Tanshinone II A Sulfonate, but Not Tanshinone II A, Acts as Potent Negative Allosteric Modulator of the Human Purinergic Receptor P2X7[S]

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ABSTRACT

Tanshinone II A sulfonate (TIIAS) was identified as a potent, selective blocker of purinergic P2X7 in a compound library screen. In this study, a detailed characterization of the pharmacologic effects of TIIAS on P2X7 is provided. Because TIIAS is a derivative of tanshinone II A (TIIA) and both compounds have been used interchangeably, TIIA was included in some assays. Fluorometric and electrophysiologic assays were used to characterize effects of TIIAS and TIIA on recombinantly expressed human, rat, and mouse P2X7. Results were confirmed in human monocyte-derived macrophages expressing native P2X7. In all experiments, involvement of P2X7 was verified using established P2X7 antagonists. TIIAS, but not TIIA, reduces Ca2+ influx via human P2X7 (hP2X7) with an IC50 of 4.3 μM. TIIAS was less potent at mouse P2X7 and poorly inhibited rat P2X7. Monitoring of YO-PRO-1 uptake confirmed these findings, indicating that formation of the hP2X7 pore is also suppressed by TIIAS. Electrophysiologic experiments revealed a noncompetitive mode of action. TIIAS time-dependently inhibits hP2X7 gating, possibly by binding to the intracellular domain of the receptor. Inhibition of native P2X7 in macrophages by TIIAS was confirmed by monitoring Ca2+ influx, YO-PRO-1 uptake, and release of the proinflammatory cytokine interleukin-1β. Fluorometric experiments involving recombinantly expressed rat P2X2 and human P2X4 were conducted and verified the compound’s selectivity. Our data suggest that hP2X7 is a molecular target of TIIAS, but not of TIIA, a compound with different pharmacologic properties.

Introduction

The purinergic P2 receptor family comprises G protein–coupled P2Y receptors as well as P2X receptors that are ATP-gated ion channels [nomenclature follows (Collingridge et al., 2009)]. Unlike other P2X family members, P2X7 exhibits a low affinity for ATP and comprises a large C-terminal domain (Jiang et al., 2013). Prolonged agonist stimulation induces pore formation in P2X7, thereby allowing passage of organic cations of up to 900 Da (Khakh and North, 2012). P2X7 is mainly expressed on immune cells, such as monocytes and macrophages, but also on osteoclasts, osteoblasts, and distinct types of glial cells (Volonté et al., 2012). It has been related with various pathologic conditions. Inflammatory processes are enhanced by stimulation of P2X7 by triggering caspase-1–mediated interleukin (IL)-1β maturation and release (Carroll et al., 2009). Microglial P2X7 receptors are involved in neuroinflammation, which can be attenuated using P2X7 antagonists (Chu et al., 2012).

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Abbreviations:

A438079, 3-[5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl][methyl]pyridine hydrochloride; AZ10606120, N-[2-[[2-hydroxyethyl]amino]ethyl]amino]-5-quinolinyl]-2-tricyclo[3.3.1.13,7]dec-1-yacetamide dihydrochloride; [Ca2+], intracellular free Ca2+ concentration; DIC, divalent cations; DMEM, Dulbecco’s modified Eagle medium; DMSO, dimethylsulfoxide; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HBS, HEPES-buffered saline; HEK, human embryonic kidney; hMDM, human monocyte-derived macrophage; hP2X7, human P2X7; IL, interleukin; LPS, lipopolysaccharide; MITT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; OATP, organic anion-transporting polypeptide; ORAC, oxygen radical absorption capacity; TIIA, tanshinone II A; TIIAS, tanshinone II A sulfonate.
changes of pharmacodynamic properties. Low micromolar concentrations of TIIAS lacked effects on human P2X4 and rat P2X2. Comparison of TIIAS effects on human, mouse, and rat P2X7 revealed a species-prevalent inhibitory effect on the human P2X7, which was characterized in more detail applying electrophysiologic methods. Inhibition of ATP-triggered Ca^{2+} entry, YO-PRO-1 uptake, and IL-1β release demonstrated that TIIAS also acts on native P2X7 in human macrophages. Thus, TIIAS has a distinct pharmacologic profile and might be a promising agent for further testing in models of P2X7-related diseases.

Materials and Methods

Cell Culture Procedures. Human embryonic kidney (HEK) 293 cells stably transfected with the human P2X7 (hP2X7) receptor (HEKhP2X7) or the human P2X4 receptor (HEKhP2X4) were cultured at 37°C and 5% CO2 in Dulbecco's modified Eagle medium (DMEM; c c pro, Oberdorla, Germany) containing 4.5 mM d-glucose, 10% fetal calf serum (FCS; Biochrom, Berlin, Germany), 2 mM l-glutamine (PAA, Pasching, Austria), and 0.05 mg ml^{-1} geneticin (Invitrogen, Carlsbad, CA). Mouse P2X7 cDNA was cloned into a pCDNA3.1/Zeo vector. The cloned cDNA was sequenced and stably expressed in HEK cells to obtain the HEKmP2X7 cell line. HEKmP2X7 were maintained at 37°C and 5% CO2 in DMEM containing 4.5 mM d-glucose, 10% FCS, 2 mM l-glutamine, and 100 µg ml^{-1} zeocin (Invitrogen). Stably transfected Flp-In T-Rex cell lines inductively expressing the rat P2X7 (HEKrP2X7) or rat P2X2 (HEKrP2X2) receptor were maintained at 37°C and 5% CO2 in DMEM containing 4.5 mM d-glucose, 10% FCS, 2 mM l-glutamine, 100 µg ml^{-1} hygromycin B (Invivogen, San Diego, CA), and 15 µg ml^{-1} blasticidin (Invivogen). Expression of the integrated gene was induced by adding 1 µg ml^{-1} tetrycyclin to the medium 24 hours before the experiments.

Preparation of Human Monocyte-Derived Macrophages. All procedures involving human materials had been approved by the local ethical committee. Mononuclear cells were prepared from buffy coats by density gradient centrifugation, as described elsewhere (Nörenberg et al., 2011). Human monocyte–derived macrophages (hMDM) were prepared by culturing human monocytes obtained from peripheral blood of healthy donors for 8 days in RPMI-1640 (Gibco, Life Technologies, Germany) supplemented with 5% FCS, 10 ng ml^{-1} monocyte macrophage colony-stimulating factor (M-CSF), 10% dextran-coated charcoal-treated FCS, and 200 Units ml^{-1} recombinant human IL-4. A total of 1 million monocytes per 25-cm² cell culture flask was seeded and cultured for 8 days, and the resulting monocyte-derived macrophages were used within the first 24 hours. Prior to experimentation, hMDM were cultured for 24 hours in phenol red-free medium containing 0.1% (v/v) dimethyl sulfoxide (DMSO). Absorption was read in a plate-reader device (Polarstar Omega; BMG Labtech, Offenburg, Germany). YO-PRO-1 (1 µM) and P2X7 modulators were directly applied to the bath chamber.

Cytotoxicity of TIIAS. Cellular viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Briefly, approximately 25,000 HEKhP2X7 cells/well were seeded into 96-well culture plates and incubated overnight. After incubation in the presence of the modulators for 24 hours, cells were washed and incubated for 4 hours in phenol red-free culture medium, containing 0.5 mg ml^{-1} MTT. Formazan crystals were dissolved in dimethylsulfoxide (DMSO). Absorption was read in a plate-reader device (Polarstar Omega; BMG Labtech, Offenburg, Germany). YO-PRO-1 uptake in cultured hMDM, coverslips were rinsed with low DIC HBS and transferred to a bath chamber. Experiments were carried out in low DIC HBS and in the digital video microscopy setup described for single-cell Ca^{2+} imaging. Fluorescence was excited at 475 nm, and emission was recorded through a 515-nm long-pass filter with a cooled CCD camera (PCO Senscam, Kelheim, Germany). YO-PRO-1 (1 µM) and P2X7 modulators were directly applied to the bath chamber.

Antioxidative Capacity of TIIAS and TIIA. TIIA equivalent antioxidant capacities of modulators were determined with the oxygen radical absorption capacity (ORAC) assay. Assays were conducted in a plate-reader device (Polarstar Omega; BMG Labtech), according to an established protocol (Bernaret et al., 2012). Capabilities to prevent loss in fluorescent fluorescence were assessed for various concentrations (0.78–50 µM) of TIIA (trolox) and modulators. Measurement values were corrected for blanks corresponding to the respective concentration of solvent in buffer. Linear regression analysis of concentration-response curves was performed, and Trolox equivalents were calculated from their slopes.

Electrophysiologic Procedures. Whole-cell recordings were performed at 22–24°C and, unless otherwise stated, at a holding potential of −60 mV using an EPC9 amplifier controlled by a Pulse software (HEKA, Lambrecht, Germany). The extracellular solution contained 147 mM NaCl, 2 mM KCl, 13 mM d-glucose, and 10 mM HEPES (pH 7.3 with NaOH), supplemented with 1 mM MgCl₂, 2 mM CaCl₂ (standard DIC), or low DIC, as outlined above. The whole-cell configuration was always obtained in standard DIC before switching to low DIC bath solutions. Patch pipettes had a resistance of 2–6 MΩ when filled with an intracellular solution, containing 147 mM KCl, 10 mM HEPES, 10 mM EGTA (∼300 mM l^{-1}; pH 7.3 with KOH), and in some experiments also 3 mM MgCl₂. Drugs were applied to patched cells by means of a pressurized superfusion system (DAD-12; ALA Scientific Instruments, Farmingdale, NY). Experiments during which series resistance (compensated by 60–80%) changed by more than 20% were discarded. ATP-induced currents, filtered at 1.7 kHz and sampled at 5 kHz, were measured as peak amplitudes and normalized for membrane capacitance to obtain current densities (pA pF^{-1}).
et al., 1992). All experiments were conducted at 37°C. Cleared supernatants (1000g for 10 minutes) were stored at −20°C until assayed. IL-1β was determined using the human IL-1β Quantikine ELISA kit (R&D Systems, Minneapolis, MN), according to manufacturer’s instructions.

**Materials and Compounds.** P2X7 antagonists A438079 (3-[[5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl]methyl]pyridine hydrochloride) and AZ10606120 (N-[2-[[2-(2-hydroxyethyl)amino]ethyl]amino]-5-quinolinyl]-2-tricyclo[3.3.1.13,7]dec-1-ylacetamide dihydrochloride) were from Tocris (Bristol, UK), and TIIA was from MicroSource Discovery Systems (Gaylordsville, CT). TIIAS was obtained from Bosche Scientific (New Brunswick, NJ). Unless otherwise stated, all other chemicals were from Sigma-Aldrich (St. Louis, MO).

Stock solutions of drugs were prepared in standard or low DIC bath solutions (ATP), or in DMSO (A438079, AZ10606120, TIIA, TIIAS, nigericin). Aliquots of stock solutions were stored at −20°C and freshly diluted on the day of the experiment. The DMSO concentration in bath solutions never exceeded 0.1%, a concentration that had no effects on ATP-induced currents in HEKhP2X7 cells. ATP stock solutions were routinely readjusted to pH 7.3.

**Data Evaluation and Statistical Analysis.** Predictions of physicochemical properties of TIIA (CAS 69659-80-9) were computed with the Chemicalize software tool (ChemAxon, Budapest, Hungary).

Concentration-response curves were obtained by fitting the experimental data to a Hill equation:

\[
E = E_{\text{min}} + \left(\frac{E_{\text{max}} - E_{\text{min}}}{1 + (|M|/EC_{50})^{-nH}}\right)
\]

where \(E_{\text{min}}\) and \(E_{\text{max}}\) are the extrapolated minimum and maximum effects of the tested compound, modulator, or agonist; |\(M\)| is the actual concentration of the compound; EC_{50} is the compound concentration producing 50% of the response; and \(nH\) is the Hill coefficient, indicating the cooperativity of the effect.

To quantify the impact of TIIAS on the hP2X7 on-kinetics, we compared the rise times from 10 to 90% of the ATP-induced peak current densities in the absence and presence of the compound. To extract fast and slow time constants of hP2X7 current decay (Yan et al., 2010; Nörenberg et al., 2012), biexponential fits of the decaying current phase were performed by applying the function

\[
I_{\text{ATP}_t} = A_0 + A_1 \exp\left[-\frac{t}{\tau_{\text{fast}}}\right] + A_2 \exp\left[-\frac{t}{\tau_{\text{slow}}}\right],
\]

where \(I_{\text{ATP}_t}\) is the ATP-induced current at time \(t\) and \(A_0\), \(A_1\), and \(A_2\) denote the extrapolated effect levels at infinite time and the initial signal amplitude at \(t = 0\) second. A monoexponential function \(I_{\text{ATP}_t} = A_0 + A_1 \exp(-t/\tau_{\text{on}})\) was used to determine time constants for the onset (\(\tau_{\text{on}}\)) and offset (\(\tau_{\text{off}}\)) of TIIAS effects.

All data were expressed as mean ± S.E.M., obtained in n cells or experiments. After proofing normal distribution and homogeneity of variance, statistical significance between two experimental groups was tested by Student’s t test. In the case of multiple comparisons, one-way analysis of variance followed by the Bonferroni procedure was applied. \(P < 0.05\) was accepted as minimum level of significance.

**Results**

**TIIAS Selectively Inhibits Human P2X7.** After TIIAS has been identified as an inhibitor of P2X7 in our compound library screen, its potency was assessed applying a Ca^{2+} assay that enabled us to monitor ATP-triggered Ca^{2+} entry through heterologously expressed human P2X7 in a stably transfected HEK_{hP2X7} cell line. A strong and concentration-dependent inhibition was exerted by TIIAS (Fig. 1, A and B; n = 7, independent experiments). The half-maximally inhibitory concentration IC_{50} of increases in the fluo-4 fluorescence was 4.3 ± 0.8 \mu M. Similar results were achieved in YO-PRO-1 uptake assays, indicating P2X7 pore formation. The IC_{50} of TIIAS to suppress YO-PRO-1 uptake through hP2X7 was 5.7 ± 0.7 \mu M (Fig. 2; n = 4 independent experiments). Ca^{2+} entry and YO-PRO-1 uptake were reduced by approximately 80% by 10 \mu M TIIAS.

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**Fig. 1.** Concentration dependence of TIIAS-mediated inhibition of calcium influx via P2X7 and selectivity over P2X2 and P2X4. Cell lines HEKhP2X7, HEKrP2X7, HEKmP2X7, and HEKmP2X4 were loaded with fluo-4/AM. Ca^{2+} influx was triggered with 1 mM or 3 \mu M ATP, respectively, in the presence of different concentrations of TIIA, TIIAS, or 10 \mu M A438079. Fluorescence increase was analyzed and normalized to control measurements without modulators. n = 5 for HEKhP2X7 (TIIA), n = 7 for HEKhP2X7 (TIIAS), n = 8 for HEKrP2X7 and HEKmP2X7, and n = 4 for HEKmP2X4 and HEKmP2X2. (A) Representative traces for HEKhP2X7 (left), HEKrP2X7 (center), and HEKmP2X4 (right). Light gray lines represent positive controls, that is, cells stimulated with ATP in the absence of antagonists (control [ctr]). Black lines illustrate measurements in the presence of 12.5 \mu M TIIAS, whereas dark gray lines show those recorded in the presence of 10 \mu M A438079 (A43). (B) Concentration-response curves for TIIA- and TIIAS-mediated modulation of Ca^{2+} influx in HEKhP2X7. (C) Concentration-response curves for TIIAS-mediated reduction of Ca^{2+} influx in HEKhP2X2 and HEKhP2X4.
TIIAS was not reached in either cell line (Fig. 1, A and C; experiments (Fig. 2). A438079, a known P2X7 antagonist of TIIAS at rat P2X7 was confirmed in YO-PRO-1 uptake independent experiments, each). The weak inhibitory activity ATP-stimulated HEKmP2X7 cells and was essentially inactive in vitro exert no strong cytotoxic effect.

Effects of TIIAS on the viability of HEK293 cells were assessed using the MTT metabolic activity assay. Whereas TIIAS reduced cell viability at concentrations exceeding 500 μM (78.1 ± 11.4% and 23.2 ± 5.2% for 500 μM and 1 mM, respectively, compared with solvent controls), viability of cells treated with concentrations up to 25 μM TIIAS did not affect the Ca2+ response upon stimulation with ATP in HEKp2X7 cells; an impaired Ca2+ influx could only be seen after exposure to the highest test concentration of TIIAS. An IC50 of 35 μM TIIAS was determined for this cell line. TIIAS lacked effects on Ca2+ influx in HEKp2X4 cells in the considered concentration range (Fig. 1D; n = 4 independent experiments, each). Effects of TIIAS on different targets are summarized in Table 1.

**TIIAS Is Not Cytotoxic in Micromolar Concentrations.** Effects of TIIAS on the viability of HEK293 cells were assessed using the MTT metabolic activity assay. Whereas TIIAS reduced cell viability at concentrations exceeding 500 μM (78.1 ± 11.4% and 23.2 ± 5.2% for 500 μM and 1 mM, respectively, compared with solvent controls), viability of cells treated with concentrations up to 25 μM TIIAS did not significantly differ from that of untreated controls. For instance, cells exposed to 250 μM TIIAS showed 98.8 ± 7.6% viability compared with solvent controls (n = 8). We conclude that low micromolar concentrations of TIIAS that suffice to strongly suppress hP2X7 activity in vitro exert no strong cytotoxic effect.

**TIIAS Bears Antioxidative Properties.** Oxidative degradation of fluorescein entails a loss of fluorescence intensity. The inhibition of this process indicates antioxidant capacities of modulators in the ORAC assay. Trolox, a well-established antioxidant, but also TIIAS and TIIA, reduced the 2,2’-azobis(2-methylpropionamide) dihydrochloride−induced decay of fluorescein fluorescence in a concentration-dependent manner. In the ORAC assay, a linear concentration dependence was obtained for the indicated concentration range of the modulators. Thus, the slopes, also referred to as Trolox equivalents, indicate the antioxidative capacity of the tanshinone derivatives. The Trolox equivalent of TIIAS was 0.28 ± 0.02, thereby confirming previous reports (Liu et al., 2011). The antioxidative capacity of TIIA was lower than that of TIIAS (0.04 ± 0.02 Trolox equivalents, P < 0.001; n = 8 in 4 independent experiments).

**TIIAS Acts as Noncompetitive Antagonist and Delays the ATP-Induced hP2X7 Gating.** To characterize the impact of TIIAS on ionic currents through P2X7, we performed electrophysiologic experiments in the whole-cell configuration. When applied for 4 seconds and at 60-second intervals, repetitive application of 1 mM ATP to HEKp2X7 cells led to a characteristic run-up of hP2X7 currents, reaching a steady state after six to seven challenges, irrespective of the absence and presence of TIIAS (10 μM), which was added to the superfusion medium 15 minutes prior to first pulse of ATP stimulation (Fig. 3, A and B). After current run-up had been accomplished, we obtained concentration-response curves by applying ATP at concentrations of 10 μM to 30 mM (Fig. 3, A and B). TIIAS (10 μM) reduced the maximal current densities at almost saturating ATP concentrations (30 mM) by about 60% (n = 13−14; P < 0.01; Fig. 3C). By contrast, the calculated EC50 and nH values remained almost unchanged in the presence of TIIAS (Fig. 3, C and D). The nonsurmountable inhibition of ATP-induced hP2X7 currents and the unchanged EC50 of ATP are indicative of a noncompetitive, allosteric inhibitory mechanism.

In the presence of TIIAS, the onset of ATP-induced P2X7 currents was slowed down (Supplemental Fig. 1A). In HEKp2X7 cells repeatedly stimulated with 1 mM ATP, rise times from 10% to 90% of the peak responses after channel run-up were 207.1 ± 13.7 milliseconds. Exposure of the same cells to 10 μM TIIAS for 6 minutes resulted in slower rise times of 536.4 ± 43.9 milliseconds (n = 7; P < 0.001; Supplemental Fig. 1, A and C). The channel deactivation upon washout of the activator was not affected by TIIAS (Supplemental Fig. 1, B and D). When calculated for data obtained at various ATP concentrations, the typical effect of ATP itself on rise and decay times (Yan et al., 2010) was observed in the absence of TIIAS (open symbols in Fig. 3, E and F). In the presence of TIIAS, again the rise times were delayed by up to 3-fold (filled symbols in Fig. 3E) with no discernible effect on current decay rates (Fig. 3F). Remarkably, the TIIAS-dependently-delayed rise times of currents did not differ between 10 and 30 mM ATP (365.5 ± 9.0 milliseconds and 366.1 ± 12.8 milliseconds; P = 0.832). This effect suggests that gating transition, but not the agonist-binding reaction, is rate-limiting under these conditions.

**TIIAS Inhibits hP2X7 Currents in a Time-Dependent, but Not Use-Dependent, Manner.** The effect of TIIAS on ATP-triggered hP2X7 currents was slowly developing and reversible. To resolve the time constants of current inhibition and reversibility of TIIAS effects, ATP (1 mM) was repeatedly applied for 1.5 seconds at short (30-second) intervals. Following run-up of hP2X7 currents, a steady state was reached after 9–12 challenges (Fig. 4). Upon addition of 10 μM TIIAS,
the inhibitory effect progressively developed with a time constant $\tau_{on}$ of 38.8 ± 6.6 seconds ($n = 7$; Fig. 4, A and B). In the continuous presence of TIIAS, current amplitudes were reduced within 5–6 minutes by 66.4% ± 4.3% and fully recovered after removal of the inhibitor. This recovery was again slow, developing with $\tau_{off} = 53.9 ± 8.7$ seconds.

In accordance with our Ca$_{\text{2+}}$ imaging and YO-PRO-1 fluorescence measurements, TIIAS was less efficient or essentially

### Table 1

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**Fig. 3.** TIIAS affects ATP efficacy, but not potency possibly by decelerating hP2X7 current activation. Shown are representative whole-cell currents (A and B), corresponding ATP concentration-response curves without normalization (C) or when normalized with respect to the effects of 30 mM ATP (D), as well as plots of current rise times from 10% to 90% of the peak response (E) or fast ($\tau_{off, \text{fast}}$) and slow ($\tau_{off, \text{slow}}$) time constants of current decay versus ATP concentration (F), obtained in the absence (A, open circles in C–F; n = 13) or presence of 10 $\mu$M TIIAS, which was preapplied for 6 minutes before and throughout the experiments (B, filled circles in C–F; n = 14). Whole-cell recordings were performed in HEK-P2X7 cells at a holding potential of −60 mV in a low DIC bath solution. Cells were repetitively stimulated for 4 seconds at 60-second intervals, first with 1 mM ATP until current run-up was completed (see superimposed responses to the first, sixth, and seventh ATP application in A and B) and then with subsequently increasing concentrations of the agonist. The long dashed line in A and B marks the 50% level of the maximally achievable ATP response. In this and subsequent electrophysiologic figures, short dashed lines indicate the peak level of ATP-induced currents after run-up was completed. Dotted lines indicate the zero current level, and bars above and below the current traces depict the times of stimulation with ATP and addition of TIIAS, respectively. (E) *$P < 0.05$, significant difference between current rise time in absence and presence of TIIAS; ns, not significant (for the procedures used to estimate rise times and decay time constants, see Supplemental Fig. 1).
inactive in suppressing currents through mouse or rat P2X7 (Supplemental Fig. 2). The reversibility of TIIAS effects on hP2X7 indicates that it does not covalently bind to the channel. The slow onset of inhibition by TIIAS and recovery from the block may either point to an involvement of signaling cascades or indicate a restricted accessibility of the binding site. However, TIIAS (10 μM) suppressed ATP (1 mM)-evoked hP2X7 currents by 55.8 ± 7.5% and 58.0 ± 4.9% in the absence and presence of internal Mg2+, respectively (n = 7 each; P = 0.830), suggesting a G protein–independent mode of action (Birnbaumer, 2007).

Regarding the effects of TIIA on hP2X7 currents, our electrophysiologic data are in agreement with the results of fluorometric Ca2+ assays. No inhibitory effect on hP2X7 currents was observable with TIIA applied at concentrations of 10 μM (n = 10; Fig. 4, C and D).

Notably, TIIAS also inhibited hP2X7 in the presence of more physiologic extracellular divalent cation concentrations. As expected, ATP (1 mM)-induced currents in standard DIC solutions were about 10-fold smaller in amplitude and showed no run-up upon repetitive stimulation (Supplemental Fig. 3, A and B). The TIIAS (10 μM)-induced hP2X7 inhibition developed and recovered once more with slow time courses and reached a similar inhibitory efficiency (54.6% ± 4.4%; n = 11; Supplemental Fig. 3, C and D), which was paralleled by a doubling of current rise times from 480.6 ± 50.7 milliseconds to 972.2 ± 46.4 milliseconds in the absence and presence of 10 μM TIIAS, respectively. Because the activity of TIIAS was independent of the used bath solution, we performed all following electrophysiologic experiments in low DIC solutions, which is typically applied to assess ionic currents through P2X7 (Yan et al., 2011).

The slow onset and offset, as well as the partial inhibitory effect of TIIAS, may be explained by a use-dependent or time-dependent mode of action. When the repetitive ATP application was paused for a 6-minute period after hP2X7 run-up had
been completed, the nucleotide-induced current amplitudes remained at the sensitized level \( (n = 7; \text{Fig. 5, A and B}) \). Continuous superfusion with TIIAS during this pause inhibited the subsequent response to ATP with an efficiency not significantly differing from that obtained applying the continuous stimulation paradigm \( (n = 5; P = 0.202; \text{Fig. 5, C and D}) \). We conclude that the TIIAS-induced inhibition of P2X7 is time-, but not use-dependent. This assumption was further corroborated by a lack of inhibitory TIIAS effects on ATP-induced P2X7 currents when tested in an acute coapplication protocol, in which cells were only exposed to the drug during the stimulation with 1 mM ATP \( (n = 7; \text{Supplemental Fig. 4}) \).

**TIIAS Inhibits hP2X7 Currents by a Nonpore Block Mechanism and via an Intracellular Point of Attack.** At physiologic pH, the sulfonate moiety of TIIAS is negatively charged (see Fig. 6A). Although Kubick et al. (2011) have shown that hP2X7 is normally neither permeable for nor blockable by anions, TIIAS may access a binding site within the electrical field of the membrane. Applying voltages ranging from \(-80\) to \(+40\) mV at 20-mV increments for a longer time span (30 seconds) before and during ATP stimulation, we found an apparent voltage dependence of P2X7 inhibition by TIIAS. Current inhibition was more efficient at low negative and positive membrane potentials compared with a holding potential of \(-80\) mV \( (P < 0.001, n = 8; \text{Fig. 6C}) \). Of note, TIIAS did not cause shifts of the reversal potential (Fig. 6, A and B), indicating no gross effects on the ion selectivity of P2X7 currents. The more efficient block at low negative or positive potentials may imply that an electrical driving force pushes TIIAS from the extracellular solution into the conductive path (Hille, 1992). However, a voltage-dependent block would typically develop in the ms time range, which is not compatible with the observed slow and use-independent onset of hP2X7 inhibition by TIIAS (see Fig. 4B). Accordingly, no voltage dependence of P2X7 inhibition was seen in a voltage step

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**Fig. 5.** TIIAS inhibits hP2X7 currents in a time- but not use-dependent manner. Shown are representative whole-cell currents (A and C) and corresponding plots of ATP-evoked peak current densities versus time obtained in the absence (B) or presence of 10 \( \mu M \) TIIAS (D; \( n = 7 \) each). Whole-cell recordings were acquired essentially as shown in Fig. 4, but with a 6-minute interruption of the repetitive stimulation protocol that began immediately after the twelfth pulse of ATP.
or in a fast voltage ramp protocol (1-second duration; Supplemental Fig. 5, D and E) that would resolve a voltage-dependent behavior in the subsecond time range.

Alternatively, the slowly (>1 second) developing voltage-dependent inhibition may rely on a restricted and voltage-dependent access of the negatively charged inhibitor to an intracellularly located binding site. In agreement with this assumption, we observed not only a more than 3-fold stronger inhibition of hP2X7 currents, but also a more than 5-fold acceleration in $t_{on}$ for the TIIAS effects, when HEK cells were clamped to $140 \text{ mV}$ ($n = 10$) instead of $280 \text{ mV}$ ($n = 8$; Supplemental Fig. 6). The acceleration of TIIAS effects at more positive membrane potentials also argues against signal transduction cascades relaying the effects of TIIAS to P2X7 inhibition. When intracellularly perfusing HEK-hP2X7 with a pipette solution containing 10 $\mu$M TIIAS, ATP (1 mM)-triggered P2X7 currents reached a lower density compared with experiments performed with standard pipette solutions (Fig. 7, A and B; compare Fig. 4, A and B). In TIIAS-perfused cells, extracellular superfusion with TIIAS (10 $\mu$M) exerted only an inhibition by 13.7% ± 8.1% (Fig. 7, C and D), indicating that a majority of binding sites may have been preoccupied by the intracellularly applied drug. Interestingly, intracellular TIIA (10 $\mu$M) did not prevent the inhibitory action of superfused TIIAS ($n = 8$; Supplemental Fig. 7), further supporting the importance of the sulfonate moiety for the interaction with hP2X7, as well as excluding artifacts potentially caused by DMSO as the solvent of both TIIAS and TIIA.

**TIIAS Inhibits Native hP2X7 on Primary Cells.** Monocyte-derived macrophages express P2X4 and P2X7, but also P2Y$_2$ and P2Y$_6$ receptors (Gendaszewska-Darmach and Kucharska, 2011). Although adenine nucleotides are essentially inactive at P2Y$_6$ (von Kügelgen and Harden, 2011), we took advantage of the higher potency of ATP to stimulate P2Y$_2$ and P2X4 with EC$_{50}$ values of 0.2 and 10 $\mu$M, respectively (Lazarowski et al., 1995; Garcia-Guzman et al., 1997). Superfusion of LPS-primed hMDM with 50 $\mu$M ATP, a subthreshold concentration at hP2X7 (Fig. 3), caused a rapid and transient rise in $[\text{Ca}^{2+}]_i$, presumably triggered via P2X4- and P2Y$_2$-like receptors (Fig. 8A). After intracellular Ca$^{2+}$ concentration returned close to

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**Fig. 6.** Effects of membrane voltage on the modulation by TIIAS of hP2X7 currents. (A) ATP (1 mM) was repetitively applied to the cells for 1.5 seconds and at 30-second intervals. After completing current run-up at a holding potential ($V_h$) of −60 mV (superimposed responses to the first, eleventh, and twelfth response in the left panel), the membrane voltage was changed stepwise (range −80 to 40 mV) for the 30 seconds between subsequent ATP pulses to record a current-voltage (I-V) relation under control conditions ($I_{ctrl}$; middle panel). TIIAS (10 $\mu$M) was then preapplied for 6 minutes, and the voltage step protocol was repeated during the subsequent pulses of ATP application ($I_{TIIAS}$; right panel). Inset: chemical structure of TIIAS, an anion when dissolved in aqueous solution at pH values >2. (B) I-V plots from peak current densities obtained in $n = 8$ cells. The connecting lines are derived from fitting the data to linear ($I_{ctrl}$) or third-order polynomial regression functions ($I_{TIIAS}$). (C) Percentage of inhibition of ATP-induced currents by TIIAS at different $V_h$ calculated from the data in B.
basal values, brief application of 1 mM ATP again led to increases in 
\( \text{[Ca}^{2+}\text{]} \) in the absence of TIIAS (Fig. 8A, left panel). TIIAS (10 μM) and the selective P2X7 antagonist A438079 (10 μM), added after the first application of 1 mM ATP, abolished the second ATP-induced rise in 
\( \text{[Ca}^{2+}\text{]} \) (\( P < 0.01; n = 19–20; \text{Fig. 8, A and B})\), indicating an efficient inhibition of native P2X7 in hMDM. Likewise, the formation of a dye-permeable pore was significantly and concentration-dependently prevented, when hMDM were preincubated with either 3 or 10 μM TIIAS (\( n = 8; \text{Fig. 8, C and D})\).

**TIIAS Inhibits IL-1β Secretion by hMDM.** Because P2X7 activation in hMDM has been shown to induce maturation and release of proinflammatory cytokines, we determined the impact of TIIAS on the ATP-dependent release of IL-1β into the culture supernatants. As depicted in Fig. 9, priming of hMDM with LPS alone (100 ng ml\(^{-1}\)) was sufficient to induce a slight and statistically significant release of this cytokine (\( n = 7); \), which was, however, more than 3-fold augmented, when cells were additionally exposed to 1 mM ATP, with responses reaching 16% of the maximally releasable IL-1β pool, as judged by the effects of the K\(^+\) ionophore nigericin (Perreagaux and Gabel, 1994). TIIAS (10 μM) and the P2X7 antagonist AZ10606120 (300 nM) (Michel et al., 2008) prevented the ATP-induced augmentation and restored IL-1β release to similar levels as measured in response to LPS alone. We therefore conclude that anti-inflammatory effects of TIIAS may involve inhibition of P2X7-dependent cytokine release from immune cells.

**Discussion**

TIIAS species-specifically inhibited human P2X7 with an IC\(_{50}\) of about 4 μM. Our electrophysiologic experiments suggested that TIIAS acts as a noncompetitive inhibitor that interferes with the ATP-induced gating transition after binding to an intracellular domain of the receptor. TIIA was biologically inactive at human and rat P2X7. Thus, although TIIAS has initially been predicted to act in a similar fashion as TIIA, we want to express the caveat that the range of possible medical indications should not a priori be considered to be identical for both drugs. To our knowledge, this is the first study providing evidence that the purinergic receptor P2X7 is a molecular target of TIIAS. Human P2X4, the most closely related purinergic receptor (Volonté et al., 2012), was not modulated by TIIAS and concentrations of 50 μM were...
necessary to affect rat P2X2. Our findings may constitute a starting point for target-oriented studies using TIIAS to treat P2X7-related diseases in models comprising human receptor isoforms. Based on our findings, beneficial results obtained after treatment with TIIAS cannot be attributed to an inhibition of P2X7. Regarding TIIAS, few studies have suggested the drug to exert anti-inflammatory properties in rodent models of hepatitis and sepsis (Xu et al., 2008; Zhu et al., 2012) and to be beneficial in animal models of ischemic heart disease (Wu et al., 1993; Yang et al., 2008a). At large, data available are very scarce. The drug has been used in humans (Wang et al., 2013), but controlled clinical trials have not yet been carried out (Raja, 2013).

Currently known targets of TIIAS include Ca^{2+}-activated potassium channels and carboxylesterase 2, which are affected at similar or higher TIIAS concentrations as needed to strongly inhibit P2X7 (Yang et al., 2008b; Hatfield et al., 2013). In addition, a protection from LPS-induced acute lung injury by TIIAS has been demonstrated by Xu et al. (2009). In this study, indirect inhibition of LPS-dependent phospholipase A2 activation in alveolar macrophages was proposed as a possible intermediate. Of note, stimulation of immune cells with LPS involves the auto- or paracrine activation of P2X7 (Ferrari et al., 1997), possibly due to release of endogenous ATP. It is therefore tempting to speculate that the protective effect of TIIAS in the LPS-induced lung injury model may at least partially be attributed to the inhibition of P2X7.

The precise location of the interaction between TIIAS and hP2X7 is unknown. Several lines of evidence point to an intracellular localization of the TIIAS binding site, as follows: 1) the delayed on- and offset of hP2X7 inhibition with extracellularly applied TIIAS, 2) the activity of intracellularly perfused TIIAS, and 3) the voltage dependence of the inhibition with regard to onset times and efficiency. An intracellular binding site would distinguish TIIAS from other gating and/or binding modifiers of hP2X7, such as clemastine or perazines, which presumably act via an extracellularly accessible point of attack (Nörenberg et al., 2011; Hempel et al., 2013), or ivermectin, which has been proposed to intercalate between transmembrane helices of P2X4 and possibly also human P2X7 (Silberberg et al., 2007; Nörenberg et al., 2012). Binding of TIIAS to the intracellular portion of the receptor may require positively charged amino acids to neutralize the negative charge of the drug.

A still unresolved question pertains to the route by which TIIAS may enter the cells to attack its putative intracellular binding site. According to known predictors of membrane permeability elaborated by Pham-The et al. (2013), calculated
physicochemical properties of TIIAS (logD = 1.55 at pH 7.3, polar surface area = 104.5 Å², molecular mass = 396.4 g mol⁻¹) hint to a limited passive lipid membrane permeability of TIIAS. Because TIIAS is negatively charged at pH > 2, specific transporter proteins, such as organic anion-transporting polypeptides (OATPs), may be required to facilitate the cellular uptake of the compound. OATPs have a rather broad substrate profile (TIIAS or AZ10608120 (AZ)). Nigericin was used as a positive control. n = 7; significant differences are indicated (*P < 0.05; **P < 0.01).

Fig. 9. IL-1β secretion by human macrophages. IL-1β secretion by human monocyte-derived macrophages was detected using an ELISA kit. All samples but negative control were primed with 0.1 μg ml⁻¹ LPS for 24 hours. Unprimed macrophages released significantly less IL-1β than those exposed to LPS. IL-1β secretion further augmented upon treatment with 1 mM ATP. This could be prevented applying P2X7 antagonists TIIAS or AZ10608120 (AZ). Nigericin was used as a positive control. n = 7; significant differences are indicated (*P < 0.05; **P < 0.01).

To prove their effectiveness with regard to treatment of rheumatoid arthritis.

At present, possible beneficial effects of TIIAS cannot be judged due to the fact that the drug has not been tested in human pathologies that have been linked to P2X7. Proinflammatory actions of P2X7 mediated by macrophages contribute to bone and cartilage diseases (Baroja-Mazo and Pelegrín, 2012). Our results show that the P2X7-specific antagonist A438079 or TIIAS largely inhibited the ATP-induced Ca²⁺ influx and YO-PRO-1 uptake in hMDM. Accordingly, a P2X7-induced release of the proinflammatory cytokine IL-β from LPS-primed macrophages (Carroll et al., 2009) was counteracted by TIIAS. It has also been described that P2X7 activation induces release of matrix metalloproteinase 9 from mononuclear cells (Gu and Wilely, 2006), which in turn degrades extracellular matrix in rheumatoid arthritis (Itoh et al., 2002). Because reactive oxygen species exacerbate joint inflammation, the antioxidative properties of TIIAS may add to its potential therapeutic value. Thus, the bimodal activity of TIIAS may provide a treatment option for inflammatory joint diseases that involve P2X7 activation. Other human diseases in which P2X7 activation or polymorphisms have been implicated include inflammatory or neuropathic pain, neurodegenerative diseases, and depression (North and Jarvis, 2013).

Because human P2X7 is among the most potently TIIAS-modulated molecular targets identified to date, its inhibition by the drug may guide the future clinical development of TIIAS.

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Authorship Contributions

Participated in research design: Fischer, Schaefer, Nörenberg.
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