Identification and Characterization of a Selective Allosteric Antagonist of Human P2X4 Receptor Channels

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Abstract

P2X4 is an ATP-gated nonselective cation channel highly permeable to calcium. There is increasing evidence that this homomeric purinoceptor, which is expressed in several neuronal and immune cell types, is involved in chronic pain and inflammation. The current paucity of unambiguous pharmacological tools available to interrogate or modulate P2X4 function led us to pursue the search for selective antagonists. In the high-throughput screen of a compound library, we identified the phenylurea BX430 (1-(2,6-dibromo-4-isopropyl-phenyl)-3-(3-pyridyl)urea, molecular weight = 413), with antagonist properties on human P2X4-mediated calcium uptake. Patch-clamp electrophysiology confirmed direct inhibition of P2X4 currents by extracellular BX430, with submicromolar potency (IC50 = 0.54 μM). BX430 is highly selective, having virtually no functional impact on all other P2X subtypes, namely, P2X1–P2X3, P2X5, and P2X7, at 10–100 times its IC50.

Unexpected species differences were noticed, as BX430 is a potent antagonist of zebrafish P2X4 but has no effect on rat and mouse P2X4 orthologs. The concentration–response curve for ATP on human P2X4 in the presence of BX430 shows an insurmountable blockade, indicating a noncompetitive allosteric mechanism of action. Using a fluorescent dye uptake assay, we observed that BX430 also effectively suppresses ATP-evoked and ivermectin-potentiated membrane permeabilization induced by P2X4 pore dilation. Finally, in single-cell calcium imaging, we validated its selective inhibitory effects on native P2X4 channels at the surface of human THP-1 cells that were differentiated into macrophages. In summary, this ligand provides a novel molecular probe to assess the specific role of P2X4 in inflammatory and neuropathic conditions, where ATP signaling has been shown to be dysfunctional.

Introduction

The P2X receptor channels are ATP-gated cation channels with high calcium permeability (Jiang et al., 2003; Egan and Khakh, 2004; Khakh and North, 2012). They are formed by the assembly of three subunits belonging to a single gene family (P2X1–P2X7) in homomeric or heteromeric channels (Hattori and Gouaux, 2012; Saul et al., 2013). P2X channel subtypes display different sensitivities to ATP, with EC50 values ranging from nanomolar to submillimolar concentrations (North, 2002; Coddou et al., 2011). They are expressed in excitable as well as nonexcitable cells, where they control both membrane potential and intracellular calcium homeostasis, and increasing evidence points to their major involvement in sensory transduction, neuro-immune communication, and inflammatory response (Burnstock, 2011).

The homomeric P2X4 channel is widely expressed in central neurons, where its role remains to be fully elucidated (Rubio and Soto, 2001; Surprenant and North, 2009). This purinoceptor is also present at the surface of vascular endothelial cells, and knockout mice lacking P2X4 have deficits in shear stress–induced vasodilation, indicating a significant contribution to the regulation of vascular tone (Yamamoto et al., 2006). P2X4 is also expressed in monocytes, macrophages, and microglia, where it is involved in the release of inflammatory mediators (Bowler et al., 2003; Tsuda et al., 2003; Raouf et al., 2007; Ullmann et al., 2010). It is upregulated at the surface of spinal microglia following peripheral nerve injury, and P2X4-mediated brain-derived neurotrophic factor release from microglia has been shown to be a key step in the development of abnormal spinal excitability underlying tactile allodynia in conditions of chronic neuropathic pain (Trang et al., 2012; Tsuda et al., 2012).

Recent reports of the crystal structure of zebrafish P2X4 solved at high resolution in closed as well as ATP-bound open configurations (Kawate et al., 2009; Hattori and Gouaux, 2012) provided a breakthrough in the understanding of the structure-activity relationships that underlie gating mechanisms by specific nucleotides or ion selectivity of P2X receptor channels; however, the lack of potent and selective antagonists of P2X4 hinders conclusive investigations of its specific roles using pharmacological approaches in vitro and in vivo.

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ABBREVIATIONS: BBG, Brilliant Blue G; 5-BDBD, 5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]1,4-diazepin-2-one; BX430, HTS-03052, 1-(2,6-dibromo-4-isopropyl-phenyl)-9-(3-pyridyl)urea; BzATP, 2′(3′)-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate; HTS, high-throughput screening; PSB-12054, N-(benzoylcarbonyl)phenoxazine; PSB-12062, N-(p-methylphenyl)sulfonylphenoxazine; TNP-ATP, 2′,3′-O-(2,4,6-trinitrophenyl)adenosine-5′-triphosphate; YO-PRO1, 4′-[2′-([3′-methyl-1,3-benzoxazol-2′(3′H)-ylidene)methyl]-1′-[3′(trimethylammonio)propyl]quinolinium diiodide.
Nonselective antagonists for P2X4 include the extensively used nucleotide 2′,3′-O-(2,4,6-trinitrophenyl)adenosine-5′-triphosphate (TNP-ATP) with nanomolar potency for P2X1, P2X3, and P2X2+3 subtypes (Lewis et al., 1998; Virgínio et al., 1998), the dye Brilliant Blue G (BBG) with higher potency for P2X7 (Jiang et al., 2000), and the monoamine uptake blocker paroxetine with indirect inhibitory effects on the translocation of P2X4 to the cell surface (Nagata et al., 2009). The tricyclic antidepressant amitriptyline modestly inhibits rat and mouse P2X4 at 10 μM and has no effect on human P2X4 (Sim and North, 2010). Recently, the N-substituted phenoxazines PSB-12054 [N-(benzoylcarbonyl)phenoxazine] and PSB-12062 [N-[(methylphenyl)sulfonyl]phenoxazine] were identified as potent and noncompetitive, albeit poorly soluble, blockers of P2X4, with significant subtype selectivity (Hernandez-Olmos et al., 2012). The same group reported a carbamazepine derivative displaying the properties of a nonselective human P2X4 antagonist that also inhibits P2X1 and P2X3 subtypes (Tian et al., 2014). The benzodiazepine 5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-c]-1,4-diazepin-2-one (5-BDBD) has been described in a patent as a selective blocker of P2X4 channels with high potency, but its full characterization is not reported (Fisher et al., 2005). Competitive inhibitory effects of 5-BDBD on P2X4 appear to be similar in potency to that of TNP-ATP (Casati et al., 2011; Wu et al., 2011; Balázs et al., 2013), while its P2X subtype selectivity remains unknown.

The current paucity of pharmacological tools available to interrogate and modulate P2X4 function led us to pursue the search for small-molecule compounds capable of selectively inhibiting P2X4 receptor channels. Here, we report the results of a high-throughput screening (HTS) of a library of chemical compounds using an intracellular calcium readout on a stable human P2X4-expressing cell line, which allowed us to discover the phenylurea BX430 [1-(2,6-dibromo-4-isopropyl-phenyl)-3-(3-pyridyl)urea] with allosteric antagonist properties on P2X4 receptor channel activity. Our characterization of this novel potent and subtype-selective P2X4 blocker involves patch-clamp recording, fluorophore uptake assay, and single-cell calcium imaging in recombinant preparations as well as human monocytic THP-1 cells.

Materials and Methods

Cell Culture. HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium and 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA) containing penicillin and streptomycin. Stable HEK293 cells transfected with human His-tagged P2X4 channels (hP2X4-HEK293 cells) were a kind gift from Dr. R. Alan North (University of Manchester). This stable cell line was kept in Dulbecco’s modified Eagle’s medium/F12 (1:1) containing 10% fetal bovine serum and 100 units/ml of penicillin/streptomycin supplemented with G-418 (250 μg/ml) for selection. For selectivity studies, HEK293 cells were transiently cotransfected with mCherry and human P2X1–P2X6, P2X7, or P2X4 of mouse, rat, and zebrafish origin in a pCDNA3 vector. Transfected cells were used for electrophysiological recordings 48 hours after transfection.

High-Throughput Screening. To 384 well plates, 25 μl of extracellular fluid buffer was dispensed using WellMate (Thermo Fisher Scientific, Ottawa, ON, Canada). Library compounds were pipetted into the buffer using PlateMate Plus (Matrix Technologies, Maumee, OH) affixed with the FP3 pin tool to deliver 70 nl of compound to each well. The hP2X4-HEK293 cells were loaded with Fluo-2 AM high affinity buffer using WellMate Plus (Matrix Technologies, Maumee, OH) and incubated at 37°C, 5% CO2 and incubated at 37°C, 5% CO2 on a shaker. After incubating, cells were spun at 1300 rpm for 4 minutes to remove excess Fluo-2, and the pellet was resuspended in an extracellular fluid buffer. Cells were seeded at 10,000 cells/well in 384-well plates using WellMate. A 10-μl volume of 50% phosphate-buffered saline was dispensed to one control column of the plate using the Multidrop Combi 836 (Thermo Fisher Scientific) liquid dispenser. P2X4-mediated calcium entry was then stimulated by dispensing 10 μl of ATP to the remaining wells for a final ATP concentration of 100 μM, and plates were read again for Fluo-2–based intracellular calcium. A total of 26,240 compounds from the Canadian Chemical Biology Network library were initially screened at 7 μM for inhibition of 100 μM ATP-evoked P2X4-mediated calcium entry. Promising hits from several chemical families were selected for further pharmacological characterization in patch-clamp electrophysiology and single-cell ratiometric calcium imaging. The compound HTS-03052...
[1-(2,6-dibromo-4-isopropyl-phenyl)-3-(3-pyridyl)urea], soluble in dimethylsulfoxide, was purchased from Maybridge (Thermo Fisher Scientific) for confirmation of BX430 activity.

Ratiometric Measurement of Intracellular Calcium. Stable hP2X4-HEK293 and THP-1 cell lines were loaded with calcium-sensitive fluorescent dye Fura-2 AM (2.5 μM; Molecular Probes/Life Technologies, Burlington, ON, Canada) in Ringer’s solution containing (in mM) 140 NaCl, 5 KCl, 2 CaCl2, 2 MgCl2, 10 HEPES, and 10 glucose (pH 7.4) with 1% bovine serum albumin for 30–40 minutes at 37°C in the dark. Cells were washed twice to remove extracellular Fura-2 AM and left for at least 30 minutes for complete hydrolyzation of the acetoxymethyl ester before imaging. Fluorescence measurements of intracellular calcium in individual cells were performed on an inverted TE2000-U microscope (Nikon, Tokyo, Japan) with 40× oil immersion objective (CFI super(S) fluor; Nikon). Fura-2 was alternatively excited at 340 and 380 nm with a λ 10-2 filter wheel (Sutter Instruments, Novato, CA) coupled to a xenon arc lamp (XPS-100; Nikon). Fluorescence was monitored at 510 nm and recorded with a high-resolution cooled charge-coupled device camera (Cool Snap-HQ; Roper Scientific/Photometrics, Tucson, AZ). Pairs of 340- and 380-nm images were acquired every 2 seconds, and ratio images were calculated using MetaFluor 7.0 software (Molecular Devices, Sunnyvale, CA). Intracellular calcium measurement in 96-well black plates was performed on a Victor31420 Multilabel Plate Reader (Perkin Elmer, Waltham, MA), and the ratio of fluorescence intensity was measured (excitation, 491 nm; emission, 509 nm) following YO-PRO1 uptake were monitored in individual healthy cells initially impermeable to YO-PRO1 were selected for recording. Fluorescence changes (excitation, 491 nm; emission, 509 nm) following YO-PRO1 uptake were monitored in individual cells with a inverted TE2000-U microscope (Nikon) equipped with a 40× oil-immersion objective and coupled to a xenon arc lamp (XPS-100; Nikon). Images were captured every 10 seconds on a high-resolution cooled charge-coupled device camera (Cool Snap-HQ; Roper Scientific/Photometrics) and processed using MetaFluor 7.0 software (Molecular Devices). Each experiment was repeated at least three times.

Electrophysiology. Whole-cell patch-clamp recording of stable hP2X4-HEK293 cells and transiently transfected HEK293 cells (Vhada = −60 mV) was performed using pipettes filled with internal solution, pH 7.2, containing (in mM) 120 K-gluconate, 1 MgCl2, 5 EGTA, and 10 HEPES. The recording solution, pH 7.4, comprised (in mM) 140 NaCl, 5 KCl, 2 CaCl2, 2 MgCl2, 10 HEPES, and 10 glucose. Membrane currents were recorded using an Axopatch 200B amplifier and digitized at 500 Hz with a Digidata 1330 interface (Axon Instruments/Molecular Devices). Only recordings with a series resistance below 10 MΩ that were stable for the duration of the recording were considered for analysis. The liquid junction potential was calculated (Clampex; Axon Instruments/Molecular Devices) to be 3.7 mV and was not compensated. Drugs were dissolved in a recording solution and applied using a SF-77B fast perfusion system (Warner Instruments, Hamden, CT) at a rate of 1 ml/min. All experiments were performed at 22°C. For each individual experiment, current amplitudes or total currents (areas under the current traces) before and after drug treatment were compared and expressed as a percentage.

Statistical Analysis. Current amplitudes or ΔF/FO fluorescence values recorded during agonist application were measured, and their

![Fig. 2. Structure of BX430 and blockade of ATP-evoked calcium responses.](image)
average values ± S.E.M. were calculated for comparative analysis. Data are presented as mean ± S.E.M. unless indicated otherwise and analyzed using Student’s t test, two-tailed distribution, paired and nonpaired, or one-way analysis of variance and two-way analysis of variance followed by a Bonferroni’s multiple comparisons test. Dose-response data, including IC\textsubscript{50} or EC\textsubscript{50} values, were analyzed using GraphPad Prism (GraphPad Software Inc., San Diego, CA) by fitting data to the following logistic equations:

\[
Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{(1 + 10^{\left[\frac{\log IC_{50} - X}{\text{HillSlope}}\right]})}
\]

or

\[
Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{(1 + 10^{\left[\frac{\log EC_{50} - X}{\text{HillSlope}}\right]})}
\]

Results

Taking advantage of the high calcium permeability of human P2X4 channels expressed in a stable HEK293 cell line (Young et al., 2008), we defined experimental conditions yielding a robust signal-to-noise ratio for Fura-2–based fluorescence measurements of the rise of intracellular calcium levels evoked by ATP. As shown in Fig. 1A, ATP-evoked calcium responses were sensitive to TNP-ATP, a nonselective P2X4 antagonist, at 50 μM. Due to the expression of endogenous metabotropic ATP receptors, we treated the cells with 1 μM thapsigargin to...
deplete intracellular calcium stores and thus evaluate the contribution of non-P2X4 channel-mediated ATP responses. The amplitude of ATP-evoked responses showed only a small decrease following thapsigargin treatment (Fig. 1A), suggesting a minor contribution of Gq-coupled purinoceptors to calcium responses in the hP2X4-HEK293 cell line. Based on this experimental strategy, an HTS assay ($Z' = 0.59$; signal to noise ratio = 13) was designed to search for small organic compounds endowed with antagonistic properties toward human P2X4. Figure 1B illustrates the frequency distribution of the inhibitory and facilitatory effects of the 26,240-compound library from the Canadian Chemical Biology Network. A set of 30 candidate antagonists showing $>70\%$ inhibition (a threshold chosen visually based on the distribution of hits) and belonging to five distinct chemical families was identified (hit rate = 0.4%). Concentration-response curves were generated to confirm activity using compounds directly from the library plates. Compound 1-(2,6-dibromo-4-isopropyl-phenyl)-3-(3-pyridyl)urea (molecular weight = 413.1; $X\log P = 4.23$) showed superior potency, with an IC$_{50}$ of 0.85 $\mu$M (Fig. 1C). This compound was purchased commercially and retested in triplicate, yielding a similar IC$_{50}$ of 0.78 $\mu$M (Fig. 1C).

We selected this potent inhibitor (see structure in Fig. 2A), termed BX430, as an acronym of Blocker of P2X4 compound 3.0, for further characterization. We first tested it in single-cell calcium imaging with Fura-2 as a ratiometric fluorescence method on the hP2X4-HEK293 cells treated with thapsigargin to deplete the intracellular calcium store (Fig. 2B). ATP-evoked intracellular calcium responses from vehicle or thapsigargin were quite similar, confirming that contribution of the intracellular calcium store sensitive to ATP is minor in this cellular background. Human P2X4-expressing cells treated with thapsigargin plus BX430 showed a significant reduction in the intracellular calcium rise evoked by ATP (Fig. 2C). To verify if the treatment with 1 $\mu$M thapsigargin was sufficient to empty intracellular calcium stores, we challenged the hP2X4-HEK293 cells with the muscarinic agonist carbachol instead of ATP. Under this experimental condition, carbachol-evoked responses were strongly suppressed (data not shown). Moreover, no inhibitory effect of BX430 on the intracellular calcium response was observed in nontransfected HEK293 cells following ATP application, indicating that endogenous metabotropic ATP receptors, mainly the P2Y2 subtype in this background, are not sensitive to BX430 (data not shown).

Fig. 5. BX430 acts on P2X4 channels from the extracellular side. (A) BX430 (5 $\mu$M) showed a pronounced inhibitory effect on P2X4 channel currents in hP2X4-HEK293 cells when coapplied with ATP extracellularly without preincubation. The quantitative results on peak versus total current reflect the effect of BX430 blockade on desensitization kinetics. **$P < 0.01$ and ***$P < 0.001$ ($n = 5–6$) versus vehicle-treated cells. (B) When BX430 (5 $\mu$M) is applied intracellularly via the patch pipette, no change in the current amplitude was observed when the pipette solution is expected to have reached equilibrium with the intracellular milieu. (C) Fast antagonist effect of extracellular BX430 (5 $\mu$M) on ATP-evoked P2X4 current when applied during the phase of desensitization in representative control (1), test (2), and washout (3) conditions.
The calcium uptake measurements used as the basis of our screening readout can have limitations, including the detection of indirect artifactual effects of the tested compounds on ATP-sensitive pathways regulating intracellular calcium levels. Therefore, we performed patch-clamp recordings in the hP2X4-HEK293 cell line to document a direct antagonistic effect of BX430 on P2X4 receptor channels.

Figure 3A shows human P2X4 current responses following ATP application 2 minutes apart. The compound BX430 was then initially tested at a 5 μM concentration in preapplication and coapplication with the agonist ATP. BX430 potently inhibited ATP-evoked current responses, and P2X4 currents showed a complete recovery to baseline values after a washout period, indicating a reversible blockade (see also the quantitative results in the right panel of Fig. 3A). For comparison, we tested the competitive antagonist 5-BDBD under the same experimental conditions (Fisher et al., 2005; Balázs et al., 2013). Although 5-BDBD (10 μM) showed a slight decrease in the current amplitude evoked by ATP in the stable hP2X4-HEK293 cell line, the effect was not found significant (Fig. 3B; \(P = 0.218; n = 4–5\)). Therefore, BX430 is definitely more potent than 5-BDBD under the same experimental conditions (current amplitude in control = 90%; \(P < 0.05; n = 8\) versus current amplitude with BX430 = 10%; \(P < 0.001; n = 8\)).

We next assessed the potency (IC\(_{50}\)) of BX430 on the P2X4-mediated ATP-evoked currents in patch clamp recordings in hP2X4-HEK293 cells. The sigmoidal BX430 dose-response curves for inhibition of the maximal current amplitude and total current were fitted to the logistic equation, giving an IC\(_{50}\) of 0.55 μM, with a slope \(n_H\) of –1.00, and an IC\(_{50}\) of 0.54 μM, with a slope \(n_H\) of –0.86, respectively (Fig. 4, A and B). The compound BX430 binds to a single site, and the binding follows the law of mass action, as the time constant of P2X4 current decay is linearly related to its concentration, with an association rate of 0.96 μM\(^{-1}\) ms\(^{-1}\) and a dissociation rate of 0.53 ms\(^{-1}\) (\(R^2 = 0.95\); data not shown).

To further characterize the antagonist effect of BX430 on the P2X4 receptor channel response, we asked if the compound exerts its effect extracellularly or intracellularly, i.e., on intracellular domains of the P2X4 channel or some intracellular components important for P2X4 function. BX430 (5 μM) was found to significantly reduce both peak (about –29%) and total current (about –75%) of the P2X4 channel response when coapplied with ATP without preincubation (Fig. 5A), strongly suggesting that the antagonist acts on the receptor channel from the extracellular space by binding on either the ectodomain or transmembrane domains.

We also recorded P2X4 currents with BX430 applied intracellularly through the patch pipette. No major effect on P2X4 current amplitude or kinetics was observed when the pipette solution reached equilibrium with the intracellular milieu, as shown by the consecutive ATP-evoked current responses (Fig. 5B). The size of the initial ATP responses was identical to that recorded in cells with control intracellular solution without BX430 (Fig. 5, A and B). Moreover, application of extracellular BX430 (5 μM) during the desensitizing phase of the P2X4 current evoked an immediate acceleration of the relaxation (Fig. 5C). The inhibitory effect of 1 μM BX430 was not sensitive

![Figure 6](https://example.com/figure6.png)
to increasingly hyperpolarizing membrane potentials, from −20 mV (49 ± 2% blockade; n = 4) to −80 mV (52 ± 2% blockade; n = 6) (data not shown). These results indicate that the compound BX430, uncharged at physiologic pH, does not sense membrane voltage and is not a direct pore channel blocker.

To investigate whether the inhibition of P2X4 activity is competitive or noncompetitive, electrophysiological recordings were performed in hP2X4-HEK293 cells at different concentrations of ATP with a fixed concentration of the antagonist (1 μM). Concentration-response curves in the absence and presence of BX430, in which the current amplitude was normalized to the current activated by the highest concentration of ATP (1 mM), gave an EC50 value of 7.3 μM, with a slope nH of 0.99 in the absence of BX430, and an EC50 of 187 μM, with a slope nH of 0.49 in the presence of 1 μM BX430 (Fig. 6). The inhibitory effect of BX430 could not be overcome with increasing concentrations of ATP of up to 1 mM. These two effects, i.e., a shift in ATP EC50 and a reduction in the maximal response at saturating concentrations of ATP, indicate that BX430 is a non-competitive antagonist binding to an allosteric site on the P2X4 receptor channel.

The human P2X channel family is composed of six homomeric functional subtypes. We tested the potential effects of BX430 on current amplitude, kinetics, and recovery of the subtypes P2X1–P2X5 and P2X7 in transiently transfected HEK293 cells. We observed a high degree of selectivity of BX430 toward P2X4, as no difference in current amplitude or phenotype was detected on all other human P2X channel subtypes when used at 10 times its IC50 for human P2X4 (Fig. 7). We confirmed in these experiments that BX430 showed similar potency between wild-type human P2X4 in transiently transfected HEK293 cells and the His-tagged version used for HTS in the stable HEK293 cell line. As P2X4 and P2X7 are natively coexpressed in many cell types, a potential inhibition of human P2X7 by 50 μM BX430 (around 100 times its IC50 for human P2X4) was also tested, without any significant effect (data not shown).

The primary sequence of P2X4 subunits is significantly conserved between vertebrates, from fish to primates; therefore, we investigated the sensitivity of several P2X4 channel orthologs to BX430. Unexpectedly, mouse P2X4 (87% amino acid identity with human P2X4) in transiently transfected HEK293 cells did not show any inhibition when BX430 was applied at 10 μM (Fig. 8, A and B), a concentration that produces a strong blockade of human P2X4 (Fig. 7). BX430 did not have any significant effect on the rat P2X4 either (87 and 94% amino acid identity with human and mouse P2X4, respectively) under the same experimental conditions (data not shown). However, we observed that zebrafish P2X4, despite a weaker sequence homology
(57% amino acid identity with human P2X4), is effectively blocked by BX430 (Fig. 8, B and C), with an IC$_{50}$ of 1.89 μM (data not shown).

Ivermectin, an antiparasitic agent extensively used in human and veterinary medicine, is also used as a pharmacological tool in basic research and as a potent positive allosteric modulator to pharmacologically identify P2X4 receptor channels in native preparations (Khakh et al., 1999; Burkhart, 2000; Priel and Silberberg, 2004). Using a patch-clamp recording, we confirmed that 3 μM ivermectin potentiates 50 μM ATP-evoked human P2X4 currents (Fig. 9). Treatment with 10 μM BX430 significantly inhibited the ivermectin-potentiated P2X4 current response (67%), as shown quantitatively in Fig. 9A. A typical biophysical property of several slowly desensitizing P2X channel subtypes, including P2X4, is their ability to generate a large pore that allows the passage of molecules of up to 900 Da under continuous stimulation with ATP (Khakh and Lester, 1999; Bernier et al., 2012). We took advantage of a robust fluorescence assay based on ATP-evoked YO-PRO1 uptake to test whether BX430 has an inhibitory effect on dilation of the P2X4 pore in the presence of ivermectin to facilitate pore dilation in normal extracellular divalents. In line with our previous results, we observed that P2X4-mediated YO-PRO1 uptake was significantly blocked by 5 μM BX430 (Fig. 9B).

Lastly, we tested the inhibitory effects of BX430 on native P2X4 receptor channels expressed in the THP-1 cells, a human monocytic cell line that can be differentiated in macrophages with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate. As depicted in Fig. 10, ATP applied at 50 μM to prevent activation of P2X7 (EC$_{50}$ = 300 μM) was able to evoke robust intracellular calcium responses in phorbol ester–differentiated THP-1 cells. When the cells were exposed to BX430 (5 μM), the amplitude of ATP-evoked intracellular calcium responses was markedly reduced (quantitative data in Fig. 10B). We recorded calcium responses evoked by 100 μM BzATP [2’(3’)-O-(4-benzoylbenzoyl)adenosine-5’-triphosphate], a more potent P2X7 agonist (EC$_{50}$ = 30 μM), confirming the coexpression of P2X7 in differentiated THP-1 cells. Treatment with the selective P2X7 antagonist BBG (1 μM) was without any effect on 50 μM ATP-evoked calcium responses (Fig. 10C), but significantly blocked BzATP-evoked P2X7-mediated calcium responses (Fig. 10D). This indicated that 50 μM ATP was selectively activating BX430-sensitive P2X4 receptor channels without evoking significant BBG-sensitive BX430-insensitive P2X7-mediated calcium responses in THP-1 cells.

**Discussion**

High-throughput screening of a library of compounds using an ATP-evoked calcium uptake assay in a stable hP2X4-HEK293 cell line allowed us to identify BX430, a phenylurea endowed with potent (IC$_{50}$ = 0.54 μM) and selective antagonist properties on recombinant as well as native human P2X4 receptor channels.

Confirming the high calcium permeability of human P2X4 channels reflected by a fractional calcium current close to 15% (Egan and Khakh, 2004), ATP-evoked calcium responses were robust and reproducible, with only a minor contribution by endogenous P2Y receptors in this hP2X4-HEK293 cell line. Therefore, the sensitive screening method described here has the potential to identify novel lead compounds that block P2X4 receptor channels. Moreover, with simple adjustments of the recording parameters, such a calcium-based cellular assay can also be used to screen for positive allosteric modulators of P2X4 function (see Fig. 1B). The blocker BX430, which was selected among other inhibitory lead compounds in the Canadian Chemical Biology Network library for its potency and selectivity for P2X4, was found to be highly soluble in physiological solutions and stable at room temperature, and it showed the same inhibitory effects during several hours of recording.
Another compound with reported inhibitory properties is ivermectin (IVM), a positive allosteric regulator of P2X4 channels, which was reported to block P2X4 currents in recombinant preparations, with an IC$_{50}$ of 0.5 μM (Fisher et al., 2005). Unfortunately, the results were described in a corporate patent and details of the experimental procedures are not available. Baláz et al. (2013), using both calcium imaging and patch-clamp electrophysiology, reported that 5-BDBD is a slightly potent (IC$_{50}$ = 1.6 μM) but competitive antagonist against human P2X4 in HEK293 cells when it is activated by 0.25–1 μM ATP. In our hands, 5-BDBD, tested at 10 μM on human P2X4 responses evoked by 50 μM ATP, shows a minor effect, with a less than 20% decrease in the currents. These striking differences in the efficacy of 5-BDBD demonstrate the advantage of noncompetitive ligands like BX430, in which inhibitory effects on P2X4 responses do not depend on ATP levels.

When coapplied with ATP without preincubation, BX430 showed a remarkable effect on the phenotype of the human P2X4 current. This fact and the observation that BX430 does not induce any inhibition when it is applied intracellularly suggest that BX430 acts from the extracellular side of the plasma membrane. The dose-response curve of ATP on human P2X4, with and without BX430, showed an insurmountable blockade of up to 1 mM ATP, clearly indicating a noncompetitive mechanism of action. Also, knowing that the efficacy of BX430 is not voltage dependent, we can discard the possibility that BX430 acts as a channel blocker by plugging the cationic pore of P2X4.

To test the potential involvement of P2X4 in any physiological process using pharmacology, a highly selective tool is needed. The ligand BX430 seems to be remarkably selective for P2X4, as no effect (inhibitory or facilitatory) was observed on currents mediated by the human P2X1–3, P2X5, and P2X7 subtypes when tested at 10 times its IC$_{50}$ for P2X4, either on peak amplitude, total current, or recovery time. As the P2X7 and P2X4 purinoceptors are known to be coexpressed in many non-neuronal cell types, including monocytes, macrophages, microglia, and airway ciliated cells (Bowler et al., 2003; Xiang and Burnstock, 2005; Ma et al., 2006; Guo et al., 2007), BX430 was retested on human P2X7 at about 100 times its IC$_{50}$ for P2X4, without showing any effect.

Regarding the sensitivity of P2X4 from other species to BX430, we noticed that this compound effectively inhibited zebrafish P2X4, while it had little or no effect on rodent P2X4 current responses. Several members of the P2X family, including P2X4 and P2X7, have been reported to show large species differences in antagonistic potencies between human and rodent receptors (Buell et al., 1996; Jones et al., 2000; North and Surprenant, 2000; Gever et al., 2006; Michel et al., 2009). Indeed Hernandez-Olmos et al. (2012) recently observed that the compound PSB-12054 was much more potent on human P2X4 than on rodent P2X4 orthologs. From a structural point of view, it will be interesting to analyze sequences and protein alignments between a number of P2X4 orthologs sensitive and insensitive to BX430 as a potential strategy to identify the amino acids/domains responsible for the effects of this novel antagonist.

Ivermectin, a positive allosteric regulator of P2X4 channels, strongly increased ATP-evoked human P2X4 currents, as previously reported (Khakh et al., 1999; Priel and Silberberg, 1999). As a channel blocker by plugging the cationic pore of P2X4. Therefore, we conclude from these data that the most likely mechanism of action of BX430 on P2X4 receptor channel activity is a change of subunit conformation triggered by its binding to an allosteric site located on the ectodomain or the transmembrane domains, with access from the extracellular space.

Fig. 9. Blockade of ivermectin potentiation and P2X4 large pore formation by BX430. (A) Using a patch-clamp recording, we observed strong inhibitory effects of BX430 on P2X4 currents potentiated by ivermectin (IVM) (3 μM), as illustrated here by the quantitative results. White bars 1 and 3 correspond to normalized control ATP-evoked currents before ivermectin and after washout, respectively. Black bars 4 and 6 correspond to normalized control ATP-evoked currents before ivermectin + BX430 (5 μM) and after washout, respectively. *P < 0.05; ***P < 0.001 (n = 4). (B) ATP-evoked YO-PRO1 entry reflecting P2X4 pore formation in the hP2X4-HEK293 cell line in the presence of IVM and significant blockade by BX430 (5 μM) (n = 16–25).
and its potentiating effect was suppressed by BX430. Prolonged stimulation of P2X4 with ATP, facilitated by ivermectin, causes the ion channel to dilate, leading to the formation of a pore that is permeable to large molecules (Bernier et al., 2012; Rokic and Stojilkovic, 2013). Interestingly, in the presence of BX430, ATP-evoked P2X4 pore dilation was almost completely abolished. To our knowledge, this is the first time that a P2X4 antagonist is used to inhibit pore dilation.

Tsuchiya et al. (1980) established the human THP-1 cell line, which became increasingly used as a source of primary monocytes and macrophages. After exposure to phorbol esters, THP-1 cells, initially rounded and in suspension, start to adhere to culture plates accompanied by differentiation into a macrophage phenotype, with marked morphologic changes in cell shape and organelles (Auwerx, 1991; Park et al., 2007). Signaling via extracellular ATP alters the outcome of inflammation by mounting appropriate macrophage responses against foreign agents or tumors or, conversely, by promoting chronic inflammation during ischemia or chronic pain (Idzko et al., 2014). It has been reported that human macrophages expressed several ATP-sensitive P2 receptors, including P2X1, P2X4, P2X7, and P2Y2. In our experiments, ATP-evoked calcium responses in differentiated THP-1 cells were very sensitive to BX430. Therefore, based on the absence of any effect of BX430 on P2X1 and P2X7 currents or on P2Y2-mediated calcium responses, we confirmed the expression of functional P2X4 receptors at the surface of THP-1–derived macrophages (Li and Fountain, 2012).

Guo et al. (2007) provided convincing evidence for the existence of functional P2X4+7 heteromeric channels sensitive to the antagonist BBG. The absence of the inhibitory effect of BBG on ATP-evoked calcium responses strongly suggests that the population of P2X4+7 heteromers, if present, is a minor component in differentiated THP-1 cells. This is in agreement with several studies in which coexpressed homomeric P2X4 and P2X7 receptor channels have been shown to constitute the main P2X populations in immune cells (Boumechache et al., 2009) as well as in heterologous expression systems (Antonio et al., 2011).

In summary, our study describes the identification of the phenylurea BX430, with high potency and selectivity for P2X4 receptor channels. This ligand will provide a novel pharmacological tool to specifically investigate the role of human P2X4 in physiologic and pathologic conditions where ATP signaling has been shown to be dysfunctional, including inflammatory diseases.

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Authorship Contributions

Participated in research design: Ase, Honson, Pfeifer, Séguéla.

Conducted experiments: Ase, Honson, Zahgdane.

Contributed new reagents or analytic tools: Zahgdane.

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