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ORIGINAL ARTICLE

Therapeutic Effects of AF219 on Interstitial Cystitis/Bladder Pain Syndrome Induced by Cyclophosphamide or Water Avoidance Stress in Rats

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Abstract

Introduction and hypothesis To evaluate the effect of AF219, a P2X3 receptor antagonist, in animal models of interstitial cystitis/bladder pain syndrome (IC/BPS) induced by cyclophosphamide (CYP) or water avoidance stress (WAS).

Methods Thirty-two adult female Wistar albino rats were used in each IC/BPS model. Assessment of nociception and anxiety and severity of inflammation in the bladder were assessed by behavioral experiments and histopathological examinations respectively. The contraction responses of the bladder were evaluated in vitro and protein levels of P2X3, P2X7, Trk-A, TRPV1, and TRPA1 were analyzed by Western blot.

Results The IC/BPS groups had shorter response times to noxious stimuli, exhibited more anxiety-like behavior, had higher inflammation-based histological scores, and showed greater increased contraction responses to carbachol, adenosine triphosphate, and electrical field stimulation in in vitro bladder strips than controls for both models ($p < 0.05$). The improvements in behavioral and bladder contraction responses and inflammation scores in the IC/BPS + AF219 groups were similar to control findings ($p > 0.05$). Exposure to WAS or CYP increased P2X3 expression in the bladder compared with the controls ($p < 0.05$). Apart from TRPA1, the levels of P2X7, Trk-A, and TRPV1 were also higher in the IC/BPS groups than in the controls ($p < 0.05$). No significant differences were observed between IC/BPS + AF219 and controls regarding P2X3, P2X7, Trk-A, and TRPV1 in the WAS model ($p > 0.05$). Moreover, P2X3 and P2X7 levels were significantly lower in IC/BPS + AF219 than in the AF219-untreated WAS model ($p < 0.05$).

Conclusions These findings suggest that P2X3 receptors play a significant role in bladder functional responses, nociception, and also the pathogenesis of IC/BPS. AF219 may be a promising therapeutic strategy for IC/BPS. Comparing AF219 with current IC/BPS treatment agents in future studies may yield valuable insights into its efficacy.

Keywords AF219 · Bladder pain syndrome · Interstitial cystitis · P2X3 · Therapeutic efficacy

Introduction

Interstitial cystitis/bladder pain syndrome (IC/BPS) is a symptom complex in which unpleasant bladder-related sensations, such as pain, pressure, and discomfort, are perceived and accompanied by lower urinary tract symptoms [1]. Diagnosing the condition can be difficult owing to variable presentation, and a lack of a clear understanding of the mechanism of IC/BPS may lead to ineffective treatment. Moreover, the prevalence of depression and anxiety has been reported to be significantly higher in IC/BPS, and high-psychological stress levels were associated with an aggravation of bladder symptoms [2, 3]. Recent theoretical developments have shown that the sensitivity of

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the bladder afferent pathways to physiological stimuli is a crucial component of the underlying mechanism of IC/BPS. As a result of the hyperactivity and mechanosensitive of C-fiber afferents, bladder distention may induce the sensation of pain. Chemical interactions between urothelial cells, afferent nerves, and myofibroblasts in the bladder and the common receptors expressed on them may cause changes in the expression of inflammation-related proteins/receptors in bladder afferent pathways. These changes can lead to afferent neuroplasticity, resulting in pain symptoms in IC/BPS [4, 5]. The urothelium has become a primary focus for several lower urinary pathological conditions, including IC/BPS, because of its multifunctionality, including its role as a barrier as well as acting as a sensory organ by transmitting physical and chemical stresses to the relevant afferent nervous system and underlying smooth muscles.

The urothelium is a coordination center for communication between bladder functional components, linking neuron terminals, detrusor, and interstitial cells bordering the basal surface [6]. In response to an increase in bladder distension, adenosine triphosphate (ATP) released from the urothelium causes an increase in intracellular Ca^{2+} through its natural ligands, the ionotropic P2X3 and heterotypic $P2X_{2/3}$ purinoceptors. Thus, ATP mediates the local sensation of bladder filling as a component of sensory transmission and plays a crucial role in the physiology of the lower urinary tract [7, 8]. Additionally, in patients with IC/BPS, there is a more significant increase in stretch-mediated ATP release and P2X3 expression in the urothelium compared with controls. This excessive upregulation of P2X3 and increase in ATP signaling have been linked to bladder sensitivity and pain in IC/BPS [9]. Therefore, P2X3 receptors (P2X3Rs) expressed on the urothelium and neuron terminals may be important in IC/BPS.

The modification of ATP, a natural ligand, led to the discovery of TNP-ATP, a potent but nonselective P2X3R antagonist. A-317491 was the first selective and competitive P2X3R antagonist, but its polar structure resulted in poor oral bioavailability. As a result, the AF219 molecule, a highly selective allosteric antagonist for the P2X3R with high oral availability from diaminopyrimidine derivatives, was developed. In this study targeting the purinergic pathway via P2X3Rs, AF219 was used as the treatment agent owing to its superior properties over other P2X3R antagonists [10].

The present study investigated whether the level of P2X3 increased and whether any increased expression was associated with pathological findings in two animal models of IC/BPS, water avoidance stress (WAS) or cyclophosphamide (CYP) exposure. In addition, the effect of AF219, a P2X3R antagonist, was investigated in terms of pathological findings in these IC/BPS models.

Materials and Methods

Experimental Animals

Sixty-four young-adult female Wistar albino rats (10–12 weeks old, weighing 200–250 g) were obtained from the Kocaeli University Experimental Medicine Research Center. We chose these experimental animals with characteristics that reflect the disease epidemiology and the characteristics of IC/BPS patients and are commonly used in IC/BPS animal models. Animals were housed in a controlled ambient temperature of $22 \pm 2°C$ and relative humidity at $55 \pm 10\%$ on a 12-h dark–light cycle with free access to food and water. All procedures were conducted under the Guide for the Care and Use of Laboratory Animals of the European Council Directive (2010/63/EU) and were approved by the Animal Research Ethics Committee of Kocaeli University (Approval Number: KOU-HADYEK.5/1–2021).

Experimental Design

IC/BPS is complex with distinct subgroups or phenotypes with unique pathophysiology and treatment responses. Therefore, two different animal models with distinct mechanisms were selected for this preclinical therapeutic evaluation instead of a single model that reflects a particular patient group. The two models commonly used in modeling IC/BPS are the bladder-centric model (CYP) and the psychological and physical stressors/natural model (WAS) [11]. In particular, the WAS is a model that the Multidisciplinary Approach to the Study of Chronic Pelvic Pain (MAPP) Research Network has frequently favored in recent preclinical studies of chronic pelvic pain because it accurately mimics findings in patients with IC/BPS [12]. Each IC/BPS model, CYP or WAS, used 32 rats. For both models, four groups were established: Control, Control + AF219, IC/BPS, and IC/BPS + AF219. Eight animals were assigned at random to each group. The weights of the animals were measured at the start and end of the experiment. The experimental design is shown in Fig. S1.

Model 1–IC/BPS Induced by CYP

Three doses of 75 mg/kg/ml of CYP were administered intraperitoneally to the IC/BPS and IC/BPS + AF219 groups every three days (on days 1, 4, and 7) as previously described [13]. Placebo treatment consisted of 1 ml of 0.9% NaCl administered intraperitoneally to the Control and Control + AF219 groups with the same dosing schedule.

Model 2–IC/BPS Induced by WAS

The WAS IC/BPS and IC/BPS + AF219 groups were exposed to WAS for 1 h/day for 10 consecutive days, as recently described [14]. Briefly, the rats were placed on a platform (10 × 8 × 8 cm) standing in the middle of plastic containers (30 × 25 × 40 cm) filled with water at room temperature up to 1 cm below the top of the platform. Rats in the Control and Control + AF219 groups were placed on the platform in waterless containers. The experiments were conducted between 10:00 a.m. and 12:00 a.m. to minimize circadian effects.

Administration of AF219

To our knowledge, AF219 has not previously been used as a therapeutic agent in any IC/BPS model. In a rat model of osteoarthritic pain, AF219 was given orally, and the optimal dose of 10 mg/kg was determined [15]. Thus, an identical oral dose was used in the current study. AF219 (MedChemExpress, Monmouth Junction, NJ, USA) was suspended in 1 ml of sodium carboxymethyl cellulose (CMC; Sigma-Aldrich, St. Louis, MO, USA) at 10 mg/kg. In both models, the Control + AF219 and IC/BPS + AF219 groups, were administered orally once daily for three days; on days 6, 7, and 8 for the CYP model and days 9, 10, and 11 for the WAS model. On the same day, 1 ml of CMC (vehicle) was orally administered to the Control and IC/BPS groups of both models. The administration of AF219 occurred at 9:00 a.m., and behavioral experiments began 1 h after the last dose was administered.

Behavioral Experiments

There is no direct method to measure pain in animals, so nociception was evaluated by observing their pain-like behavior. The anxiety-like consequences of chronic pain were measured using the open field and the elevated plus maze tests. A withdrawal response based on applying a noxious stimulus was measured by tail-flick and hot plate tests, which are stimulus-evoked methods. Also, locomotor scores were obtained by automatically analyzing locomotor activity to determine the effects of IC/BPS and AF219 on motor functions. Experiments on behavior were conducted on the 8th and 11th days in the CYP and WAS models respectively. Resting (lack of evidence of motor activity) for more than 50% of the locomotor activity test period was considered an exclusion criterion. Animals in this condition were excluded from behavioral experiments owing to the potential for inaccurate outcomes.

Locomotor Activity and Open Field Tests

Locomotor activity was evaluated with an animal activity monitoring system (May AMS 9701, Commat Ltd., Ankara, Turkey), including a plexiglass box (40 × 40 × 30 cm), a computer, and open-field activity software. The total locomotor activity was defined as the sum of the animals' vertical, ambulatory, and stereotypic movements for 5 min (Fig. S2A). For the open field test, video tracking software, Ethovision XT 11.5 (Noldus Information Technology, Leesburg, VA, USA), was used to analyze the movement times of rats in the central square (20 × 20 cm^2) and at the periphery for 5 min (Fig. S2B).

The Elevated Plus Maze Test

On the platform consisting of two open arms, two closed arms, and a central area (EMP01-R/M, Commat Ltd.) the same video tracking system identified and calculated the time that animals placed in the central square spent in the arms for 5 min (Fig. S2C).

Tail-flick Test

A radiant heat device (May TF 211-C1, Commat Ltd.) was applied to the tail through a focused light beam to evaluate the response to noxious stimuli. On both day 0 (baseline) and the day of the behavioral experiments, the time to tail-flick (latency) was measured three times for each animal. The percentage of latency relative to baseline was calculated (Fig. S2D).

Hot Plate Test

The rats were placed on a metal surface maintained at a constant temperature of 54 ± 2°C inside a plexiglass cylinder (May AHP 9601, Commat Ltd.). The withdrawal of the hind paw was considered an indicator of nociception. The evaluation was conducted on day 0 and on the day of the behavioral experiments. The response latency, which is the time taken to observe nociceptive behavior, was determined by a blinded researcher by reviewing video records (Fig. S2E).

Tissue Collection and Preparation

The animals were sacrificed under ketamine 50 mg/kg and xylazine 5 mg/kg anesthesia, and the bladder was removed through a midline abdominal incision. The tissue was weighed and isolated in Krebs solution. Then, it was opened from the base to the dome to form a flat plate. Each lateral segment, one of which corresponds to approximately 20% of the plate, was dissected and randomly allocated for histological evaluation and molecular experiments. The remaining bladder strip was used in isolated tissue bath studies.

Isolated Tissue Bath Studies

The bladder strips were placed in 20-ml tissue bath chambers containing Krebs solution at pH 7.4, aerated with carbogen and maintained at 37°C. The strips were washed every 15 min and the baseline tension was adjusted to 1 g after each wash. Isometric force measurements were recorded with a force transducer (May FDT-10A, Commat Ltd.) connected to a data acquisition system (MP 30; Biopac Systems, Santa Barbara, CA, USA) and software (BSL Pro 3.7; Biopac Systems). Tissue viability was tested by adding KCl (80 mM) directly to the bath for 5 min or until a plateau response was reached.

The cholinergic and purinergic contraction responses were evaluated by cumulative concentration–response curves to varying concentrations of carbachol (CCh, 10^{-9}-10^{-4} M) and ATP (10^{-8}-10^{-4} M). The contraction responses induced by CCh or ATP were normalized to each strip's weight. Electrical field stimulation (EFS) was performed to obtain nerve-evoked contractions with a stimulator (May ST95PT, Commat Ltd.) utilizing wave pulse trains (50 V, 1 ms, 10 s) at varying frequencies (1–64 Hz) through platinum wire electrodes placed on opposite ends of the baths, as previously described [16]. In addition, nerve-evoked contractions were obtained in the presence of atropine (10^{-6} M) and debrisoquine (3×10^{-6} M) to prevent cholinergic and adrenergic mechanisms from contributing to neurotransmission. Finally, all tissues were incubated with AF219 (10^{-5} M) to determine the effects of purinergic P2X3R on EFS-induced contraction responses. The EFS-induced contraction responses were normalized to the weight of each strip. In experiments, nonresponsive tissues to standard pharmacological tests were excluded. CCh, ATP, atropine, and debrisoquine were acquired from Sigma-Aldrich (St. Louis, MO, USA).

Histological Evaluation

Tissue samples were fixed in 10% formalin for 24 h. The specimens were then mounted in paraffin blocks using standard procedures. Sections of 5 µm in thickness were produced and stained with hematoxylin–eosin (H&E). The sections were viewed and photographed using an Olympus BX50 light microscope (Olympus Life-Science, Tokyo, Japan). Histological examination was performed with an inflammation-based scoring system previously defined in an experimental cystitis model [17]. Criteria for histological evaluation of bladder inflammation are presented in Table S1.

Western Blot Experiments

The protein extraction reagent lysis buffer T-PER (Thermo Fisher Scientific, Waltham, MA, USA) containing 1×protease inhibitor cocktail (Pierce, Waltham, MA, USA) was added to each tissue pellet. The tissue pellets were homogenized using Next Advance homogenizer (Next Advance, Troy, NY, USA). Homogenates were centrifuged at 10,000×g and 4°C for 15 min. Then the supernatant was centrifuged at 15,000×g for a further 30 min to obtain protein extracts. Protein concentrations were determined by the modified Bradford method (Bradford Protein Assay; Bio-Rad, Hercules, CA, USA). An equal concentration of extract from each sample was pooled into eight groups in total. Utilizing the SDS-PAGE method, 25 µg of protein from each group pool was transferred to nitrocellulose membranes (GE Healthcare, Chicago, IL, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, SC-137179; Santa Cruz Biotechnology, Dallas, TX, USA) was used as the internal control protein for normalization experiments and membranes were subjected to Western blot analysis with anti-GAPDH antibody. The bands were visualized with high-sensitivity films using an electrochemiluminescence (ECL; Bio-Rad) detection system. Image-J software was used to calculate band intensities, and the protein concentrations in the protein pools were re-determined.

Equal concentrations of proteins were separated by molecular weight on 12% SDS-PAGE gel and transferred to the nitrocellulose membrane by a semi-dry transfer cell (Bio-Rad). The membrane was blocked for 1 h at room temperature in TBS-T (Tris–HCl 25 mM pH 7.2, NaCl 150 mM, and 0.1% Tween 20) containing 5% nonfat dry milk powder. After blocking and washing three times in TBS-T for 10 min, primary antibodies were applied. Primary antibodies were: P2X3 (SC-390572; Santa Cruz Biotechnology), P2X7 (SC-514962; Santa Cruz Biotechnology), Trk-A (MA5-32,123; Thermo Fisher Scientific), TRPV1 (NBP1-97,417; Novus Biologicals, Centennial, CO, USA), TRPA1 (NB110-40763; Novus Biologicals), and GAPDH (SC-137179; Santa Cruz Biotechnology). Antibodies were administered using appropriate dilutions. Then the membrane was washed three times with TBS-T and incubated with horseradish peroxidase labeled secondary antibodies at a dilution of 1:10,000 at room temperature for 1 h. The protein bands were visualized using ECL. Western blot experiments were repeated three times for each antibody. The image-J program was used for the relative quantitative evaluations of band intensities.

Statistical Analysis

Statistical analysis was performed and graphics were produced using Prism 6 software (GraphPad, San Diego, CA, USA). Power and sample size were determined using G*Power statistical software. The Shapiro–Wilk test was used to confirm that the data were distributed normally. The data are presented as mean ± standard deviations and were analyzed using a one-way ANOVA followed by Tukey's post hoc test for comparison. A p value <0.05 was considered to be statistically significant. The number of animals used is indicated in the legends.

Results

Evaluation of Nociception and Changes in Behavioral Responses

The outcomes of the groups' weight changes and behavioral experiments are shown in Tables 1, 2, 3, and 4. There was no significant difference in the baseline mean weights between the groups in the two models. Although all groups' end-of-experiment weights were lower than the respective baseline results, the animals in the IC/BPS groups experienced a more significant decrease ($p < 0.01$). There was no evidence of an adverse effect of AF219 and of CYP- and WAS-induced IC/BPS on motor functions based on total locomotor activity scores. Rats with induced IC/BPS by either method spent significantly less time in the central square than rats in the other groups, but there was no significant difference in the mean percentages of time spent in

Table 1 The outcomes of the weight changes of the groups in the cyclophosphamide model

Body weights	Control ($n=8$)	Control + AF219 ($n=8$)	IC/BPS ($n=8$)	IC/BPS + AF219 ($n=8$)	*p*
Baseline body weight (g)	229.75 ± 8.379	230.25 ± 9.036	227.88 ± 7.900	229.38 ± 8.879	0.951
Final body weight (g)	224.00 ± 9.289	225.13 ± 8.442	216.50 ± 11.747	218.63 ± 9.590	0.598
The change in body weight (%)	-2.51 ± 1.411	-2.21 ± 1.302	-5.04 ± 2.034**	-4.70 ± 1.119*	**< 0.001**

The data were presented as the mean ± SD

Data were analyzed using a one-way ANOVA followed by Tukey's post hoc test for comparison

The symbols * and # represent IC/BPS or IC/BPS + AF219 vs Control and IC/BPS + AF219 vs IC/BPS respectively

The significance level was set at $p < 0.05$ (* or #), $p < 0.01$ (** or ##), and $p < 0.001$ (*** or ###)

The bold *p* values indicate $p < 0.05$, that is, a statistically significant difference

Table 2 The outcomes of the behavioral experiments of the groups in the cyclophosphamide model

Behavioral responses	Control ($n=7$)	Control + AF219 ($n=7$)	IC/BPS ($n=7$)	IC/BPS + AF219 ($n=7$)	*p*
Total locomotor activity scores	1,085.29 ± 94.615	1,062.73 ± 111.645	998.00 ± 75.193	1,045.14 ± 131.374	0.472
The mean percentage of time spent in the central square	4.39 ± 1.131	4.26 ± 1.080	2.72 ± 0.627*	3.79 ± 1.405	**0.036**
The mean percentage of time spent in the open arms	13.48 ± 3.221	13.53 ± 4.087	7.89 ± 1.910*	10.93 ± 4.847	**0.026**
The mean percentage of changes in tail-flick withdrawal latency	4.36 ± 3.051	6.25 ± 6.796	-5.15 ± 2.743**	6.36 ± 10.120 ##	**0.007**

The data were presented as the mean ± SD

Data were analyzed using a one-way ANOVA followed by Tukey's post hoc test for comparison

The symbols * and # represent IC/BPS or IC/BPS + AF219 vs Control and IC/BPS + AF219 vs IC/BPS respectively

The significance level was set at $p < 0.05$ (* or #), $p < 0.01$ (** or ##), and $p < 0.001$ (*** or ###)

The bold *p* values indicate $p < 0.05$, that is, a statistically significant difference

Table 3 The outcomes of the weight changes of the groups in the water avoidance stress model

Body weights	Control ($n=8$)	Control + AF219 ($n=8$)	IC/BPS ($n=8$)	IC/BPS + AF219 ($n=8$)	*p*
Baseline body weights (g)	229.13 ± 13.293	223.88 ± 11.470	234.88 ± 12.873	227.88 ± 11.532	0.455
Final body weights (g)	223.75 ± 16.456	217.75 ± 11.720	221.50 ± 11.796	220.38 ± 11.600	0.831
The change of body weights (%)	-2.42 ± 2.069	-2.74 ± 1.274	-5.32 ± 1.792*	-3.30 ± 0.830	**0.004**

The data were presented as the mean ± SD

Data were analyzed using a one-way ANOVA followed by Tukey's post hoc test for comparison

The symbols * and # represent IC/BPS or IC/BPS + AF219 vs Control and IC/BPS + AF219 vs IC/BPS respectively

The significance level was set at $p < 0.05$ (* or #), $p < 0.01$ (** or ##), and $p < 0.001$ (*** or ###)

The bold *p* values indicate $p < 0.05$, that is, a statistically significant difference

Table 4 The outcomes of the behavioral experiments of the groups in the water avoidance stress model

Behavioral responses	Control ($n=7$)	Control + AF219 ($n=7$)	IC/BPS ($n=7$)	IC/BPS + AF219 ($n=7$)	p
Total locomotor activity scores	1,082.86 ± 76.443	1,072.71 ± 73.373	1,098.57 ± 70.595	1,110.14 ± 75.959	0.789
The mean percentages of time spent in the central square	4.76 ± 0.956	4.57 ± 0.787	3.18 ± 0.624**	3.82 ± 0.527	**0.002**
The mean percentage of time spent in the open arms	13.55 ± 3.927	13.70 ± 2.530	7.71 ± 3.548*	11.12 ± 3.993	**0.018**
The mean percentage of changes in tail-flick withdrawal latency	2.17 ± 6.302	-7.59 ± 2.307	-19.75 ± 6.801**	-7.93 ± 0.839#	**< 0.001**
The mean percentage of changes in hind paw withdrawal latency	-1.03 ± 1.655	8.83 ± 2.385	-13.71 ± 1.390**	4.92 ± 9.157##	**< 0.001**

The data were presented as the mean ± SD

Data were analyzed using a one-way ANOVA followed by Tukey's post hoc test for comparison

The symbols * and # represent IC/BPS or IC/BPS + AF219 vs Control and IC/BPS + AF219 vs IC/BPS respectively

The significance level was set at $p<0.05$ (* or #), $p<0.01$ (** or ##), and $p<0.001$ (*** or ###)

The bold p values indicate $p<0.05$, that is, a statistically significant difference

the central square between the IC/BPS + AF219 and control groups. In the elevated plus maze test, the mean percentage of time spent in the open arms was significantly lower in the IC/BPS groups of both models, whereas again there was no difference between the IC/BPS + AF219 groups and the controls. When the tail-flick and hind paw withdrawal latencies were compared with baseline, the response times were slower in the IC/BPS groups of both models compared with the other groups ($p<0.01$). Furthermore, the percentage change in latency was significantly greater in the IC/BPS + AF219 groups than in the respective IC/BPS groups ($p<0.05$), but there was no difference between the IC/BPS + AF219 and control groups.

Contraction Responses of In Vitro Bladder Smooth Muscle

There was no difference in the KCl-induced contraction forces between the groups in either model ($p=0.628$ and $p=0.430$ for CYP and WAS models respectively). When incubated with CCh, in the IC/BPS groups, increased contraction responses were greater than for controls, beginning at a concentration of 10^{-6} M for the CYP model and 10^{-7} M for the WAS model. The increase in the contraction responses of the IC/BPS + AF219 groups was significantly less than in the IC/BPS groups (Fig. 1A, B). At cumulative concentrations of ATP, more increased contraction responses were observed in the IC/BPS groups than in the control groups, starting from 10^{-7} M ATP concentrations for both models. The increase in contraction responses of the IC/BPS + AF219 groups was significantly lower than in the IC/BPS groups but did not differ from controls (Fig. 1C, D). In the IC/BPS groups, more increased contraction responses were observed at all EFS frequency ranges for both models, whereas the increase in contraction responses of the IC/BPS + AF219 groups was less than that of the IC/BPS groups. However, there was no significant difference in contraction responses between the IC/BPS + AF219 and control groups at increasing frequency ranges in both models (Fig. 1E, F). In addition, when the bladder strips from all groups from both models were incubated with 10^{-5} M AF219, no significant difference was observed between the groups regarding contraction responses (Fig. 1G, H).

Inflammation-based Histological Scores

The weights of bladder tissues from the IC/BPS groups and the ratio of these weights to body weights were significantly higher ($p<0.05$, Fig. 2A, B) than in the controls. In addition, both models' IC/BPS groups had higher inflammation-based histological scores than the other groups ($p<0.05$, Fig. 2C, D). Although the scores decreased in the IC/BPS + AF219 groups, this decrease was significant only for the WAS model (for CYP, 1.63 ± 0.52 vs 2.00 ± 0.00, $p=0.114$; for WAS, 1.75 ± 071 vs 2.63 ± 0.52, $p=0.025$). In both models, there were no significant differences in histological scores between the IC/BPS + AF219 and control groups.

Protein Levels Determined by Western Blot

Levels of the proteins P2X3, P2X7, Trk-A, and TRPV1 were significantly higher in both CYP and WAS IC/BPS groups when compared with their respective control groups. There was no significant difference in TRPA1 levels between groups in either model ($F(3, 8)=[1.410]$, $p=0.309$ for the CYP model, $F(3, 8)=[1.761]$, $p=0.232$ for the WAS model). In the CYP model, P2X3, Trk-A, and TRPV1 protein levels were significantly higher in the IC/

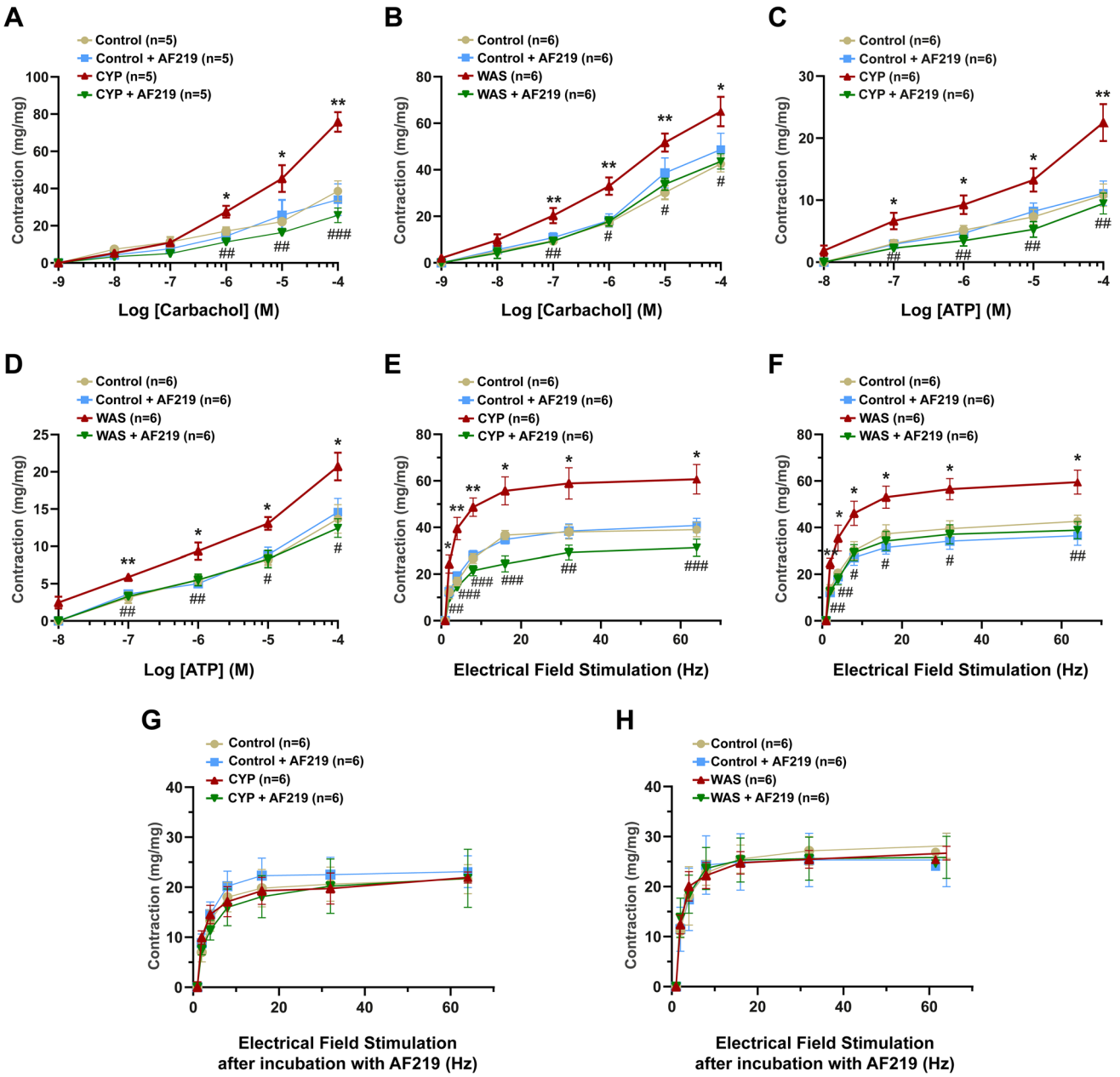


Fig. 1 The in vitro contraction responses of bladder smooth muscles from the cyclophosphamide (*CYP*) and the water avoidance stress (*WAS*) model groups are illustrated in **A, C, E, G** and **B, D, F, H)** respectively. Cumulative concentration–response curves are shown for carbachol in **A, B** and adenosine triphosphate (*ATP*) in **C, D**. The contraction-response curves induced by electrical field stimulation (*EFS*) are shown in **E, F**, and contraction-response curves induced by EFS after incubation with AF219 are shown in **G, H**. The data are presented as the mean ± SEM and were analyzed using a one-way ANOVA followed by Tukey's post hoc test for comparison. The *asterisks* and *hash symbols* represent IC/BPS or IC/BPS + AF219 vs Control and IC/BPS + AF219 vs IC/BPS respectively. The significance level was set at $p < 0.05$ (*asterisk* or *hash symbol*), $p < 0.01$ (*two asterisks* or *two hash symbols*), and $p < 0.001$ (*three asterisks* or *three hash symbols*). The term IC/BPS refers to the WAS and CYP groups

BPS + AF219 group than in the control group ($p < 0.05$), whereas no difference was observed in terms of P2X7 ($p > 0.05$). In contrast, in the WAS model, no significant difference was observed between the IC/BPS + AF219 group and the control group in terms of P2X3, P2X7, Trk-A, and TRPV1 levels. Furthermore, in the WAS model, the P2X3 and P2X7 levels were significantly lower in the IC/BPS + AF219 group than in the IC/BPS group. Figure 3 shows protein quantification bar chart results normalized by GAPDH and the gel images.

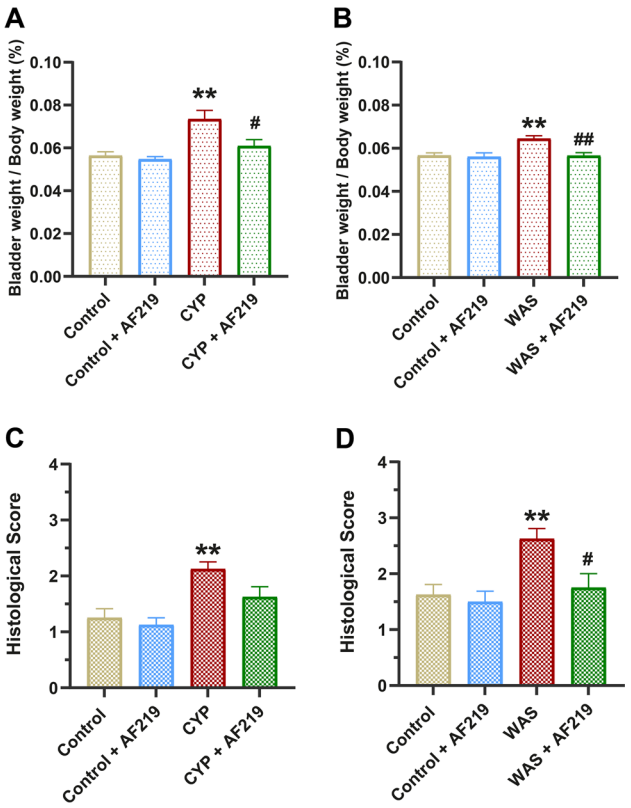


Fig. 2 The results of the comparison between the groups regarding the bladder-to-body weight ratios and inflammation-based histological scores are shown in **A, B** and **C, D**. The data are presented as the mean ± SEM and were analyzed using a one-way ANOVA with Tukey' post hoc test for comparison. The *asterisks* and *hash symbols* represent IC/BPS or IC/BPS + AF219 vs Control and IC/BPS + AF219 vs IC/BPS respectively. The significance level was set at $p < 0.05$ (*asterisk* or *hash symbol*), $p < 0.01$ (*two asterisks* or *two hash symbols*), and $p < 0.001$ (*three asterisks* or *three hash symbols*). The term IC/BPS refers to the WAS and CYP groups

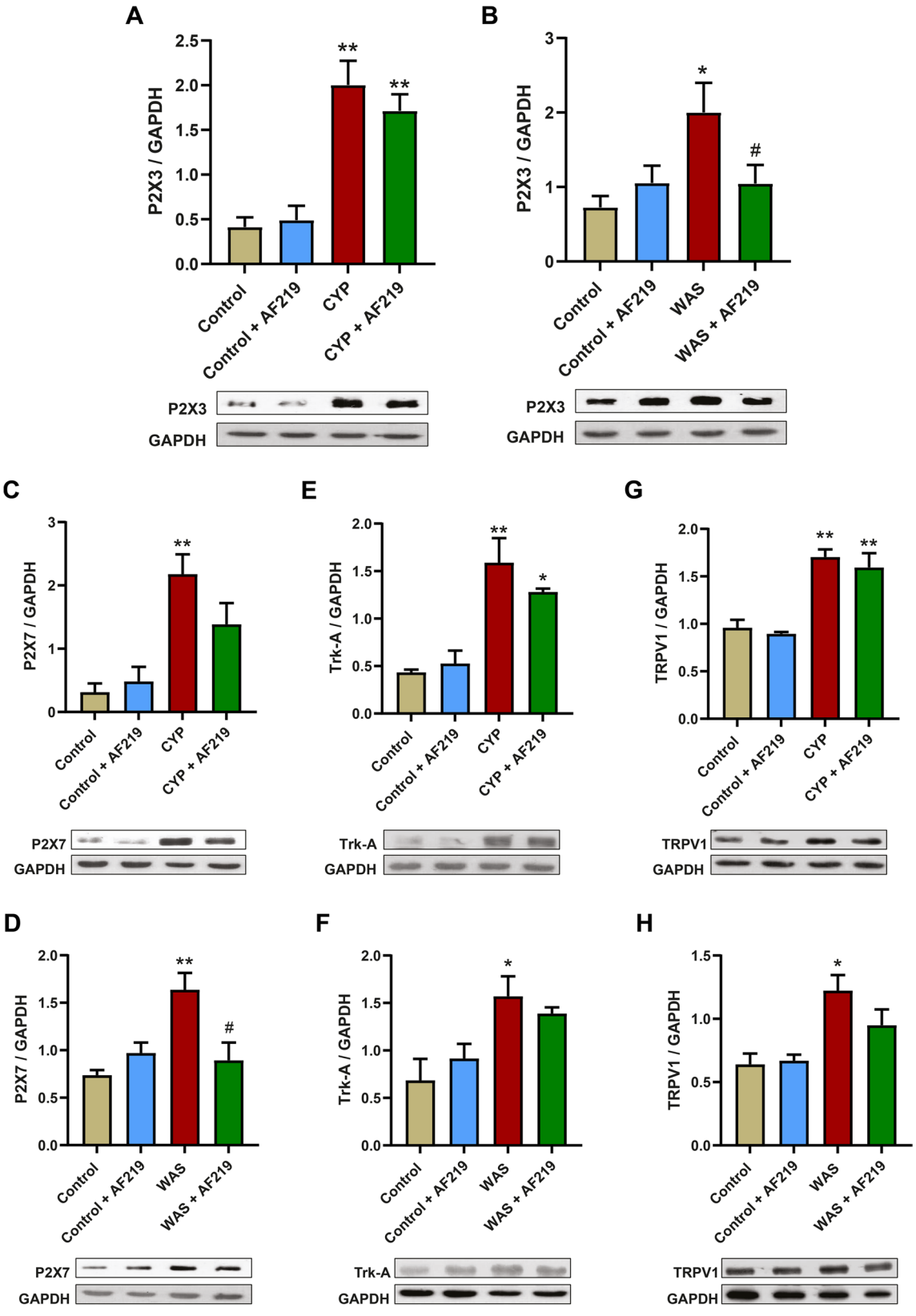
Fig. 3 The protein quantification results and the gel images from the cyclophosphamide (*CYP*) and the water avoidance stress (*WAS*) model groups are illustrated in **A, C, E, G** and **B, D, F, H** respectively. Comparisons of protein levels between groups are shown for P2X3 in **A, B**, P2X7 in **C, D**, Trk-A in **E, F**, and TRPV1 in **G, H**. The data are presented as mean ± SEM and were analyzed using a one-way ANOVA with Tukey's post hoc test for comparison. The *asterisks* and *hash symbol* represent IC/BPS or IC/BPS + AF219 vs Control and IC/BPS + AF219 vs IC/BPS respectively. The significance level was set at $p < 0.05$ (*asterisk* or *hash symbol*), $p < 0.01$ (*two asterisks* or *two hash symbols*), and $p < 0.001$ (*three asterisks* or *three hash symbols*). The term IC/BPS refers to the WAS and CYP groups

Discussion

The structure of the urothelium allows expansion and contraction, adjustment to tissue changes, and regulation of bladder sensation and contractions, while also serving as a barrier [18]. Recent research indicates that IC/BPS may be caused by peripheral and central sensitization, with stress affecting this mechanism [19]. The present study used two IC/BPS models, CYP-IC/BPS and WAS-IC/BPS, to better understand the etiopathology of IC/BPS and to identify potential treatment targets. The study also investigated the relationship between P2X3Rs and expression levels in IC/BPS, bladder function, and changes in nociceptive responses. For the first time, this preclinical study also evaluated the effect of AF219, a new-generation P2X3R antagonist, at a therapeutic dose in two different IC/BPS animal models.

Several tests were performed to evaluate pain-related behavior patterns and locomotor activity was assessed to determine the impact of IC/BPS and AF219. Our analysis revealed that exposure to CYP and WAS and treatment with AF219 did not have any negative effect on locomotor activity. Previous reports showed that rats with elevated anxiety levels spent less time in the central square during the open field test and more time in the closed arms during the elevated plus maze test [20, 21]. In the present study, rats with CYP- and WAS-induced IC/BPS showed increased anxiety-like behaviors. This suggested that pain may play a role in the development of anxiety, as evidenced by similar behavioral responses in the CYP model. In terms of hyperalgesia, assessed by the time to tail-flick and hind-paw withdrawal tests, the IC/BPS groups had the shortest latency times in both tests for both models. In a study on the effects of chronic stress on rats, it was reported that exposure to chronic stress was associated with shorter reaction times than controls in the hot plate test, suggesting that chronic stress might cause hypersensitivity to painful stimuli [22]. In the current study, CYP- and WAS-induced IC/BPS had a hyperalgesic effect on rats, which was reversed by AF219, suggesting that the P2X3 pathway might play a role in the pathogenesis of this hyperalgesia in IC/BPS. AF219 did not appear to convey any analgesic effect. A possible mechanism for this may be a decrease in the post-treatment activity of the purinergic pathway rather than a direct analgesic effect of AF219.

To assess the effects of IC/BPS on bladder functional response, bladder smooth muscle strips were tested in vitro. The bladders of rats with IC/BPS had increased contraction responses to cholinergic-CCh and purinergic-ATP compared with the control groups in both models, indicating that both pathways are active in IC/BPS. We also observed a potential regulation effect of AF219 on the cholinergic pathway, as the cholinergic and purinergic responses in the IC/BPS + AF219 groups were similar to those in controls and were significantly different from those of the IC/BPS groups. Moreover, the detrusor may be more sensitive to cholinergic and purinergic stimuli via the ATP-P2X3 pathway, as the nerve-evoked contraction responses of the IC/BPS groups were increased in cholinergic and adrenergic blocking but not after co-treatment with AF219. In a study looking at


A
P2X3 / GAPDH
2.5
2.0
1.5
1.0
0.5
0.0
**
**
Control
Control + AF219
CYP
CYP + AF219
P2X3
GAPDH
B
P2X3 / GAPDH
3
2
1
0
*
#
Control
Control + AF219
WAS
WAS + AF219
P2X3
GAPDH
C
P2X7 / GAPDH
3
2
1
0
**
Control
Control + AF219
CYP
CYP + AF219
P2X7
GAPDH
E
Trk-A / GAPDH
2.0
1.5
1.0
0.5
0.0
**
*
Control
Control + AF219
CYP
CYP + AF219
Trk-A
GAPDH
G
TRPV1 / GAPDH
2.0
1.5
1.0
0.5
0.0
**
**
Control
Control + AF219
CYP
CYP + AF219
TRPV1
GAPDH
D
P2X7 / GAPDH
2.0
1.5
1.0
0.5
0.0
**
#
Control
Control + AF219
WAS
WAS + AF219
P2X7
GAPDH
F
Trk-A / GAPDH
2.0
1.5
1.0
0.5
0.0
*
Control
Control + AF219
WAS
WAS + AF219
Trk-A
GAPDH
H
TRPV1 / GAPDH
1.5
1.0
0.5
0.0
*
Control
Control + AF219
WAS
WAS + AF219
TRPV1
GAPDH

the effects of P2X3Rs on detrusor contractions, contraction responses were evaluated in cystometric and in vitro bladder strips by administering a P2X3R antagonist (AF353) intravesically to groups with and without urothelium. Removing the urothelial layer prevented the P2X3R antagonist from increasing the contraction range and decreasing the contraction amplitude. The absence of urothelium did not affect contraction responses to KCl or EFS, but inhibition of the P2X3R decreased contraction responses to EFS in bladder strips containing only urothelium. It was concluded that alterations in ATP release through P2X3Rs on the urothelium might increase sensitivity to P2X1-mediated detrusor contractions [23]. Based on comparable results, the results of the present study suggest that the activated P2X3 pathway might increase ATP release from both the urothelium and nerve terminals innervating the detrusor, which may lead to increased activation of P2X1 and cholinergic receptors on the detrusor. We also speculate that AF219 might have a modulatory effect on the cholinergic pathway and the potential to improve bladder function by preventing the increase in ATP release from the urothelium and sensitization of the detrusor to this pathway.

Based on previous results using both models, it was shown that IC/BPS causes histological changes in the bladder, including edema, inflammatory infiltration, congestion, and urothelial erosion. In this study, bladder changes were assessed using an inflammation-based scoring system [17]. The results of the present study showed that the bladder weights and ratios to body weights were greater in the IC/BPS groups in both models, whereas the addition of AF219 produced bladder weights and ratios similar to those of controls. Our results indicate that we successfully induced IC/BPS as the scores were higher in the IC/BPS groups than in the control groups, consistent with previous research [24–26]. Furthermore, AF219 alleviated the inflammation aggravated by IC/BPS as the scores were similar to those of the controls in the IC/BPS + AF219 groups.

The role of P2X3, P2X7, Trk-A, TRPV1, and TRPA1 located on the urothelium and bladder afferent nerve terminals in the pathogenesis of acute cystitis (single high-dose CYP) and IC/BPS (repeated low-dose CYP) has been investigated in animal models. These studies have demonstrated that the upregulation of these proteins was associated with the function and histology of the bladder and nociception alterations [27–31]. Although exposure to CYP in a single dose can lead to bladder inflammation in animals, it has limitations in mimicking the full range of symptoms associated with IC/BPS. Changes in receptor expression vary depending on whether the exposure is acute or chronic. In a study examining the effects of acute and chronic CYP exposure on TRPV1 expression in the bladder, it was shown that a single dose of CYP administration did not affect the TRPV1 expression level, whereas repeated doses of CYP caused overexpression of this receptor [32]. The present study is the first to evaluate the levels of all these proteins in WAS in addition to IC/BPS induced by repeated low-dose CYP. In both models, IC/BPS increased the expression of P2X3, P2X7, Trk-A, and TRPV1 proteins in the bladder. In a study examining the therapeutic effect of estrogen on IC/BPS, P2X3R was upregulated in animals with IC/BPS and that estrogen improved bladder overactivity by downregulating this pathway [27]. It was also reported that the bladder contractility/excitability potential was directly proportional to the P2X3R expression level, suggesting that our study's results were consistent with those in the literature.

In the present study, the decrease in these proteins after AF219 administration was significant only in the WAS-IC/BPS model, possibly because CYP causes more damage to the bladder than intended, or the number of days treated with AF219 was insufficient. However, P2X7 levels, which were significantly elevated in the IC/BPS groups in both models, were significantly lower in the IC/BPS + AF219 groups. A higher level of P2X7 protein was detected in the bladders of mice with single-dose CYP-induced acute cystitis, compared with controls, and it was concluded that bladder inflammation regressed through inhibition of the P2X7R. It was proposed that the primary mechanism in cystitis-induced bladder inflammation might be increased activation of the ATP-P2X7 pathway [28]. However, P2X7Rs, unlike other purinergic receptors, are activated by higher concentrations of ATP. For the expression of P2X7Rs to increase, ATP in the environment must reach very high levels [33]. In the present study, AF219 treatment did not significantly reduce P2X3R expression in CYP-induced IC/BPS, in contrast to the WAS-induced IC/BPS group. However, the IC/BPS + AF219 groups of both models had inflammation scores that were statistically similar to those of controls. Thus, AF219 may have indirectly decreased the expression of P2X7Rs because AF219 decreased ATP release below the threshold for increased P2X7R expression in the urothelium and afferent nerve terminals.

In conclusion, the results of the present study strongly suggested that P2X3Rs play a significant role in bladder functional responses, nociception, and the pathogenesis of IC/BPS. Furthermore, AF219 may be an effective treatment modality for patients with IC/BPS based on encouraging results in these animal models. In order to better understand the pathophysiology of IC/BPS and to determine prognostic factors and treatment targets, future preclinical and clinical studies on P2X3Rs and AF219 may clarify the pathogenesis of IC/BPS and the clinical utility of this agent in human disease.

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Authors' contributions I.E. Avci: project development, data collection, manuscript writing; K. Teke: project development, manuscript writing; S.S. Gocmez: project development, data analysis, manuscript writing; M. Kasap: project development, data analysis, manuscript editing; G. Akpinar: data collection, data analysis, manuscript writing; B.Yaprak Bayrak: data analysis, manuscript editing; G. Celebi: data collection, data analysis; M. Sarihan: data collection, data analysis; T. Utkan: project development, manuscript editing; C. Ozkurkcugil: project development, manuscript writing.

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Data Availability Data available on request from the authors.

Declarations

Conflicts of interest None.

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