharmacological activity profile was AGN 211377. This compound was shown to produce a superior effect on cytokine release from human macrophages compared with cyclooxygenase (COX) inhibitors.³ Moreover, this favorable activity profile translated into living animal studies.⁴ AGN 211377 profoundly inhibited S-antigen-induced uveitis and laser-induced retinal neovascularization and exceeded the inhibition afforded by a COX inhibitor.⁴

Although AGN 211377 exhibits pronounced anti-inflammatory effects in models of ocular inflammation, it lacks certain qualities that would be desirable in a drug. For ophthalmic use, it has poor ocular bioavailability and would need to be derivatized to an ester in the same way that prostaglandin pharmacokinetics were optimized to provide delivery to the ocular anterior segment.⁵ Esterification would dramatically decrease aqueous solubility, making formulation into an eye drop very difficult. AGN 211377 also exhibited poor oral bioavailability, which would not necessarily be improved by esterification. Clearly, a much wider scope of therapeutic applications could only be achieved by considerable simplification of the AGN 211377 structure, with retention of the key pharmacological profile. This was achieved as a series of pyrazole based scaffolds, the primary candidate compound being AGN 225660. The structural differences between AGN 211377 and AGN 225660 are depicted in Figure 1. The effect of AGN 225660 was studied in a range of cellular and ocular inflammation models. AGN 211377 and a combination of DP₁, EP₁, EP₄, FP, and TP antagonists were routinely tested as positive controls. These pharmacological comparisons were made since the interactions of such diverse structures with the individual prostanoid receptors may vary with respect to orthosteric and allosteric binding and resultant signal bias.

RESULTS AND DISCUSSION

AGN 225660 and AGN 211377 are structurally dissimilar and were designed from entirely different scaffolds (Figure 1). They are, however, in the same pharmacological class³ being almost identical with respect to potent pro-inflammatory prostanoid receptor antagonism and possessing no meaningful activity at anti-inflammatory EP₂, EP₃, and IP receptors. Prostanoid receptor characterization was achieved by employing a Ca²⁺ signaling (FLIPR) assay. The carrier cells were HEK-293 cells, stably transfected with each individual receptor. A chimeric G protein cDNA was cotransfected, as required according to each receptor subtype, thereby permitting a Ca²⁺ signal for each receptor subtype, as previously described.³ The IC₅₀ values (nM) for AGN 225660 (intracellular Ca²⁺ release) activity at human recombinant receptors stimulated by relevant selective agonists were as follows: DP₁ = 220, EP₁ = 70, EP₂ = 2900, EP₃ = 7100, EP₄ = 70, FP = 180, IP = 1100, TP = 5. The data are presented and compared to those of the prototypical compound in Table 1.

As in previous studies,³ the effects of AGN 225660 on chemokine secretion from human monocyte-derived macrophages were determined. AGN-211377 and a combination of separate Selective PG Antagonists were compared at the same time in the same assays to provide authentic positive controls. AGN 225660 dose-dependently inhibited LPS-induced RANTES and TNF α induced release of IL-8, MCP-1, and RANTES from human primary macrophages, essentially as effectively as the first generation pan PG antagonist AGN 211377 (Figure 2). To further investigate the anti-inflammatory effects of pan PG antagonists, their polypharmacological profile was created in a different way using a combination of PG antagonists selective for each of the receptors susceptible to AGN-211377 and AGN 225660. The combination of PG receptor selective antagonists was as follows; DP₁ (BW A868C), EP₁ (SC-51322), EP₄ (GW627368X), FP (AS604872), and TP (SQ29548). This combination of separate antagonists was tested on the production of LPS-induced RANTES, as well as TNF α -induced IL-8, MCP-1, and RANTES (Figure 2). The results demonstrated that the

Figure 2. Effects of AGN 225660, AGN 211377, and a combination of single PG antagonists on cytokine release from human monocyte-derived macrophages. Effects of graded doses of 1 μ M, 3 μ M, 10 μ M of (a) AGN 225660, (b) AGN 211377, (c) a combination of selective PG antagonists that mimic the pharmacological profiles of AGN 225660 and AGN-211377 at identical doses, and comprising DP₁ (BW A868C), EP₁ (SC-51322), EP₄ (GW627368X), FP (AS604872), and TP (SQ29548). Percentage change in LPS induced cytokine/chemokine secretion was compared to the vehicle control (broken line), normalized by cell viability of the no stimulus control. $N = 3$ donors tested in triplicate experiments with 3 technical replicates per condition/donor; mean $\pm$ SD. P values were generated using a one-way ANOVA with Dunnett's post-test compared with the vehicle control: $*P \leq 0.05$, $**P \leq 0.01$. Statistically significant changes for each treatment group are summarized as follows. (a) $*1 \mu$ M, $*10 \mu$ M AGN 225660 vs vehicle on IL-8 secretion; $*10 \mu$ M AGN 225660 vs vehicle on MCP-1 secretion; $*1 \mu$ M, $*3 \mu$ M, $*10 \mu$ M AGN 225660 vs vehicle on RANTES secretion. (b) $**1 \mu$ M, $**3 \mu$ M, $*10 \mu$ M AGN 211377 vs vehicle on IL-8 secretion; $**10 \mu$ M AGN 211377 vs vehicle on MCP-1 secretion; $*1 \mu$ M, $*3 \mu$ M, $*10 \mu$ M AGN 211377 vs vehicle on RANTES secretion. (c) $**1 \mu$ M, $**3 \mu$ M, $**10 \mu$ M antagonist combination vs vehicle on IL-8 secretion; $*3 \mu$ M, $*10 \mu$ M antagonist combination vs vehicle on MCP-1 secretion; $*1 \mu$ M, $*3 \mu$ M, $*10 \mu$ M antagonist combination vs vehicle on RANTES secretion. No other statistically significant differences were obtained.

combination of five separate receptor selective-PG antagonists inhibited TNF α -induced IL-8, MCP-1, and RANTES release as effectively as AGN 211377 and AGN 225660.

Beyond human recombinant prostanoid receptors and human monocyte derived macrophages experiments, the previous studies were extended to include chemokine/cytokine release from human monocyte derived macrophages and dendritic cells. The most intriguing finding was the considerable reduction in the release of T-cell skewing IL12p70 and IL-23 from human monocyte derived dendritic cells. This inhibitory effect was a function of the inherent pharmacological properties of AGN 225660 and AGN 211377, since IL12p70 and IL-23 release was similarly reduced by a combination of prostanoid receptor antagonists selective for DP₁, EP₁, EP₄, FP, and TP receptors (Figure 3).

Quantitatively, AGN 225660 produced a pronounced decrease in the secretion of the T cell skewing cytokines IL-12p70 and IL-23 from human MoDCs, which was in the vicinity of 65–75% over the concentration range employed

(Figure 3). AGN 225660 appeared to produce a greater effect on IL-12p70 and IL-23 release compared to AGN 211377 and the combination of antagonists individually selective for DP₁, EP₁, EP₄, FP, TP receptors, which tended to exhibit an inverted but nonsignificant dose–response profile. In marked contrast to IL12p70 and IL-23 release, AGN 225660 significantly and dose-dependently increased MCP-1 secretion from human monocyte-derived dendritic cells (Figure 3).

In contrast to human monocyte derived macrophages, pharmacological inhibition of multiple prostanoid receptors did not typically alter cytokine/chemokine secretion from human MoDCs (Figure 3). Differences between human cell types is expected. Although all three treatment groups produced similar results in most instances, a few results could be ostensibly considered as outliers. AGN 211377 and the antagonist combination dose dependently reduced ENA-78 release from human MoDCs, although AGN 225660 had surprisingly little effect. The antagonist combination reduced IL-10 release from human MoDCs, but this effect was not

Figure 3. Effects of AGN 225660, AGN 211377, and a combination of single PG antagonists on cytokine release from human monocyte-derived dendritic cells. The effects of graded doses of 1 μ M, 3 μ M, 10 μ M, of (a) AGN 225660, (b) AGN 211377, (c) a combination of selective PG antagonists that mimic the pharmacological profiles of AGN 225660 and AGN-211377 at identical doses, and comprising DP₁ (BW A868C), EP₁ (SC-51322), EP₄ (GW627368X), FP (AS604872), and TP (SQ29548). Percentage change in cytokine/chemokine secretion was compared to the vehicle control (broken line), normalized by cell viability of the no stimulus control. $N = 3$ donors tested in triplicate experiments with 3 technical replicates per condition/donor; mean $\pm$ SD. The P values were generated using a one-way ANOVA with Dunnett's post-test compared with the vehicle control: * $P \leq 0.05$, ** $P \leq 0.01$. Statistically significant changes for each treatment group are summarized as follows. (a) **1 μ M, **3 μ M, **10 μ M AGN 225660 vs vehicle on IL-12p70 secretion; *1 μ M, **3 μ M, **10 μ M AGN 225660 vs vehicle on IL-23 secretion; *1 μ M, **3 μ M, **10 μ M AGN 225660 vs vehicle on MCP-1 secretion. (b) *1 μ M, *3 μ M, ** AGN 211377 vs vehicle on IL-12p70 secretion; *1 μ M, *3 μ M, *10 μ M AGN 211377 vs vehicle on IL-23 secretion; *1 μ M, *3 μ M, *10 μ M AGN 211377 vs vehicle on ENA-78 secretion. (c) *3 μ M, *10 μ M antagonist combination vs vehicle on IL-10 secretion; **1 μ M, **3 μ M, **10 μ M antagonist combination vs vehicle on ENA-78 secretion. No other statistically significant differences were obtained.

apparent for AGN 225660 or AGN 211377 (Figure 3). IL-6, IL-8, MCP-1, TNF α , and MIP1- β secretion from human MoDCs was unaltered by any of the treatment modalities employed (Figure 3). In monocyte derived dendritic cells (MoDCs), MCP-1 hypersecretion occurred in response to AGN 225660 treatment, while AGN 211377 and the selective antagonist combination were inactive. There is no definitive explanation for these AGN 225660 related differences as yet. It may be related to the residual antagonism reported for AGN 225660 at the EP₂ receptor (2900 nM) blunting anti-inflammatory effects of endogenously released PGE₂ at its target anti-inflammatory EP₂ receptor. Among many anti-

inflammatory properties, EP₂ receptor stimulation inhibits neutrophil chemotaxis.⁶ Prostanoid EP₂ receptors inhibit T-cell proliferation and regulate antigen-presenting cell function,⁷ inhibit TNF α release from bone marrow-derived dendritic cells,⁸ inhibit major histocompatibility complex class II expression in dendritic cells,⁹ suppress IFN α release by natural killer cells,¹⁰ inhibit Th1 and Th2 polarized antigen-specific T-cell responses,¹¹ and augment the signaling and function associated with the anti-inflammatory cytokine IL-10.¹²

At a glance, AGN 225660 seems a much more drug-like structure than AGN 211377. This supposition is affirmed by the much better ocular biodisposition of AGN 225660

compared to that of AGN 211377 following eye drop administration to the ocular surface (Table 2). The result

Table 2. Ocular Bioavailability^a

time (h)	iris/ciliary body ng/g tissue	aqueous humor ng/mL	cornea ng/g tissue	sclera ng/g tissue	bulbar conjunctiva ng/g tissue	palpebral conjunctiva ng/g tissue
(a) AGN 225660						
1	87.9	54.3	1489.1	721.2	380.8	602.3
2	96.4	60.1	951.6	359.6	505.1	2516.0
4	8.2	BLQ	133.5	28.7	50.6	159.0
8	BLQ	BLQ	55.5	26.3	46.0	335.5
24	BLQ	BLQ	63.9	34.2	41.5	853.9
(b) AGN 211377						
1	BLQ	14.9	1882.5	1348.5	4359.6	1278.6
2	BLQ	31.9	1959.8	2649.5	7353.7	7700.1
4	BLQ	BLQ	21.6	22.3	190.9	7375.4
8	BLQ	BLQ	BLQ	BLQ	38.6	10530.4
24	BLQ	BLQ	BLQ	BLQ	BLQ	817.9

^aThe ocular tissue bio-availabilities of (a) AGN 225660 and (b) AGN 211377 are compared following their topical administration to the ocular surface of rabbits. BLQ indicates below quantifiable level. *N* = 5 for each compound on study.

most relevant to the anti-inflammatory activity of AGN 225660 and AGN 211377 was the tissue concentrations in the iris/ciliary body. AGN 225660 was measurably present in the iris/

ciliary body tissue for at least 4 h postdosing. In marked contrast to AGN 225660, AGN 211377 was not detected in the iris/ciliary body at any time point. AGN 211377 remained on the ocular surface tissues (cornea, sclera, bulbar, and palpebral conjunctivae) with the exception of a brief presence in the aqueous humor. These data are provided in their entirety in Table 2

All previous studies with AGN 211377 on models of inflammation have relied on systemic administration of the compound to reach ocular tissues. Diseases of the front of the eye are preferably treated by eye drops and AGN 225660 is entirely suited to this purpose. The effects of AGN 225660, AGN 211377, and ketorolac, administered topically to the eye as drops, on LPS induced uveitis in rats are shown in Figures 4 and 5. AGN 225660 (1%) and AGN 211377 (1%) both significantly reduced protein exudation, with only AGN 225660 reducing the protein concentration to <20 mg/mL (Figure 4). Only AGN 225660 statistically significantly reduced leukocyte infiltration into the anterior chamber (Figure 5). Ketorolac (0.4%) produced no significant inhibition in protein exudation (Figure 4) and no effect whatsoever on leukocyte emigration (Figure 5).

AGN 211377 produced a more modest effect than AGN 225660 on protein exudation and no effect on leukocyte infiltration. The disappointing performance of AGN 211377 in the LPS induced rat uveitis model reflects its far from optimal penetration into the iris/ciliary body. The fact that AGN 211377 exhibited any activity at all in the rat uveitis model is perhaps due to the small size of the rat eye, which favors access

Figure 4. Effect of AGN 225660 on LPS-induced protein exudation in rat eyes. The effect of (a) AGN 225660, (b) AGN 211377, and (c) ketorolac on protein exudation into the aqueous humor in the LPS model of uveitis in rats. Values are mean $\pm$ SD. Open symbols represent vehicle treated eyes, filled symbols represent compound treated eyes; *N* = 8; **P* < 0.05, ***P* < 0.01 according to Student's nonpaired *t* test; (a) **1% AGN 225660 vs vehicle, (b) *1% AGN 211377 vs vehicle, (c) 0.4% ketorolac, no significant difference.

Figure 5. Effects of AGN 225660 on LPS-induced leukocyte infiltration in rat eyes. The effect of (a) AGN 225660, (b) AGN 211377, and (c) ketorolac on leukocyte infiltration present in the aqueous humor in the LPS model of uveitis in rats. Values are mean $\pm$ SD. Open symbols represent vehicle treated eyes, filled symbols represent compound treated eyes $N = 8$; $*P < 0.05$, according to Student's nonpaired t test. Statistically significant difference was obtained on for (a) $*1\%$ AGN 225660. No other statistically significant differences were obtained.

to the globe and may overestimate clinical outcomes in much larger eyes, such as those of nonhuman primates and humans. For this reason, AGN 211377 was not tested in experiments involving the much larger eyes of rabbits and nonhuman primates. Ketorolac (0.4%) exerted no statistically significant activity in the rat LPS uveitis model, which is consistent with previous studies in models of ocular inflammation in rats.⁴

In the rabbit eye, arachidonic acid is converted to pro-inflammatory prostaglandins. Thus, in the arachidonic acid induced uveitis model in rabbits, the prostanoid mediated inflammatory response may be formed in a very discrete fashion.¹³ This permitted the effectiveness of prostanoid biosynthesis inhibition and blockade of multiple target prostanoid receptors to be definitively compared. Graded doses of AGN 225660 (0.1%, 0.3%), 0.4% ketorolac, and a combination of 0.3% doses of BW A868C, SC-51322, GW627368X, AS604872, and SQ 29548 were examined in this model. AGN 211377 was not included as a test substance due to its poor penetration into the ocular anterior segment. AGN 225660 produced dose dependent inhibition of arachidonate induced uveitis; a 0.1% dose was marginally effective whereas a 0.3% dose totally inhibited arachidonate induced leukocyte infiltration (Figure 6). A combination of receptor selective PG antagonists and Ketorolac (0.4%) also abolished arachidonic acid induced leukocyte filtration into the anterior chamber (Figure 6).

Since the cyclo-oxygenase inhibitor and AGN 225660 both totally inhibited arachidonic acid induced inflammation in the rabbit eye, it follows that any prostanoid mediated inflammatory component may be removed equi-effectively by either of

these therapeutic modalities in uveitis and other ocular inflammatory conditions.

This outcome occurred in a surgical inflammation model in monkeys, namely phacoemulsification surgery induced inflammation. The peak laser flare responses, a measure of anterior chamber protein concentrations, occurred on day 1 following surgery. Treatment twice daily with 0.3% AGN 225660 and 0.4% ketorolac statistically significantly lowered photometry measures relative to the PBS treated control group by $\sim 50\%$ (Figure 7).

In such cases the only advantage of antagonist therapy would be relatively rapid reversal of an ongoing prostanoid mediated inflammatory event. Cyclo-oxygenase inhibition would prevent only de novo prostanoid biosynthesis and prostaglandins are long-lived in the eye with no deactivating oxidative metabolism¹⁴ to terminate their protracted effects. This may be part of the reason why the prototypical compound AGN 211377 was much more effective than a cyclo-oxygenase inhibitor in preventing experimental choroidal neovascularization and, importantly, autoimmune uveitis.⁴

The *in vivo* data reported to date has been exclusively ophthalmological. This is because the prototypical compound (AGN 211377) has poor ocular bioavailability as an eye drop, although intraocular drug delivery could perhaps be achieved by incorporation into an erodible injectable pellet.¹⁵ AGN 211377 also has very low oral bioavailability. AGN 225660 has satisfactory ocular bioavailability, more than sufficient to be the active pharmaceutical ingredient in eye drops. It also showed good oral bioavailability in nonhuman primates (data not shown), which opens up nonocular disease states. On the basis

Figure 6. AGN 225660 reduces leukocyte infiltration in arachidonic acid-induced uveitis in rabbit eyes. Comparison of the effects of (a) 0.1% AGN 225660, (b) 0.3% AGN 225660, (c) a combination of 0.3% BW 868C, SC-51322, GW627368X, AS 604872, SQ 29548 (d) 0.4% ketorolac on arachidonate induced leukocyte infiltration into the rabbit ocular anterior chamber. Values are median $\pm$ SD. Open symbols represent vehicle treated eyes, filled symbols represent compound treated eyes $N = 4$; $**P < 0.01$, according to Student's paired t test compared to contralateral vehicle treated control (open symbols). Statistically significant changes are summarized as follows: (b) $**0.3\%$ AGN 225660, (c) $**0.3\%$ antagonist combination t (d) $**0.4\%$ ketorolac. No other statistically significant difference were obtained.

Figure 7. Effects of AGN 225660 on phacoemulsification surgery-induced flare in African green monkey eyes. The effect of 0.3% AGN 225660 (■) and 0.4% ketorolac (▲) on the anterior segment flare response associated with phacoemulsification surgery in African green monkeys. Values are mean $\pm$ SD. On day 1, both AGN 225660 and ketorolac produced significant reductions relative to the PBS control (●): $**P < 0.01$, according to two-way ANOVA followed by Tukey HSD, $n = 5$ per group.

of the cytokine-chemokine release profiles, human monocyte-derived macrophages, and dendritic cells, AGN 225660 could

prove useful in rheumatoid arthritis, psoriasis, and many other diseases.

The newly discovered effects of AGN 211377 and AGN 225660 on IL-12p70 and IL-23 release from MoDCs further expands the potential therapeutic utility of this class of compounds. It also provides explanation for the inhibitory effects in a model of autoimmune uveitis, since IL-23 has been previously considered a cytokine relevant to the pathogenesis of experimental and clinical uveitis.^{16,17} The effectiveness of AGN 225660 and identical pharmacological modalities on IL-23 release is not entirely surprising since the prostanoid EP₄ receptor has previously been reported as critical for IL-23 release from dendritic cells.^{17–19} The EP₄ antagonism afforded by AGN 211377 and AGN 225660 is likely fortified by concomitant inhibition of facilitating EP₁ receptors^{3,4} and the EP₂ receptor being open, since EP₂ receptors down-regulate IL-23 release at PGE₂ levels exceeding 50 nM.²⁰ Several additional cytokines/chemokines that have been claimed as potentially important in uveitis and in LPS induced uveitis and decreases in MCP-1, IL-6, I-CAM, and PGE₂ correlate with novel treatments that reduce intraocular inflammation.^{21,22}

METHODS

Cell Studies. Ca²⁺ Signaling Using FLIPr Instrumentation. Stable cell transfectants overexpressing human prostanoid DP₁, EP_{1–4}, FP, IP, and TP receptors have been previously reported.³ Responses of G_s and G_i coupled prostanoid receptors were measured as a Ca²⁺ signal, by employing chimeric G protein cDNAs. Ca²⁺ signaling studies were performed using a FLIPR system (Molecular Devices, Sunnyvale, CA, USA) in a 96-well format. Briefly, cells were seeded at a density of 5×10^4 cells/well in Biocoat poly-D-lysine-coated blackwall, clear-bottom 96-well plates (BD Biosciences, Franklin Lakes, NJ) and allowed to attach overnight in an incubator at 37 °C. The cells were then washed twice with HBSS-HEPES buffer (Hanks' balanced salt solution without bicarbonate and phenol red, 20 mM HEPES, pH 7.4). After 60 min of Fluo-4-AM (Invitrogen, Carlsbad, CA, USA) dye loading at a final concentration of 2×10^{-6} M, the plates were washed 4 times with HBSS-HEPES buffer. A 7-point serial dilution of the standard agonist for each corresponding receptor subtype was administered to give final concentrations from 10^{-11} M to 10^{-5} M in 10-fold serial dilution increments for cells expressing human recombinant DP₁, EP₁, EP₂, EP₃, EP₄, FP, and IP receptors. The concentration range for the standard agonist for human recombinant TP receptors was from 10^{-12} M to 10^{-6} M. HBSS-HEPES buffer was used as the negative control. Cells were excited with an argon laser at 488 nm, and emission was measured through a 510 nm to 570 nm emission filter. Standard agonists purchased from Cayman Chemical (Kalamazoo, MI, USA) were as follows: DP = BW 245C, EP₁–EP₄ = PGE₂, FP = 17-phenyl-PGF_{2α}, IP = carbaprostacyclin, and TP = U-46619. To obtain concentration–response curves, compounds were tested in triplicate in each plate over the desired concentration range in at least three separate experiments to give $n = 3$.

Culturing and Transformation of CD14+ Monocyte-Derived Human Macrophages and Cytokine Release. Cryopreserved human CD14+ monocytes (Lonza, Rockland, ME, USA) were seeded on day 0. To differentiate monocytes to macrophages, the cells were placed in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal calf serum (Gemini Bio-Products, West Sacramento, CA, USA), 10 ng/mL recombinant human monocyte colony-stimulating factor

(M-CSF; PeproTech, Rocky Hill, NJ, USA), and penicillin–streptomycin (Invitrogen). The medium was changed on day 3, and unattached cells were discarded. On day 7, the cells were washed and then dosed as follows: For Luminex cytokine-chemokine assays, the macrophages were treated in serum-free Opti-MEM I (Invitrogen Waltham, Massachusetts, USA) with vehicle, 1 μM, 3 μM, or 10 μM of the compounds of interest. Cells were stimulated with 20 ng/mL TNFα (R&D Systems, Minneapolis, Minnesota, USA) or 10 ng/mL lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO, USA) with unstimulated DMSO vehicle controls as the baseline for biomarker release. On day 8, after 24 h of treatment, the medium in each well was collected and stored at –70 °C until tested. For the cytokine assays, fresh, warm Opti-MEM I was immediately added back to each well, as also for the Alamar Blue cell viability assay (Invitrogen). Each condition was tested in triplicate from three donors. The viabilities of cells under all treatment conditions were above 95% compared to that for no stimulus. Alamar Blue cell viability assay was performed according to the manufacturer's instructions (Invitrogen). Luminex assays were performed using kits for Human Chemokines/Cytokines (Millipore). Each condition was tested in triplicate from three donors.

Generation of Monocyte-Derived Human Dendritic Cells and Cytokine Release. Frozen human peripheral blood mononuclear cells (PBMCs) were purchased from Biomed Supply (Carlsbad, CA) obtained from healthy donors. Monocytes were isolated using Monocyte Isolation kit II according to the manufacturer's directions (Miltenyi Biotec, Auburn, CA). The purity of the monocytes was checked by flow cytometry (CD14+, CD3–) and was found to be routinely ~95% in purity. Monocytes were cultured at 1×10^6 /mL in 6 well plates in medium containing 50 ng/mL of GM-CSF and 25 ng/mL of IL-4 (R&D Systems, Minneapolis, MN) for 6 days. Cultures were supplemented with fresh medium and cytokines on days 2 and 5. MoDCs were harvested on day 6, phenotyped by flow cytometry, and transferred to 96-well plates for stimulation assays. MoDC stimulation assays were carried out in media containing 2.5% heat inactivated, charcoal and dextran stripped FBS. MoDCs were plated at 5×10^4 /well in 96 well U-bottom plates in the presence of LPS (100 ng/mL) and pretreated with antagonist compounds for 10 min. All cultures were incubated at 37 °C in 5% CO₂ incubators for 18–20 h. Following centrifugation, the supernatants were harvested for cytokine determination. MoDCs were routinely checked for viability by the Alamar blue assay (Invitrogen, Carlsbad, CA). Cytokine determination was carried out by Luminex bead-based assay according to the manufacturer's instructions using Human cytokine panels I and II Millipore (San Jose, CA) and read on a Luminex IS-200 (BioRad, Hercules, CA). All experiments were performed in triplicate from three donors.

All cell studies were performed in adherence to prewritten protocols, many previously reported (Wang et al.³). Statistical analyses were conducted using GraphPad Prism as a one-way ANOVA with Dunnet's post-test. No data were removed from the analyses.

Animal Studies. Lipopolysaccharide (LPS) Induced Uveitis in Rats. All animal experiments adhered to the ARVO statement on the Use of Animals in Ophthalmic and Vision Research and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Animal experiment protocols were also reviewed and approved by the Animal Care and Use committees at all institutions involved in the study series. Animal studies are reported in compliance with the ARRIVE guidelines.^{23,24} All animal experiments were performed in a masked manner with respect to the identity of the drugs. Sample (group) sizes were determined according to previous experimental experiences with these drugs⁴ or by standard screening packages offered by contract research organizations.

LPS induced uveitis has previously been shown to respond to nonsteroidal anti-inflammatory agents²⁵ and was chosen partly for this reason. Male Lewis rats (LEW/SsNHsd, Harlan Laboratories) weighing between 180 and 200 g were used. Eight animals were randomly assigned to each group and the experimenter was unaware of the drug identities. The treatment groups were AGN 225660 (1%), AGN 211377 (1%), and ketorolac (0.4%). Rats were dosed topically with test drugs in one eye (25 μ L), and with vehicle (phosphate buffered saline, 25 μ L) in the contralateral eye 1 h before receiving *E. coli* LPS into both footpads (100 μ g of LPS in 0.1 mL of phosphate buffered saline). Rats were again dosed with drugs topically 30 min later and then 4 h after LPS. Thus, all rats received three doses of test drug. Approximately 22 h later the rats were euthanized with CO₂ and aqueous humor collected using a syringe attached to a 28 g needle with minimal void volume.

Cell counting was accomplished by pipetting and smearing 1.0 μ L (VWR pipet for pipetting 0.2–2 μ L) samples onto 12 well slides (Shandon Multi-Spot Slides (3 in. $\times$ 1 in. with 12 wells per slide). Slides were allowed to air-dry for 5 min followed by dehydration with 100% methanol. At this point, the slides were either frozen at -40°C and stored or stained for further examination. Staining was accomplished by rehydration with phosphate buffer, staining with Harris Hematoxylin for 5 min followed by water rinses, bluing with a basic solution, followed by additional washes and dehydration. Cells were clearly visible under the microscope (Konus Trinocular Microscope Biorex 3, equipped with a camera port). Several photographs were taken of each cell smear with a Canon EOS Rebel T2i camera attached to a computer. Images were transferred to a computer and printed. Cells were readily counted using a digital pen which marks each cell as it is counted. Averages were taken of images and results provided as total cells per 1.00 μ L smear. All rats which received the above treatment (100 μ g LPS X 2) had abundant cell numbers in the aqueous humor.

Protein concentrations were determined as follows. Following collection of aqueous humor, and pipetting of 1.0 μ L samples to slides, aqueous samples in microfuge tubes were centrifuged, and 10 μ L volumes transferred from the supernatant to tubes for protein analysis. Samples were diluted 1:50 with distilled water and frozen until analysis. Protein was analyzed using a Pierce BCA Protein Assay Kit (Dallas, TX, USA) with samples read using a plate reader set for carrying out this assay. All samples were run with an array of standards, and protein concentrations were determined using software provided by Turner Designs (San Jose, CA, USA). All aqueous samples contained elevated protein concentrations.

Arachidonic Acid Induced Uveitis in Rabbits. The arachidonate induced rabbit uveitis model was based on that previously described^{26,27} but only events in the ocular anterior chamber were determined. Conscious male or female Dutch Belted pigmented rabbits weighing 2.5 to 3 kg were used for all

in vivo slit lamp studies. Four animals were employed per test group. The right eye of each animal received 35 μ L of topically administered test material and the contralateral left eye of each animal received 35 μ L of topically administered vehicle ($t = 0$ min). This was followed by FITC (fluoroisothiocyanate) solution in physiological saline administered intravenously at a dose of 5 mg/kg via the marginal ear vein 15 min later; then, 30 min later, by treatment with 35 μ L of 0.5% sodium arachidonate onto the surface of both eyes ($t = 30$ min). Both eyes were examined by slit lamp 60 min following sodium arachidonate challenge ($t = 90$ min) at 16 $\times$ magnification under both white light and blue light illumination at an approximate angle of 45 $^{\circ}$ through 1 mm and 5 mm apertures and 10 mm slit width. Leukocyte infiltration was determined by slit-lamp (Zeiss) biomicroscopy. Readings were taken before drug administration, at 30 min postdrug administration, and at 2 h following arachidonate administration.

Anterior chamber leukocyte infiltration was measured using a numerical scoring system to estimate cell number per field defined by a 10 mm slit width: 0 = no cells per field (no response); 1 = <10 cells per field (mild); 2 = >10, <25 cells per field (moderate); 3 = >25, <50 cells per field (severe); 4 = >50 cells per field (florid). Results are reported as the mean score value $\pm$ SEM. A field was defined as the total observable field through the slit-lamp aperture employed.

Phacoemulsification Surgery Induced Inflammation in Nonhuman Primates. Fifteen in-house African green monkeys (*Chlorocebus sabaeus*), eight males and seven females and weighing 3.2–6.7 kg, were arbitrarily assigned to three groups of 5. Baseline ophthalmological and clinical examinations were conducted and performed throughout the study.

Animals were placed in a supine position for the phacoemulsification surgical procedure and anesthesia was achieved by intramuscular injection of 8 mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA, USA) and 1.6 mg/kg xylazine (Lloyd Laboratories, Shenandoah, IA, USA) to produce an effect for the short duration of the procedure. In addition to general anesthesia, local ocular surface anesthesia was provided by 0.5% proparacaine HCl (Allergan Inc. Irvine, CA, USA) applied topically 5 min before initiating the phacoemulsification procedure. The brow was shaved presurgery. The eye and periorbital region were disinfected with 5% povidone-iodine for 1 min followed by rinsing with sterile saline. A small incision in the cornea was made with a 20G MVR knife, through which 0.4–0.6 mL of viscoelastic was introduced into the anterior chamber by a viscoelastic injector to maintain the dimensions of the anterior chamber. An incision was tunneled through the cornea 1 mm anterior to the limbus using a 2.65 straight clear cornea bibeveled blade. After a capsulorhexis was performed, phacoemulsification was utilized to disrupt and aid removal of the lens. Some small fragments of lens were allowed to remain to stimulate an inflammatory response, which is not typical in the clinical procedure. Ultrasonic energy was delivered at 34% power with a Storz premier anterior phacoemulsification instrument (Bausch and Lomb, Irvine, CA, USA). Phacoemulsification energy was continued until 30 s of total exposure was achieved. Irrigation with sterile saline was maintained until 150 mL was perfused through the anterior chamber. The phaco hand-piece was then removed and one drop of 0.3% ciprofloxacin antibiotic ophthalmic solution was applied topically.

The topical test articles administered were 0.4% ketorolac tromethamine (Allergan Inc., Irvine, CA, USA) and 0.3% AGN

Figure 8. NMR spectrum for AGN 225660.

225660, 1% polysorbate 80 in normal saline was used as an inert control. Each treatment was administered topically as a single drop to the right eyes, twice daily with a 7 h intervening interval. Dosing began 1 day prior to phacoemulsification surgery and continued for the first 6 days of the postoperative period.

Slit lamp biomicroscopy and laser flare photometry were performed prior to drug dosing and at 1, 2, and 7 days postsurgery using a Kowa FM-500 instrument (Kowa Co., Tokyo, Japan). At each observation time point, measurements were repeated until seven acceptable readings were obtained. Acceptable readings were defined as a difference between two background readings of <15%. The lowest and highest readings were not included in the analyses and the mean $\pm$ standard deviation were calculated using the Kowa software. Photometry readings were expressed as photon units/msec. Anterior chamber cells, aqueous flare, and other ophthalmological findings were graded according to the Hackett–McDonald scoring system.^{28–30}

Ocular Pharmacokinetics. The pharmacokinetic methods were previously and extensively described.³¹ The pharmacokinetic studies presented herein were performed at Medicilon Preclinical Research (Shanghai) LLC (Shanghai, China). Male New Zealand white rabbits, weighing 1.79 to 2.95 kg, were used in the study. The following ocular components (aqueous humor, bulbar conjunctiva, palpebral conjunctiva, cornea, ciliary body/iris, and sclera) were collected after each animal had been euthanized by sodium pentobarbital overdose via injection in the marginal ear vein: euthanasia being confirmed by exsanguination via a femoral vein. One animal from each drug treatment group was sacrificed at 1, 2, 4, 8, and 24 h postdose. Whole tissues were harvested, excised, weighed, and then placed into one tube per tissue per animal. The samples, except the aqueous humor, were weighed, snap frozen in liquid

nitrogen, and then stored at -80°C until bioanalysis. The aqueous humor was placed on dry ice and stored at -80°C until bioanalysis.

Extra animals obtained for the study but not placed on study, were used for collection of untreated tissues (whole eye). The resulting untreated plasma and tissues were then applied to the development of the bioanalytical method and sample bioanalysis.

The liquid chromatography (LC) system comprised a Shimadzu (Shimadzu Corporation, Japan) liquid chromatograph equipped with a degasser (DGU-20A3), two pumps (LC-20AD), an autosampler (SIL-20AC), a communications bus module (CBM-20A), and a column oven (CTO-20A). Mass spectrometric (MS) analysis was performed using an API4000 (triple-quadrupole) instrument from Applied Biosystems/MDS Sciex with an ESI Ion source. The data acquisition and control system were created using Analyst 1.5.1 Software from Applied Biosystems/MDS Sciex. Other equipment included SI-A256 Vortex (Scientific Industries Inc.), Centrifuge 5417R (Eppendorf), and Millipore Academic Ultrapure-water generator system.

The LC/MS conditions were as follows. The chromatographic conditions were Thermo Hypersil GOLD, 5 μm , 50 mm $\times$ 2.1 mm, and the mobile phase in column A was 0.1% formic acid in water and in column B was 0.1% formic acid in methanol; the flow rate was 600 $\mu\text{L}/\text{min}$. The column temperature was 40°C , and injection volume was 5 μL .

Mass determination specifics were as follows: scan type, positive MRM; ion source, turbo spray; ionization model, ESI; nebulizer gas, 70 psi; gas, 2:70 psi; curtain gas, 14 psi; CAD, 8; ionspray voltage, 5500 v; temperature, 550°C .

Ocular tissues were homogenized in saline at a ratio of 1 g of tissue per 5 mL of saline. These were further diluted to a final concentration of 200 ng/mL. Seven calibration standards were

prepared by adding graded doses of drug solution to Eppendorf tubes containing whole rabbit eye homogenate. Seven calibration standards at concentrations of 5, 10, 50, 100, 500, 1000, or 5000 ng/g tissue were obtained. Quality control samples were prepared in brain homogenate to provide concentrations of 8, 800, 4000, and 10 000 ng/g tissue. Tolbutamide was used as an internal standard at a 200 ng/mL concentration. A sample without the internal standard was employed to exclude possible contamination. After a vortex of 1 min and centrifugation at 15 000 rpm $\times$ 5 min, the supernatants were individually transferred to 96 well plates. Chromatographic conditions showed that whole eye homogenates did not interfere with detection of the drugs under study or the internal standard. Linear regression analyses were obtained by plotting the peak area ratio of drug over the internal standard (*y* axis) vs the drug concentration ng/mL (*x* axis). The linear relationship between peak area ratio and drug concentration was demonstrated by the correlation coefficients obtained for the linear regression of the drug. An interassay accuracy of 90–119% for the quality control samples confirmed the reliability of the methods. The lower limits of quantification were set at 1 ng/g tissue.

Materials. AGN 211377 and AGN 225660 (Figure 1) were designed and custom synthesized by Selcia Ltd. (Fyfield, Essex, England). The NMR spectrum in for AGN 225660 is provided in Figure 8.

A short synthetic description of the last step with list of proton NMR peaks and M+1 found by LC–MS is provided as follows. To a solution of 3-{1-[5-chloro-2-(2-ethyl-butoxy)-benzyl]-5-methyl-1H-pyrazol-3-yl}-propionic acid methyl ester,¹¹ 2.05 g of (5.2 mmol) in THF (20 mL) and ethanol (10 mL) was added a solution of LiOH 1 g (41.7 mmol) in water (10 mL), and the resulting mixture was stirred at room temperature overnight. The mixture was acidified to pH 1 with 2 M HCl and extracted with EtOAc (50 mL). The organic extract was washed with brine (20 mL) and dried (MgSO₄). The volatiles were removed *in vacuo* to give AGN-225660 1.77 g (90%) as a white solid. ¹H-NMR (CDCl₃, 300 MHz) δ 7.18 (dd, 1H, ArH), 6.80 (d, 1H, ArH), 6.51 (d, 1H, ArH), 5.95 (s, 1H, ArH), 5.21 (s, 2H, ArCH₂), 3.90 (d, 2H, CH₂), 2.97 (dd, 2H, CH₂), 2.77 (dd, 2H, CH₂), 2.19 (s, 3H, CH₃), 1.71 (m, 1H, CH), 1.50 (m, 4H, CH₂), 0.96 (m, 6H, CH₃). LC–MS: *m/z* 379 M + H⁺. Purity of product > 95%.

PGD₂, PGE₂, carbaprostacyclin, 17-phenyl PGF_{2 α} , U-46619, BW 245C, and SQ 29548 were purchased from Cayman Chemical (Kalamazoo, MI, USA). The selective antagonists for DP₁ (BW A868C), EP₁ (SC-51322), EP₄ (GW627368X), and FP (AS604872) were synthesized at Target Molecules (Southampton, United Kingdom). Ketorolac 0.4% was used as Acular (Allergan Inc. Irvine, CA, USA).

AUTHOR INFORMATION

Corresponding Authors

David F. Woodward — Research and External Scientific Innovation, Allergan Inc., Irvine, California 92612, United States; orcid.org/0000-0002-3596-8578; Email: Poloso_neil@allergan.com

Neil J. Poloso — Research and External Scientific Innovation, Allergan Inc., Irvine, California 92612, United States; Email: david.woodward@imperial.ac.uk

Authors

Jenny W. Wang — Research and External Scientific Innovation, Allergan Inc., Irvine, California 92612, United States

Clayton S. Spada — Research and External Scientific Innovation, Allergan Inc., Irvine, California 92612, United States

Robert W. Carling — Discovery Department, Selcia Ltd., Ongar, Essex CM5 0GS, U.K.

Jose L. Martos — Discovery Department, Selcia Ltd., Ongar, Essex CM5 0GS, U.K.

Simon Pettit — Discovery Department, Selcia Ltd., Ongar, Essex CM5 0GS, U.K.

Jussi Kangasmetsa — Discovery Department, Selcia Ltd., Ongar, Essex CM5 0GS, U.K.

L. David Waterbury — Raven Biosolutions LLC, San Carlos, California 94070, United States

Matthew Lawrence — RxGen Inc., Hamden, Connecticut 06511, United States

Wenzheng Hu — RxGen Inc., Hamden, Connecticut 06511, United States

Complete contact information is available at:

<https://pubs.acs.org/10.1021/acsptscli.0c00118>

Author Contributions

Wrote manuscript: Woodward, Poloso, Lawrence. Research design: Woodward, Poloso, Wang, Spada, Carling, Pettit. Designed and synthesized chemical compounds: Carling, Martos, Pettit, Kangasmetsa. Designed and conducted cell experiments: Poloso, Wang. Designed and conducted or supervised animal experiments: Spada, Waterbury, Lawrence. Data analysis: Wang, Poloso, Woodward, Spada, Lawrence, Waterbury. Illustrations, Figures and design of rabbit experiments: Spada. Directors of program: Woodward, Carling, Poloso.

Notes

The authors declare the following competing financial interest(s): N. J. Poloso remains an Allergan Inc. employee.

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ABBREVIATIONS:

RANTES, regulated on activation normal T cell expressed and secreted; TNF α , tumor necrosis factor α ; LPS, lipopolysaccharide; IL, interleukin; COX, cyclo-oxygenase; PG, prostaglandin; PGI₂, prostacyclin; Tx, thromboxane; MCP-1, monocyte chemoattractant protein-1; MIP, macrophage inhibitory protein; ICAM, Intercellular Adhesion Molecule 1; CRT α 2, chemoattractant receptor homologous molecule expressed on T helper type 2 cells; M-CSF, monocyte colony-stimulating factor; FITC, fluorescein isothiocyanate; FLIPR, Fluorometric Imaging Plate Reader; DMSO, dimethyl sulfoxide; PBMC, peripheral blood mononuclear cells; MoDC, monocyte-derived dendritic cells; ENA-78, Epithelial-derived neutrophil-activating peptide 78; INF, interferon

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