Tanshinone II A sulfonate but not tanshinone II A acts as potent negative allosteric modulator of the human purinergic receptor P2X7

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Abbreviations. A438079, 3-[[5-(2,3-dichlorophenyl)-1H-tetrazoyl-1-yl]methyl]pyridine; AAPH, 2,2′-azobis(2-methylpropionamidine) dihydrochloride; AZ10606120, N-[[2-[[2-[(2-hydroxyethyl)amino]ethyl]amino]-5-quinolinyl]-2-tricyclo[3.3.1.13,7]de-1-ylacetamide; [Ca^{2+}]_{i}, intracellular free Ca^{2+} concentration; DIC, divalent cations; HBS, HEPES-buffered saline; hMDM, human monocyte-derived macrophages; LPS, lipopolysaccharide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; OATP, organic anion transporting polypeptide; PLA2, phospholipase A2; TIIA, tanshinone II A; TIIAS, tanshinone II A sulfonate; Trolox, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid

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Abstract

Tanshinone II A sulfonate (TIIAS) was identified as a potent, selective blocker of purinergic receptor P2X7 in a compound-library screen. In this study, a detailed characterization of the pharmacological effects of TIIAS on P2X7 is provided. Since TIIAS is a derivative of tanshinone II A (TIIA) and both compounds have been used interchangeably, TIIA was included in some assays. Fluorometric and electrophysiological 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 Ca\(^{2+}\) influx via human P2X7 (hP2X7) with an \(IC_{50}\) 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. Electrophysiological experiments revealed a non-competitive 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 Ca\(^{2+}\) influx, YO-PRO-1 uptake and release of the pro-inflammatory cytokine IL-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 pharmacological properties.
Introduction

The purinergic P2 receptor family comprises G-protein-coupled P2Y receptors as well as P2X receptors which 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 pathological conditions. Inflammatory processes are enhanced by stimulation of P2X7 by triggering caspase-1-mediated 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).

In order to identify possible P2X7 modulators, we have recently performed a compound-library screen and described a number of P2X7-potentiating drugs (Nörenberg et al., 2011). Among P2X7-inhibiting compounds discovered in that screen, TIIAS stood out due to its potency to fully block P2X7-mediated Ca\textsuperscript{2+} influx at low micromolar concentrations. A literature review revealed that TIIAS has been developed as a water-soluble derivative of tanshinone II A (TIIA), a pharmacologically active compound of the dried root of the Chinese red sage (Salvia miltiorrhiza). Whereas there is little data concerning the clinical effects of TIIAS, TIIA has indeed been used to treat possibly P2X7-related diseases (Dong et al., 2009; Wang et al., 2010). Furthermore, both drugs have been applied in experimental studies as comparable, interchangeable pharmacological agents (Shang et al., 2012). In this context, we decided to characterize their pharmacological effects on P2X7 and to assess their selectivity over other P2X receptor subtypes.
Suprisingly, TIIAS but not TIIA strongly and potently inhibited Ca\textsuperscript{2+} entry or ionic currents through human P2X7. Thus, the development of TIIAS did not only yield pharmacokinetic improvements such as increased water solubility and oral bioavailability (Zhu et al., 2013), but also resulted in 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 electrophysiological methods. Inhibition of ATP-triggered Ca\textsuperscript{2+} entry, YO-PRO-1 uptake and of IL-1β release demonstrated that TIIAS also acts on native P2X7 in human macrophages. Thus, TIIAS has a distinct pharmacological profile and might be a promising agent for further testing in models of P2X7-related diseases.
Material & Methods

Cell culture procedures

Human embryonic kidney 293 (HEK) cells stably transfected with the human P2X7 receptor (HEK_{hP2X7}) or the human P2X4 receptor (HEK_{hP2X4}) 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, USA). 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 HEK_{mP2X7} cell line. HEK_{mP2X7} were maintained at 37°C and 5% CO₂ in DMEM with 4.5 mM D-glucose, 10% FCS, 2 mM L-glutamine, 100 μg ml⁻¹ zeocin (Invitrogen, Carlsbad, CA, USA). Stably transfected Flp-In T-REx cell lines inducibly expressing the rat P2X7 (HEK_{rP2X7}) or rat P2X2 (HEK_{rP2X2}) 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, USA), and 15 μg ml⁻¹ blasticidin (Invivogen). Expression of the integrated gene was induced by adding 1 μg ml⁻¹ tetracyclin 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) express higher levels of P2X7 (Gudipaty et al., 2001) and were obtained after adhesion-induced differentiation of monocytes and maintained on cell culture dishes (for
IL-1β ELISA) or on 25-mm glass coverslips (for single cell [Ca²⁺]i analysis and YO-PRO-1 uptake assays) for 5-7 days prior to the experiments.

**Intracellular [Ca²⁺] analysis**

[Ca²⁺]i analysis in suspensions of HEK cells expressing human, mouse and rat P2X7, human P2X4 and rat P2X2 was conducted in a fluorescence imaging plate reader essentially as described before (Nörenberg et al., 2012) with the following modifications: fluo-4/AM-loaded cells were resuspended in HEPES-buffered saline (HBS), containing 130 mM NaCl, 6 mM KCl, 5.5 mM d-glucose, 10 mM HEPES (pH 7.4 with NaOH), supplemented with 1 mM CaCl₂ and 1 mM MgCl₂. Cells were dispensed into 384-well plates and exposed to the indicated modulators for 15 minutes. In the case of HEK₉₆P₂X₄ and HEKᵣP₂X₂, cells were also treated with 2 µM thapsigargin to deplete intracellular Ca²⁺ stores and to eliminate P2Y-triggered responses. Fluorescence intensities were monitored during injection of ATP, applied at final concentrations of 1 mM for P2X7 receptors, or 3 µM for P2X4 and P2X2. Background-corrected fluorescence intensities were normalized to the initial intensities (F₀) to compensate for uneven loading and fluorescence detection sensitivity.

Single cell [Ca²⁺]i analysis was performed as described (Nörenberg et al., 2011). Adherent hMDM were treated with 100 ng ml⁻¹ lipopolysaccharide (LPS) for 24 hours, and loaded with fura-2/AM (3 µM; biomol, Hamburg, Germany) in HBS buffer containing 2 mM CaCl₂ and 0.2% (w vol⁻¹) BSA. The coverslips were rinsed and mounted in a bath chamber filled with HBS containing no BSA. Modulators and agonists were either superfused or directly added to the bath chamber. Background fluorescence intensities were obtained by mechanically detaching the cells from the coverslips. Regions of interest were defined over single cells.
Fluorescence intensities over these regions were averaged and the background signal was subtracted prior to spectral calibration as described (Lenz et al., 2002).

**YO-PRO-1 uptake assay**

HEK cell lines were grown to confluent monolayers in 25-cm² cell culture flasks, harvested with trypsin and resuspended in HBS buffer, containing low divalent cation concentrations (no MgCl₂ and 0.1 mM CaCl₂; low DIC). YO-PRO-1 (1 µM; Life Technologies, Carlsbad, CA, USA) was added to the cell suspension before dispensing it into 384-well microtiter plates prefilled with modulating compounds. Experiments were conducted in a fluorescence imaging plate reader setup (Nörenberg et al., 2012).

To monitor 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 Ca²⁺ imaging. Fluorescence was excited at 475 nm and emission was recorded through a 515 nm long-pass filter with a cooled CCD camera (PCO Sensicam, Kelheim, 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 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. Briefly, approximately 25,000 HEK₉₉P₂X₇ cells per 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 dimethyl sulfoxide (DMSO). Absorption was read in a plate-reader device (Polarstar Omega, BMG Labtech, Offenburg, Germany) at 560 nm, and background values obtained at 670 nm were subtracted.

Antioxidative capacity of TIIAS and TIIA

Trolox-equivalent antioxidant capacities of modulators were determined with the oxygen radical absorption capacity (ORAC) assay. Experiments were conducted in a plate-reader device (Polarstar Omega, BMG Labtech, Offenburg, Germany) according to an established protocol (Ganske, 2006). Capacities to prevent loss in fluorescein fluorescence were assessed for various concentrations (0.78-50 µM) of 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.

Electrophysiological 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 (~305 mOsm l⁻¹; pH 7.3 with NaOH), supplemented with 1 mM MgCl₂, 2 mM CaCl₂ (standard DIC) or with 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 mOsm l⁻¹; pH 7.3
with KOH), and in some experiments also 3 mM of MgCl₂. Drugs were applied to patched cells by means of a pressurized superfusion system (DAD-12, Adams and List, New York, USA). 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).

**IL-1β ELISA**

LPS-primed (100 ng ml⁻¹ for 24 hours) hMDM were rinsed twice and incubated in low DIC HBS with P2X7 modulators added to the buffer 15 minutes before stimulation. ATP (1 mM) was applied for 30 minutes. Alternatively, treatment with the potassium ionophore nigericin (20 µg ml⁻¹) was used as a positive control (Perregaux et al., 1992). All experiments were conducted at 37°C. Cleared supernatants (1000 g 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, USA) according to manufacturer’s instructions.

**Materials and compounds**

P2X7 antagonists A438079 and AZ10606120 were from Tocris, Bristol, UK, and tanshinone II A from MicroSource Discovery Systems, Gaylordsville, CT, USA. Tanshinone II A sulfonate was obtained from Bosche Scientific, New Brunswick, NJ, USA. Unless otherwise stated, all other chemicals were from Sigma-Aldrich, St. Louis, MO, USA.

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 at 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 HEK_{hP2X7} cells. ATP stock solutions were routinely readjusted to pH 7.3.

Data evaluation and statistical analysis

Predictions of physicochemical properties of TIIAS (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 $E = E_{\text{min}} + (E_{\text{max}} - E_{\text{min}}) / (1 + ([M] / EC_{50})^{nH})$, 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 $I_{\text{ATP}(t)} = A_0 + A_f \exp(\tau_{\text{off}, \text{fast}} \cdot t) + A_s \exp(\tau_{\text{off}, \text{slow}} \cdot t)$, where $I_{\text{ATP}(t)}$ is the ATP-induced current at time $t$ and $A_0$, $A_f$, and $A_s$ denote the extrapolated effect levels at infinite time and the initial signal amplitude at $t = 0$ s. A monoexponential function ($I_{\text{ATP}(t)} = A_0 + A_e \exp(\tau)$) 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 (ANOVA) 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. 1A,B; \(n = 7\), independent experiments). The half-maximally inhibitory concentration \(IC_{50}\) of increases in the fluo-4 fluorescence was 4.3 ± 0.8 µM. 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 µM (Fig. 2; \(n = 4\) independent experiments). Ca\(^{2+}\) entry and YO-PRO-1 uptake were reduced by approximately 80% by 10 µM TIIAS. We set this concentration as a standard for electrophysiological measurements and experiments involving hMDM.

In order to analyze possible species differences, HEK\(_{mP2X7}\) and HEK\(_{rP2X7}\) cells were subjected to similar Ca\(^{2+}\) influx experiments. TIIAS had a low potency to inhibit Ca\(^{2+}\) signals in ATP-stimulated HEK\(_{mP2X7}\) cells and was essentially inactive at rat P2X7. With test concentrations up to 50 µM, the \(IC_{50}\) of TIIAS was not reached in either cell line (Fig. 1A,C; \(n = 8\) independent experiments, each). The weak inhibitory activity of TIIAS at rat P2X7 was confirmed in YO-PRO-1 uptake experiments (Fig. 2). A438079, a known P2X7 antagonist (King, 2007), was included in some experiments, confirming that P2X7 activation caused the observed Ca\(^{2+}\) influx (Fig. 1A) and YO-PRO-1 uptake (data not shown).

Intriguingly, TIIA in concentrations up to 50 µM failed to reduce P2X7-mediated Ca\(^{2+}\) influx in HEK\(_{hP2X7}\) (Fig. 1B; \(n = 5\) independent experiments) and in HEK\(_{rP2X7}\) cells (data not shown) stimulated with 1 mM ATP.
Selectivity of TIIAS over different P2X subtypes was evaluated in HEK<sub>p2x2</sub> and HEK<sub>h2x4</sub> cells. Concentrations up to 25 µM TIIAS did not affect the Ca<sup>2+</sup> response upon stimulation with ATP in HEK<sub>p2x2</sub> cells, an impaired Ca<sup>2+</sup> influx could only be seen after exposure to the highest test concentration of TIIAS. An IC<sub>50</sub> of 35 µM TIIAS was determined for this cell line. TIIAS lacked effects on Ca<sup>2+</sup> influx in HEK<sub>h2x4</sub> 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. While 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 to solvent controls), viability of cells treated with concentrations up to 250 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 to 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 AAPH-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 non-competitive antagonist and delays the ATP-induced hP2X7 gating**

To characterize the impact of TIIAS on ionic currents through P2X7, we performed electrophysiological experiments in the whole-cell configuration. When applied for 4 s and at 60-s intervals, repetitive application of 1 mM ATP to HEK\(_{hP2X7}\) cells led to a characteristic run-up of hP2X7 currents, reaching a steady-state after 6-7 challenges, irrespective of the absence and presence of TIIAS (10 \( \mu \)M), which was added to the superfusion medium 15 min prior to first pulse of ATP stimulation (Fig. 3A,B). After current run-up had been accomplished, we obtained concentration-response curves by applying ATP at concentrations of 10 \( \mu \)M to 30 mM (Fig. 3A,B). TIIAS (10 \( \mu \)M) 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 \( EC_{50} \) and \( nH \) values remained almost unchanged in the presence of TIIAS (Fig. 3C,D). The non-surmountable inhibition of ATP-induced hP2X7 currents and the unchanged \( EC_{50} \) of ATP are indicative of a non-competitive, allosteric inhibitory mechanism.

In the presence of TIIAS, the onset of ATP-induced P2X7 currents was slowed down (Supplemental Figure 1A). In HEK\(_{hP2X7}\) 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 ms. Exposure of the same cells to 10 \( \mu \)M TIIAS for 6 min resulted in slower rise times of
536.4 ± 43.9 ms (n = 7; P < 0.001; Supplemental Figure 1A,C). The channel deactivation upon wash-out of the activator was not affected by TIIAS (Supplemental Figure 1B,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. 3E,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 mM and 30 mM of ATP (365.5 ± 9.0 ms and 366.1 ± 12.8 ms; 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 s at short (30 s) 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 s (n = 7; Fig. 4A,B). In the continuous presence of TIIAS, current amplitudes were reduced within 5-6 min 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 s.

In accordance with our Ca$^{2+}$ imaging and YO-PRO-1 fluorescence measurements, TIIAS was less efficient or essentially inactive in suppressing currents through mouse or rat P2X7 (Supplemental Figure 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 electrophysiological data is 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. 4C,D).

Notably, TIIAS also inhibited hP2X7 in the presence of more physiological 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 Figure 3A,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 Figure 3C,D), which was paralleled by a doubling of current rise times from 480.6 ± 50.7 ms to 972.2 ± 46.4 ms in the absence and presence of 10 µM TIIAS, respectively. Since the activity of TIIAS was independent of the used bath solution, we performed all following electrophysiological 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 min period after hP2X7 run-up had been completed, the nucleotide-induced current amplitudes remained at the sensitized level (n = 7; Fig. 5A,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; Fig. 5C,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 co-application protocol, in which cells were only exposed to the drug during the stimulation with 1 mM ATP ($n = 7$; Supplemental Figure 4).

_TIIAS inhibits hP2X7 currents by a non-pore block mechanism and via an intracellular point of attack_

At physiological 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 s) 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 to a holding potential of -80 mV ($P < 0.001$, $n = 8$; Fig. 6C). Of note, TIIAS did not cause shifts of the reversal potential (Fig. 6A,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 protocol (500 ms duration; Supplemental Figure 5A-C) or in a fast voltage ramp protocol (1 s duration; Supplemental Figure 5D,E) that would resolve a voltage-dependent behavior in the sub-second time range.
Alternatively, the slowly (> 1 s) 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 $\tau_{on}$ for the TIIAS effects, when HEK cells were clamped to +40 mV ($n = 10$) instead of -80 mV ($n = 8$; Supplemental Figure 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 µM TIIAS, ATP (1 mM)-triggered P2X7 currents reached a lower density compared to experiments performed with standard pipette solutions (Fig. 7A,B, compare Fig. 4A,B). In TIIAS-perfused cells, extracellular superfusion with TIIAS (10 µM) exerted only an inhibition by 13.7% ± 8.1% (Fig. 7C,D), indicating that a majority of binding sites may have been preoccupied by the intracellularly applied drug. Interestingly, intracellular TIIA (10 µM) did not prevent the inhibitory action of superfused TIIAS ($n = 8$; Supplemental Figure 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). While 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 µM and 10 µM, respectively (Lazarowski et al., 1995; Garcia-Guzman et al., 1997). Superfusion of LPS-primed hMDM with 50 µM ATP, a subthreshold concentration at hP2X7 (Fig. 3), caused a rapid and transient
rise in [Ca$^{2+}$], presumably triggered via P2X4- and P2Y$_2$-like receptors (Fig. 8A). After intracellular Ca$^{2+}$ concentration returned close to basal values, brief application of 1 mM ATP again led to increases in [Ca$^{2+}$], 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 [Ca$^{2+}$], ($P < 0.01$; $n = 19-20$; Fig. 8A,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 µM or 10 µM TIIAS ($n = 8$; Fig. 8C,D).

**TIIAS inhibits IL-1β secretion by hMDM**

Since P2X7 activation in hMDM has been shown to induce maturation and release of pro-inflammatory 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 then 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 (Perregaux 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 \( \mu \)M. Our electrophysiological experiments suggested that TIHAS acts as a non-competitive 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 TIHAS 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 \textit{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 TIHAS. Human P2X4, the most closely related purinergic receptor (Volonté et al., 2012), was not modulated by TIHAS and concentrations of 50 \( \mu \)M were necessary to affect rat P2X2. Our findings may constitute a starting point for target-oriented studies using TIHAS to treat P2X7-related diseases in models comprising human receptor isoforms. Based on our findings, beneficial results obtained after treatment with TIIA cannot be attributed to an inhibition of P2X7. Regarding TIHAS, 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 is very scarce. The drug has been used in man (Wang et al., 2013), but controlled clinical trials have not yet been carried out (Raja, 2013).

Currently known targets of TIHAS include Ca\(^{2+}\)-activated potassium channels and carboxylesterase 2, which are affected at similar or higher TIHAS 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 TIHAS has been demonstrated by Xu et al. (2009). In this study, indirect inhibition of LPS-dependent PLA2 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: (i) the delayed on- and offset of hP2X7 inhibition with extracellularly applied TIIAS, (ii) the activity of intracellularly perfused TIIAS, and (iii) 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 (log$D = 1.55$ at pH 7.3, polar surface area = 104.5 Å$^2$, mol. wt. = 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 specificity (Roth et al., 2012), and some members (e.g. OATP2B1, OATP3A1, OATP4C1) have already been shown to be expressed in human macrophages (Moreau et al., 2011). Interestingly, OATv1, a voltage-driven organic anion
transporter that has been cloned from renal proximal tubule cells, may also fulfill basic requirements as putative TIIAS entrance route, because its inward transport rate is increased by membrane depolarization (Jutabha et al., 2003), possibly explaining the slow onset and the voltage-dependence of P2X7 inhibition by extracellularly applied TIIAS.

The biological activity of TIIAS to block human P2X7 does not qualify the use of this compound as a superior tool compound in cell-biological assays. More potent and selective P2X7 inhibitors have been developed, including the competitive antagonists A438079 (Nelson et al., 2006) and A740003 (Honore et al., 2006), or the non-competitive inhibitor AZ11645373 (Stokes et al., 2006). Of note, the potency of the competitive inhibitors to inhibit P2X7 is also slightly higher towards human and rat P2X7 orthologs compared to the mouse P2X7 (Donnelly-Roberts et al., 2009). Nonetheless, the marked species difference of TIIAS with low biological activity on rodent P2X7 as well as its non-surmountable inhibitory action more closely resemble the effects seen with AZ11645373, which is poorly active on rat, mouse or guinea pig P2X7 (Michel et al., 2009). Although a weak activity on rodent P2X7 limits their use as tools in preclinical disease models, two P2X7 inhibitors that had entered phase II of clinical trials, CE-224,535 and AZD9056 (structure undisclosed), are also non-competitive inhibitors with strong preference to block human, but not rodent P2X7 isotypes (Duplantier et al., 2011; NIH/NCATS fact sheets: www.ncats.nih.gov/files/CE-224535.pdf and www.ncats.nih.gov/files/AZD9056.pdf). However, both clinical development candidates failed 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. Pro-inflammatory 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 Ca\textsuperscript{2+} influx and YO-PRO-1 uptake in hMDM.
Accordingly, a P2X7-induced release of the pro-inflammatory cytokine IL-1β 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 Wiley, 2006), which in turn degrades extracellular matrix in rheumatoid arthritis (Itoh et al., 2002). Since 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). Since human P2X7 is among the most potently TIIAS-modulated molecular targets identified so far, its inhibition by the drug may guide the future clinical development of TIIAS.
Acknowledgements

We thank Günther Schmalzing from Aachen, Germany, for providing the rat P2X7 Flp-In T-REx cell line. Annette Nicke, Max Planck Institute for Experimental Medicine, Göttingen, Germany, kindly provided the HEK_mP2X7 cell line.
Authorship contributions

*Participated in research design:* Fischer, Schaefer, Nörenberg

*Conducted experiments:* Kaiser, Sobottka, Fischer, Nörenberg

*Performed data analysis:* Kaiser, Nörenberg

*Wrote or contributed to the writing of the manuscript:* Kaiser, Schaefer, Nörenberg
References


Footnotes

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Figure legends

Fig. 1. Concentration-dependence of TIIAS-mediated inhibition of calcium influx via P2X7 and selectivity over P2X2 and P2X4. Cell lines HEK\textsubscript{hpP2X7}, HEK\textsubscript{rpP2X7}, HEK\textsubscript{mpP2X7}, HEK\textsubscript{hpP2X4} and HEK\textsubscript{rpP2X2} were loaded with fluo-4/AM. Ca\textsuperscript{2+} influx was triggered with 1 mM or 3 µM ATP, respectively, in the presence of different concentrations of TIIA, TIIAS or 10 µM A438079. Fluorescence increase was analyzed and normalized to control measurements without modulators. n = 5 for HEK\textsubscript{hpP2X7} (TIIA), n = 7 for HEK\textsubscript{hpP2X7} (TIIAS), n = 8 for HEK\textsubscript{rpP2X7} and HEK\textsubscript{mpP2X7}, n = 4 for HEK\textsubscript{hpP2X4} and HEK\textsubscript{rpP2X2}. (A) Representative traces for HEK\textsubscript{hpP2X7} (left panel), HEK\textsubscript{rpP2X7} (center panel) and HEK\textsubscript{mpP2X7} (right panel). Light grey lines represent positive controls, i.e. cells stimulated with ATP in the absence of antagonists (ctrl). Black lines illustrate measurements in the presence of 12.5 µM TIIAS while dark grey lines show those recorded in the presence of 10 µM A438079 (A43). (B) Concentration-response-curves for TIIA- and TIIAS-mediated modulation of Ca\textsuperscript{2+} influx in HEK\textsubscript{hpP2X7}. (C) Concentration-response-curves for TIIAS-mediated reduction of Ca\textsuperscript{2+} influx in HEK\textsubscript{rpP2X7} and HEK\textsubscript{mpP2X7}. (D) Concentration-response-curves for TIIAS-mediated reduction of Ca\textsuperscript{2+} influx in HEK\textsubscript{rpP2X2} and HEK\textsubscript{hpP2X4}.

Fig. 2. Concentration-dependence of TIIAS-mediated inhibition of YO-PRO-1 uptake via P2X7. YO-PRO-1 uptake was induced in HEK\textsubscript{hpP2X7} and HEK\textsubscript{rpP2X7} with 1 mM ATP in the presence of different concentrations of TIIAS. Fluorescence increase was analyzed and normalized to control measurements without TIIAS. n = 4 for both cell lines.

Fig. 3. TIIAS affects ATP efficacy but not potency possibly by decelerating hP2X7 current activation. Shown are representative whole cell currents (A, 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 ($\tau_{\text{off, fast}}$) and slow ($\tau_{\text{off, slow}}$) time constants of current decay versus
ATP concentration (F), obtained in the absence (A, open circles in C,D,E and F; $n = 13$) or
presence of 10 $\mu$M TIIAS, which was pre-applied for 6 min before and throughout the
experiments (B, filled circles in C,D,E and F; $n = 14$). Whole cell recordings were performed
in HEK$hP2X7$ cells at a holding potential of -60 mV in a low DIC bath solution. Cells were
repetitively stimulated for 4 s at 60 s 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 electrophysiological 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 Figure 1).

**Fig. 4.** Delayed inhibition by TIIAS but not by TIIA of ATP-induced hP2X7 currents. Shown
are representative whole cell currents (A, C) and corresponding plots of ATP-evoked peak
current densities *versus* time (B, D). ATP (1 mM) was repetitively applied at a holding
potential of -60 mV and in a low DIC bath, for 1.5 s at 30 s intervals. TIIAS ($n = 7$) or TIIA
($n = 10$; 10 $\mu$M each) was added after current run-up was completed (see superimposed
responses to the first, eleventh and twelfth ATP application in (A) and (C) and remained in
the bath throughout for 6 min as illustrated in (C) and (D). (B) Monoexponential fits of the
onset of and recovery from TIIAS induced hP2X7 inhibition are superimposed to data, and the respective time constants ($\tau_{on}$, $\tau_{off}$) are depicted.

**Fig. 5.** TIIAS inhibits hP2X7 currents in a time- but not use-dependent manner. Shown are representative whole cell currents (A, C) and corresponding plots of ATP-evoked peak current densities versus time obtained in the absence (B) or presence of 10 µM 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 12th pulse of ATP.

**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 s and at 30 s 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 mV to 40 mV) for the 30 s between subsequent ATP pulses to record a current-voltage ($IV$)-relation under control conditions ($I_{ctrl}$; middle panel). TIIAS (10 µM) was then pre-applied for 6 min, 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) $IV$-plots from peak current densities obtained in $n = 8$ cells. The connecting lines are derived from fitting the data to linear ($I_{ctrl}$) or 3rd 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).

**Fig. 7.** TIIAS applied intracellularly via the patch pipette substitutes for the extracellularly applied drug. Shown are representative whole cell currents (A, C) and corresponding plots of ATP-evoked peak current densities versus time obtained in the absence (B) or presence of
10 µM of extracellular TIIAS (D; n = 7 each). Whole cell recordings were acquired essentially as shown in Fig. 4 but with a pipette solution containing also 10 µM of TIIAS. Note the only weak effect of extracellular TIIAS under these conditions (C, D).

**Fig. 8.** ATP-triggered calcium influx and YO-PRO-1 uptake in human macrophages. (A) Ca^{2+} influx mediated by P2X4 and rapidly desensitizing P2Y receptors was induced in fura-2/AM-loaded hMDM by superfusion with 50 µM ATP. Subsequent, repeated application of 1 mM ATP caused Ca^{2+} influx via P2X7 (left panel). Note that addition of P2X7 antagonists TIIAS (center panel) and A438079 (right panel) prevented an increase of \((\text{Ca}^{2+})_\text{i}\) upon the 2nd application of 1 mM ATP. (B) Statistical analysis of n = 19-20 experiments. Time-points are displayed in (A). Significant differences are indicated (** P < 0.01). (C) Representative traces of single cell YO-PRO-1 uptake in human monocyte-derived macrophages. Continuous YO-PRO-1 uptake upon stimulation with 1 mM ATP (arrow) as seen in positive controls (solvent, dotted line) and samples treated with P2X7 antagonists TIIAS or 10 µM A438079 (short-dashed and continuous lines). (D) Statistical analysis of n = 6-9 experiments. Significant differences to positive control are indicated (* P < 0.05).

**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 AZ10606120 (AZ). Nigericin was used as a positive control. n = 7. Significant differences are indicated (* P < 0.05; ** P < 0.01).
### Tables

**Table 1.** Potency of TIIAS to modulate different biological activities.

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Figure 1

(A) Time course of ATP-induced calcium responses in hP2X7, rP2X7, and mP2X7 with control, TIIAS, and A43 modulations. 

(B) Log-log plot of calcium response percentage versus log [modulator] for hP2X7 with TIIA and TIIAS modulations. 

(C) Log-log plot of calcium response percentage versus log [TIIAS] for rP2X7. 

(D) Log-log plot of calcium response percentage versus log [TIIAS] for hP2X4.
Figure 2

YO-PRO-1 response (%) against log [TlIAS] (M) for hP2X7 (black squares) and rP2X7 (gray circles).
Figure 5

A. HEK_hP2X7 - low DIC

B. ATP 1 mM

C. tanshinone IIA sulfonate 10 µM

D. tanshinone IIA sulfonate 10 µM

\[ t_{off} = 68.8 \pm 10.4 \text{ s} \]
Figure 8

A

1 mM ATP (1) (2) solvent 1 mM ATP (1) (2) 10 μM TIiAS 1 mM ATP (1) (2) 10 μM A438079

[Ca^{2+}] (nM)

50 μM ATP

Time (min)

B

\[ \text{2^{nd} peak / 1^{st} peak} \]

control 10 μM TIiAS

C

YO-PRO-1 fluorescence (arb. units)

solvent 3 μM TIiAS A438079 10 μM TIiAS

Time (min)

D

YO-PRO-1 fluorescence (arb. units)

solvent 3 μM TIiAS 10 μM TIiAS 10 μM A438079
Figure 9

IL-1β release (pg ml⁻¹)

LPS
ATP
antag.

-  -  -  -  -  -  +  +  +  +  +  +  +  +

nigerin

**

*
Tanshinone II A sulfonate but not tanshinone II A acts as potent negative allosteric modulator of the human purinergic receptor P2X7

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Fig. S1. Assessment of the kinetic properties of TIIAS-mediated hP2X7 Inhibition. Whole cell recordings were performed in HEK_Hp2X7 cells at a holding potential of -60 mV. ATP (1 mM) was repetitively applied in a bath solution containing 0.1 mM Ca\(^{2+}\) and no added Mg\(^{2+}\) (low DIC) for 1.5 s at 30 s intervals. TIIAS (10 µM) was added after current run-up was completed and remained in the bath for 6 min. (A) 10% to 90% rise times derived from the normalized activating phases of the currents, also shown in the inset, which were recorded after hP2X7 run-up (black traces, control) and after 6 min in the presence of TIIAS (grey traces). Dashed lines: levels of the indicated percentage of the maximum current obtained with ATP in the absence and presence of TIIAS. (B) Decaying phases of the same currents as in (A) with superimposed biexponential fits, as well as the fast (\(\tau_{\text{off, fast}}\)) and slow (\(\tau_{\text{off, slow}}\)) deactivation time constants derived thereof. (C) and (D) Statistical analysis of current rise times (C) and deactivation time constants (D) in the absence (open bars) and presence of TIIAS (filled bars) from \(n = 7\) independent experiments as shown in (A,B). * \(P < 0.05\), significant difference between current rise time in absence and presence of TIIAS.
Fig. S2. Currents mediated by rat, mouse and human P2X7 receptors display differential sensitivities towards TIIAS. Shown are representative whole cell currents (A, C) and corresponding plots of ATP-evoked peak current densities versus time (B, D) obtained from HEK-p2x7 (A, B) or HEK-mP2X7 cells (C, D). ATP (1 mM) was repetitively applied at a holding potential of -60 mV and in a low DIC bath, for 1.5 s at 30 s intervals. TIIAS was added after current run-up was completed (see superimposed responses to the first, fifteenth and sixteenth ATP application in (A) and (C)) and remained in the bath throughout for 6 min as illustrated in C) and D). Inset in D): statistical analysis of the effects of TIIAS (filled bars) at human, rat and mouse P2X7 in comparison to control experiments (open bars) performed essentially as those shown in A) and B), but without addition of TIIAS. TIIAS evoked inhibition was calculated as percentage of the peak current densities remaining after 6 min in the presence of TIIAS (or at the corresponding time point in control experiments), when compared with those achieved upon current run-up was completed. * $P < 0.05$, significant differences from controls; $ns$, not significantly different from control; ** $P < 0.05$, significant difference between TIIAS effects at hP2X7 and mP2X7; $n = 7 – 10$. 
Fig. S3. TIIAS exerts a delayed inhibition of ATP-induced hP2X7 currents in the presence of standard ambient divalent cation concentrations. Shown are representative whole cell currents (A, C) and corresponding plots of ATP-evoked peak current densities versus time obtained in the absence (B) and presence of 10 µM TIIAS (D; n = 11 each). Whole cell recordings were performed in HEK-hP2X7 cells at a holding potential of -60 mV. ATP (1 mM) was repetitively applied in a bath solution containing 2 mM Ca²⁺ and 1 mM Mg²⁺ (standard DIC) for 1.5 s at 30 s intervals. TIIAS was added after current run-up was completed and remained in the bath throughout for 6 min as illustrated in (C) and (D).
Fig. S4. Coapplied TIIAS lacks impact on ATP-induced hP2X7 currents. (A) Whole cell currents were recorded essentially as shown in Fig. S3 but in low DIC, while TIIAS (10 µM) was only acutely applied together with ATP (1 mM). (B) Plot of ATP-evoked peak current densities versus time from n = 7 experiments performed as illustrated in panel (A).
Fig. S5. The inhibitory impact of TIIAS on hP2X7 is not mediated by a fast pore block mechanism. Whole cell currents were recorded from HEK-hP2X7 cells in low DIC. (A) Fast (500 ms) voltage-steps (lower panels) to -80 mV, +40 mV and back to -80 mV were imposed from a holding potential of -60 mV after current run-up was completed (not shown), before (1) and around the peak response to ATP (1 mM for 4 s; (2); upper panels) in the absence (left) and presence of TIIAS (10 µM for 6 min; right). (B) Superimposed leak-corrected currents in the presence of ATP before ($I_{ctrl}$, black trace) and after superfusion with TIIAS ($I_{TIIAS}$, grey trace) from the experiment in (A), which were obtained by subtracting the respective responses to the voltage-step protocol in the absence of ATP ($2 - 1$). Note the lack of time-dependent relaxation of the TIIAS-inhibited currents at both test potentials. (C) Plot of TIIAS evoked inhibition of hP2X7 currents versus test potential, assessed just after the onset (open squares; $t = 10$ ms) or shortly before the end of the test-pulses (filled squares; $t = 490$ ms), from $n = 10$ experiments similar to that illustrated in (A) and (B), revealing in addition a similar effect level of TIIAS at -80 mV and +40 mV. (D) Experiments were performed essentially as described in (A) except that fast voltage-ramps (from -80 mV to +4 mV, 1 s in duration; inset) were used instead of the step protocol. Shown are leak-subtracted, ATP-evoked currents in the absence ($I_{ctrl}$, black trace) and presence of TIIAS ($I_{TIIAS}$, grey trace) with superimposed linear regression fits (dashed lines). (D) Plot of TIIAS evoked inhibition of hP2X7 currents versus ramp potential ($n = 10$), corroborating the lack of impact of short-term voltage variations on the TIIAS effect.
Fig. S6. Long-term depolarization markedly accelerates the Development of hP2X7 inhibition by TIIAS. Shown are representative whole cell currents (A, C) and corresponding plots of ATP-evoked peak current densities versus time (B, D) obtained from HEKhP2X7 at a holding potential ($V_h$) of either +40 mV (A, B; $n = 10$) or -80 mV (C, D; $n = 8$). ATP (1 mM) was repetitively applied in a low DIC bath, for 1.5 s at 30 s intervals. TIIAS was added after current run-up was completed (see superimposed responses to the first, fifteenth and sixteenth ATP application in (A) and (C) and remained in the bath throughout for 6 min as illustrated in (C) and (D). Note the pronounced faster onset ($\tau_{on}$) of the TIIAS effect on ATP-induced outward currents at +40 mV in comparison to that for the inward currents at -80 mV.
Fig. S7. TIIA applied intracellularly via the patch pipette does not prevent the inhibitory action of extracellularly applied TIIS. Shown are representative whole cell currents (A) and a corresponding plot of ATP-evoked peak current densities versus time (B; \(n = 8\)). Whole cell recordings were acquired essentially as shown in Fig. S6 but with a pipette solution containing also 10 µM of TIIA.