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RESEARCH ARTICLE

Pharmacological evaluation of non-nucleotide purine derivatives as P2X7 antagonists for the treatment of neuroinflammation in traumatic brain injury

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Background and purpose: Traumatic brain injury (TBI) is considered to be a leading cause of mortality and disability worldwide. After TBI, innate immunity is rapidly activated in response to damage-associated molecular patterns, such as ATP release, recognised by P2X7 receptors. The P2X7-NLRP3 inflammasome axis has been identified as one of the main players in neuroinflammation. This study aimed to validate P2X7 receptors as therapeutic target for traumatic brain injury.

Experimental approach: P2X7 receptors were studied by genetic and pharmacological approaches. Six non-nucleotide purine derivatives were evaluated as P2X7 antagonists. Compounds that prevented LPS + ATP-induced IL-1β release from primary glial cultures were investigated in the closed-head injury TBI model *in vivo* in male mice. Finally, we evaluated soluble (s)P2X7 receptor plasmatic levels in a cohort of TBI patients.

Key results: *P2rx7*$^{-/-}$ mice showed an exaggerated inflammatory response 24 h post-TBI compared to control mice. However, animals treated with the selective

Abbreviations: ASC, apoptosis associated speck-like protein containing a CARD; MSU, monosodium urate crystals; MTT, thiazolyl blue tetrazolium bromide; NSS, neurological severity score; sP2X7 receptor, soluble P2X7 receptor; TBI, traumatic brain injury; Vh, vehicle.

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P2X7 antagonist JNJ-47965567 (30 mg kg^{-1} i.p.) 30 min post-TBI showed improved neurological and inflammatory parameters. The purine derivative ITH15004 was the most potent compound reducing IL-1β production *in vitro*. When administered *in vivo* 30 min post-TBI, ITH15004 (1 mg kg^{-1} i.p.) improved both neuro-behavioural and inflammatory markers at 24 h. In TBI patients, we showed a tendency towards increase in circulating sP2X7 receptor levels at 24 and 72 h post-TBI.

Conclusions and implications: These results highlight the importance of P2X7 receptors in the acute phase of TBI and present ITH15004 as a promising pharmacological tool to counteract P2X7 receptor-dependent neuroinflammation *in vivo*.

KEYWORDS
interleukin-1 beta, neuroinflammation, P2X7 receptor, traumatic brain injury

1 | INTRODUCTION

Traumatic brain injury (TBI) is one of the leading causes of disability and even death in the global population. Traumatic brain injury is a significant public health concern worldwide, with long-term physical, cognitive and emotional consequences for those affected (Kundu & Singh, 2023). TBI is a complex process involving a cascade of molecular, cellular and physiological events that result in immediate or delayed brain damage. One of the key factors in the pathology of TBI is neuroinflammation, where an immune response leads to further brain damage. After TBI, the initial physical damage triggers a series of biochemical events that result in the activation of brain-resident immune cells, that is, microglia and astrocytes. These activated glial cells release proinflammatory cytokines, chemokines and reactive oxygen species, leading to tissue damage and neuronal cell death. Given the central role of neuroinflammation in the pathology of TBI, targeting inflammatory pathways has emerged as a promising therapeutic strategy.

P2X receptors are homo-/hetero-trimers and act as ion channels through which K^+, Na^+ and Ca^{2+} enter the cell. The homotrimeric **P2X7 receptor** is widely expressed in the central nervous system (CNS) (Coddou et al., 2011; Matute, 2008) and it is significantly less sensitive to **ATP** than other P2X receptors. Its activation requires concentrations one to two orders of magnitude higher than those of other **P2X receptors**, suggesting that P2X7 activation occurs mainly in pathological foci where extracellular ATP concentrations are high. Indeed, P2X7 receptor has been proposed as a therapeutic target for many CNS diseases, such as **Alzheimer's disease (AD)** (Calzaferri et al., 2020), cerebral ischaemia (Wilmes et al., 2022) or epilepsy (Smith et al., 2023). Furthermore, P2X7 receptor has been presented as a good biomarker for the progression of various diseases (Aivar et al., 2023; Martínez-Banaclocha et al., 2023).

Current research indicates that P2X7 receptors plays a key role in the inflammatory response after TBI. P2X7 receptor is activated by high concentrations of extracellular ATP released upon tissue injury. Activation of P2X7 receptors leads to the production of inflammatory cytokines, such as tumour necrosis factor alpha (**TNF-α**) and **interleukin-1 beta (IL-1β)**, exacerbating the neuroinflammatory response. In addition, activation of P2X7 receptors are associated with the induction of pyroptosis, a form of inflammatory cell death caused by activation of **NLR family pyrin domain containing 3 (NLRP3)** inflammasome, contributing to brain tissue damage. Studies have demonstrated that blocking P2X7 receptors in TBI models has neuroprotective effects and improves behavioural outcomes by reducing inflammation and cell death (Liu et al., 2017; Nadal-Nicolás et al., 2016). Inhibition of P2X7 receptors has been shown to reduce levels of inflammatory cytokines and oxidative stress, and to

What is already known?

- A marked inflammatory response involving P2X7 receptors occurs after traumatic brain injury (TBI).
- P2X7R-NLRP3 inflammasome axis has been identified as one of the main actors in neuroinflammation.

What does this study add?

- P2X7 receptor deficiency does not affect neurological damage but aggravates inflammation after traumatic brain injury.
- JNJ-47965567 and ITH15004 P2X7 antagonists reduce neurological damage and proinflammatory response after traumatic brain injury.

What is the clinical significance?

- Post-traumatic brain injury P2X7 blockade may be a promising therapeutical approach for traumatic brain injury.

promote tissue repair processes in the damaged brain (Kimbler et al., 2012; Wang et al., 2015). These findings suggest that targeting P2X7 receptors may be a promising therapeutic strategy in the treatment of TBI.

In this study, we aimed to contribute to the understanding of the role of P2X7 receptor in the pathophysiology of TBI. Our *in vitro* data showed that (i), among the P2X7 antagonists evaluated, **ITH15004** had the best IL-1β-reducing profile; (ii) reduction of IL-1β release was consistent with ITH15004 acting via P2X7 receptors, as it had no effect when we used NLRP3 activators other than ATP such as nigericin or monosodium urate crystals (MSU) and (iii), ITH15004 prevented **LPS** + ATP-induced apoptosis associated speck-like protein containing a CARD (ASC) specks formation as readout of NLRP3 inflammasome activation. Our results *in vivo* indicated an important role for P2X7 receptors after acute brain damage. In *P2rx7*$^{-/-}$ animals, the inflammatory response after TBI was exaggerated compared with WT mice. Furthermore, pharmacological inhibition of P2X7 receptor using **JNJ-47965567** or ITH15004 had a protective effect after TBI and plays a key role in neurological injury and inflammation. We additionally predicted *in silico* a promising safety profile for the administration of ITH15004. These results highlight the importance of P2X7 blockade after brain injury.

2 | METHODS

2.1 | Non-nucleotide purine derivatives

This series of compounds were synthesised in a new attempt to improve blood–brain barrier (BBB) penetration of P2X7 antagonists, as reported before (Calzaferri et al., 2021). In general terms, these compounds combine the essential structural features for their inhibitory capacity on P2X7 receptors, namely a heteroaromatic cycle bound to a halobenzene-type aromatic ring through a non-complex spacer. Compounds have been divided into two families: xanthinyl-halophenylsulfones and purinylarylethanones, referred to the structural mature of spacer, either sulfonyl or ethanone moieties, as shown in graphic abstract. The compounds were synthesised by our research group and evaluated *in vitro* and *in vivo*, using the method we recently reported (Calzaferri et al., 2021) and they are from which same synthesis baches as previously evaluated are used in the current project. JNJ-47965567 was used as a positive control for P2X7 antagonism.

2.2 | *In silico* toxicity predictions

To assess the safety profile of ITH15004, we use Derek Nexus v6.4.1 (2024 1.0 Knowledge Database), a knowledge-based expert system developed by Lhasa Limited for managing, searching and analysing toxicological data. This system predicts over 60 toxicological endpoints, providing valuable insights into potential safety concerns. Toxicity predictions in Derek Nexus are based on the identification of toxicophores (structural features associated with toxicity) within the ITH15004 molecular structure. The prediction process involves two key components:- alert evaluation, which detects the presence of toxicophores and likelihood estimation (Marchant et al., 2008), which assesses the probability of toxicity based on available data. The likelihood of toxicity in Derek Nexus is categorised into seven levels, ranked from highest to lowest: certain, probable, plausible, equivocal, doubted, improbable and impossible. These classifications help prioritise safety assessments and guide further experimental validation (Judson et al., 2012).

2.3 | Human samples

Venous blood samples were obtained after informed consent signature from a patient cohort included in an observational prospective study developed at the Department of Neurosurgery of Hospital Universitario La Paz (PI-2153, Ethic Committee of Hospital Universitario La Paz, Madrid, Spain). Samples were collected at 24 and 72 h post-TBI in those patients meeting the following inclusion criteria: age 18–85 years old, admitted to the Intensive Care Unit or Neurosurgery Ward upon diagnosis of closed head injury in the last 24 h. Patients with previous diagnosed neurological disease or cognitive impairment, those not legible for computed tomography, and those that did not complete the follow-up, were excluded. A group of healthy volunteers, without clinical history of TBI or major disease, was employed as control group to compare with the TBI cohort. TBI patients were evaluated by Glasgow Coma Scale (GCS) at hospitalisation for the impairment of consciousness level, rated as mild injury (13–15 score) or moderate–severe injury (3–12 score). Patients' outcome at 6 months post-TBI was assessed by Glasgow Outcome Scale Extended (GOSE), given either a favourable (6–8 points) or unfavourable (1–5 points) evaluated outcome.

2.4 | Animal model

Animal procedures are conducted under the licence PROEX 315.5/23 granted by the Ethic Committee of Universidad Autónoma de Madrid, Spain. Animal studies are also reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). All animals came from the animal facility from Universidad Autónoma de Madrid were group housed in individually ventilated cages (maximum of four mice per cage) at 21°C with a 12 h light–dark cycle and access to water and food *ad libitum* and environmental enrichment provided to each cage.

As in our previous studies (Farré-Alins et al., 2021; Lopez-Rodriguez et al., 2022; Rosa et al., 2021) and considering the higher incidence of TBI among males compared with females (Sharma et al., 2024), male C57BL6/N or *P2rx7*$^{-/-}$ mice (Kaczmarek-Hajek et al., 2018) of approximately 12 weeks and weighing 25–27 g were randomly allocated to naïve (no lesion), TBI (lesion) or treated (lesion

and post-TBI treatment) groups. Closed head injury (CHI) was performed on animals anaesthetised with inhaled isoflurane (100 mg kg^{-1}, Karisoo S.A., Barcelona, Spain), following the protocol adapted from Flierl et al. (2009). This model mimics most of the human TBI features (Flierl et al., 2009). Mice were placed under a weight drop device from which a piece of 50 g was dropped from 34 cm on the right hemisphere, taking the eye of the animal as reference to perform the lesion between sagittal and lambdoid sutures as no craniotomy was made. This TBI model produces an ipsilateral cortical severe lesion with a strong inflammatory response as well as neuromotor deficits, with an approximate 10% mortality rate within the first 5 min after the impact. Following anesthesia recovery, animals were housed separately and individually monitored. Treated animals were injected with either JNJ-47965567 (30 mg kg^{-1}, i.p.) or ITH15004 (1 mg kg^{-1}, i.p.) 30 min post-TBI. Naïve animals received i.p. vehicle injection (2% DMSO in 30% captisol in 0.9% NaCl or 30% captisol in 0.9% NaCl, respectively) and were anaesthetised but not lesioned. All animals were weighted before and 24 h post-TBI for calculation of weight loss percentage. A weight loss greater than 20% was considered as an experimental endpoint. Neurological damage was evaluated by a series or behavioural tests that rated motor performance of animals by a Neurological Severity Score (NSS) from 0 to 41 points. Tests were performed at 1 and 24 h post-TBI and consisted on a modified version of the ones employed by Flierl et al., evaluated as follows: (i) capacity to exit circle of 30 cm diameter in less than 2 min; (ii) interest on the environment and evident-seeking behaviour; (iii) alert response on a loud hand clap, (iv) walking stability, hemiparesis or homoparesis; (v) motor performance when crossing a 30 cm-long beam of 3-, 2- or 1-cm wide in less than 3 min and (vi), vertical strength keeping upside down on a grid for 1 min. After the last behavioural test at 24 h post-TBI, animals were killed by a trained individual specifically for this procedure. Animals were terminally anaesthetised with a mix of ketamine and xylazine at 1:3 ratio (Imalgene 100 mg ml^{-1}, Boehringer Ingelheim, Ingelheim, Germany; Xilagesic 20 mg ml^{-1}, Laboratorios Calier, Barcelona, Spain) and transcardial perfusion was performed with saline. Brains were removed and a punch of the right (ipsilateral) and left (contralateral) hemispheres were stored at −80°C until use.

2.5 | Cell culture

Primary mixed glial cultures were extracted from brain tissue of C57BL/6 mice pups at P0-P1. Briefly, pups were killed by neck dislocation, brains extracted and dissected for discarding olfactory bulbs and cerebellum. Brain hemispheres were cleaned out of meninges and blood vessels under a lamp. Tissue was mechanically disaggregated in DMEM-F12 medium (Cat# SH30271.01, Cytiva Spain, Barcelona, Spain) with antibiotics (streptomycin, 5 mg ml^{-1} and penicillin, 5 U ml^{-1}, Merck, Darmstadt, Germany), later filtered through 0.7-µm nylon filters and centrifuged. Finally, cell pellet was seeded in multiwell plates according to each experiment in DMEM-F12 supplemented with 20% foetal bovine serum (FBS, Cytiva Spain) and antibiotics. Cultures were maintained in an incubator at 37°C with humified atmosphere of 5% CO_2/95% air. At the fifth day, medium was changed to discard unattached cells. Experiments were performed on the tenth day. Cells were treated with **LPS** (1 µg ml^{-1}) for 4 h in DMEM-F12 with 0% FBS, followed by ATP stimulus (5 mmol l^{-1}) for 30 min. In those conditions where the compounds were evaluated, medium was changed after LPS stimulus and compounds were added 15 min before ATP. The treatment to each well was randomly distributed within each plate. At the end of the experiment, supernatant was collected and stored at −20°C until use.

In a set of experiments, BV2 immortalised mouse microglia cell line (Cat#305156, Cytion, Eppelheim, Germany, RRID:CVCL_0182) was used. BV2 cells were cultured in RPMI 1640 medium (Cat#21875, Gibco, New York, USA) supplemented with 10% FBS (Cytiva, Spain), 2.5 mmol l^{-1} L-glutamine and antibiotics (streptomycin, 5 mg ml^{-1} and penicillin, 5 U ml^{-1}, Merck). Cells were maintained at 37° in a humidified atmosphere of 5% CO_2/95% air. For *in vitro* cytotoxicity assay, cells were seeded in 48-well plates at a density of 70,000 cells per well.

2.6 | Enzyme-linked immunosorbent assay (ELISA)

IL-1β produced by glial cells in response to different treatments was measured by ELISA in cell supernatant with the commercial kit DuoSet Mouse IL-1beta/IL-1F2 (Cat#DY401, R&D Systems, Minnesota, USA). Soluble (s)P2X7 receptor levels were determined in serum samples from TBI patients and healthy volunteers following the directions of the commercial kit hP2X7R (Cat#CSB-EL017325HU, Cusabio, Texas, USA).

2.7 | Immunofluorescence

All Immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018). In some experiments, glial cells were seeded on coverslips at a density of 300,000 cells per ml. On the tenth day, cells were treated as usual and later fixed in cold 4% paraformaldehyde for 15 min and washed with PBS. Cells were then permeabilised in 0.3% Triton X-100 for 10 min, washed and blocked with 4% goat serum for 1 h at room temperature. Next, cells were incubated with the primary antibody against ASC (1/100, Cat#ADI-905-173-100, RRID:AB_2038839, Enzo Life Sciences, New York, USA) overnight at 4°C. On the next day, coverslips were washed three times in PBS and incubated with the secondary antibody anti-rabbit Alexa Fluor 568 (1/500, Cat#A-11011, Invitrogen, Massachusetts, USA) for 1 h at room temperature. After three more washes with PBS, cell nuclei were counterstained with **DAPI** and coverslips were mounted on a glass slide using Prolong mounting medium (Invitrogen). For each variable, three to five images were taken using a confocal microscope Leica TCS-SP5 with a HCX PL APO lambda blue oil immersion 63X objective (numerical aperture 1.4). ASC speck positive cells over

total cell number were quantified by a blinded observer with the cell counter tool in ImageJ software. ASC specks puncta were considered valid by evaluating its position (paranuclear), shape (toroidal, circumferential) and immunoreactivity (an additional laser channel different from the one to detect Alexa fluor 568 was evaluated to discard unspecific labelling). Data are presented as a relativisation of ASC speck positive cells on LPS + ATP treatment in each independent experiment. Two hundred total cells were quantified on average in each variable.

2.8 | *In vitro* cytotoxicity assay

MTT method was employed to evaluate acute cytotoxicity of compounds JNJ-47965567 and ITH15004. BV2 cells were treated with increasing concentrations of the compounds (JNJ-47965567: 0.01, 0.1, 1 μmol l^{-1}; ITH15004: 1, 3, 10 μmol l^{-1}) for 24 h. DMSO 10% was considered positive control for cell cytotoxicity. Thiazolyl blue tetrazolium bromide (MTT, Cat#M2128, Sigma Aldrich) dissolved in water at 5 mg ml^{-1} was added to the wells at a final concentration of 0.5 mg ml^{-1} in the last 2 h of treatment incubated at 37°C. The medium was retired and tetrazolium salts from MTT reduction were extracted with 100 μl of dimethyl sulfoxide (DMSO, Cat#BP231-1, Fisher, Massachusetts, USA). Absorbance was measured at 540 nm in a plate spectrophotometer. Cell viability was expressed as percentage relative to absorbance in non-treated cells that was considered 100% viability.

2.9 | Quantitative reverse transcription polymerase chain reaction (q-RT-PCR)

RNA was extracted from frozen murine brain sections with Trizol-chloroform method (Tri Reagent, Molecular Research Center, Inc., Ohio, USA). RNA concentration and quality were quantified using a Nanodrop 2000c spectrophotometer (Thermo Scientific, Massachusetts, USA). cDNA synthesis was performed from 500 ng of RNA as template with a commercial kit which combines a mix of oligod(T) and random primers (iScript cDNA synthesis kit, BIORAD, California, USA). q-RT-PCR was performed with SYBR green ds-DNA-intercalating fluorescent dye (Takara, Kusatsu, Japan) in a QuantStudio 5 Real-Time PCR System (Applied Biosystems, Massachusetts, USA) using specific primers for *18S* (Fw: 5′-CGCCGCTAGAGGTGAAATTCT-3′, Rv: 5′-CATTCTTGGCAAATGTCTTTCG-3′), *il1b* (Fw: 5′-AACCTG CTGGTGTGTGACGTTC-3′, Rv: 5′-CAGCACGAGGCTTTTTTGTTGT-3′), *tnfa* (Fw: 5′-GCCTCTTCTCATTCCTGCTTG-3′, Rv: 5’-CTGATGA GAGGGAGGCCATT-3′), ***il6*** (Fw: 5′-TTCTCTGGGAAATCGTGGAAA-3′, Rv: 5′-CTGCAAGTGCATCATCGTTGT-3′), ***ccl2*** (Fw: 5′-ACAAGAG GATCACCAGCAGC-3′, Rv: 5′-GGACCCATTCCTTCTTGGGG-3′) and *gfap* (Fw: 5′-CTCCAACCTCCAGATCCGAG-3′, Rv: 5′-TCCACAG TCTTTACCAGATGT-3′). The relative quantification of gene expression was determined by the $2^{-\Delta\Delta Ct}$ method and rRNA *18S* housekeeping gene was used for normalisation, because of its low variance in expression across the treatments and physiological conditions assayed in this study. mRNA levels are expressed relative to values from naïve WT animals.

2.10 | Data and statistical analysis

All the data and statistical analyses comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2025). Mice were randomly assigned to each treatment group and experiment, as well as data analyses were performed completely blind to experimental groups. First, normal distribution and homoscedasticity of each variable were checked by Shapiro–Wilk and Levene's test, respectively. No exclusion criteria were used in the statistical analysis to remove possible outliers. Post hoc test were run only when F achieved $P < 0.05$ and there was no variance in homogeneity. To assess differences between two groups, unpaired Student's *t*-test was used for parametric variables and Mann–Whitney *U*-test for non-parametric variables. In the case of comparisons between more than two groups, one-way/two-way/three-way analysis of variance (ANOVA) was applied, followed by Dunnet's or Bonferroni's post hoc tests for homoscedastic variables or Games–Howell for non-homoscedastic variables. Kruskal–Wallis' analysis followed by Dunn's post hoc analysis was used for non-parametric variables. Symbols are used in the graphs to indicate the post hoc tests performed. Statistical analysis was performed with GraphPad Prism 8.0.2 (California, USA) software and SPSS 22.0 software package (SPSS, Inc., Illinois, USA). A *P*-value ≤ 0.05 was considered statistically significant in all cases. Data are presented as means ± SEM obtained from groups of 5 to 10 animals. Group sizes were chosen according to our preliminary behavioural experiments in the Closed Head Injury TBI model. The variability observed between the experimental groups is primarily influenced by the inner mortality rate of the TBI model.

2.11 | Materials

LPS (Cat#L6143), ATP (Cat#A1852), nigericin (Cat#N7143) and captisol (Cat#H107) were purchased from Sigma Aldrich (Missouri, USA). JNJ-47965567 (Cat#5299) was acquired from Tocris Bioscience (Bristol, UK). MSU were from InvivoGen (California, USA).

Details of other materials and suppliers are provided in the specific sections.

2.12 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/24 (Alexander, Mathie et al., 2023; Alexander, Fabbro et al., 2023).

3 | RESULTS

3.1 | Evaluation of non-nucleotide purine derivatives as P2X7 antagonists *in vitro*

The inhibitory effect of sulfonyl-based (ITH15003, ITH15006) and ethanone-based (ITH15004, ITH15005, ITH15010, ITH15015) compounds was evaluated in a well-known model of P2X7 receptor-induced inflammation by measuring the release of IL-1β upon LPS priming (1 μg ml^{-1}; 4 h) and ATP stimulation (5 mmol l^{-1}; 30 min) of mixed glial cultures. JNJ-47965567 was used as a positive control for P2X7 antagonism, as it completely blunted IL-1β production to basal levels in response to LPS + ATP in a concentration-dependent manner starting from 0.1 μmol l^{-1} (Figure 1a). In contrast, none of the sulfonyl compounds reduced IL-1β release at the concentrations tested (Figure 1b,c), except for a trend towards a decrease induced by ITH15003 at high concentrations (3 μmol l^{-1}, Figure 1b). As for the ethanone-spaced derivatives, only the compound ITH15004 significantly prevented IL-1β release in a concentration-dependent manner, reaching a 38% reduction compared to LPS + ATP at 10 μmol l^{-1} (Figure 1d). Neither ITH15005, ITH15010 nor ITH15015 modified IL-1β production (Figure 1e–g). Therefore, ITH15004 was selected as hit compound for further *in vitro* and *in vivo* evaluation.

The selectivity of ITH15004 blocking P2X7 receptors was evaluated by testing its ability to block IL-1β production in response to P2X7 receptor-independent stimuli. ITH15004 did not reduce the production of IL-1β when LPS priming was followed by either 30 min of nigericin (20 μmol l^{-1}, Figure 2b) or 16 h of MSU (200 μg ml^{-1}, Figure 2d) treatment. The same effect was observed with JNJ-47965567 not reducing LPS + nigericin or MSU-induced IL-1β release at any concentration tested (Figure 2a,c). These results were consistent with a purinergic-dependent effect of ITH15004. P2X7 antagonism was further studied by analysing the formation of ASC specks in response to LPS + ATP. Adaptor protein ASC oligomerises and builds up a single three-dimensional structure within cells that entails NLRP3 inflammasome assembly and activation with concomitant IL-1β release (Stutz et al., 2013), which makes ASC speck formation a readout of NLRP3 inflammasome activation. Whereas in control non-

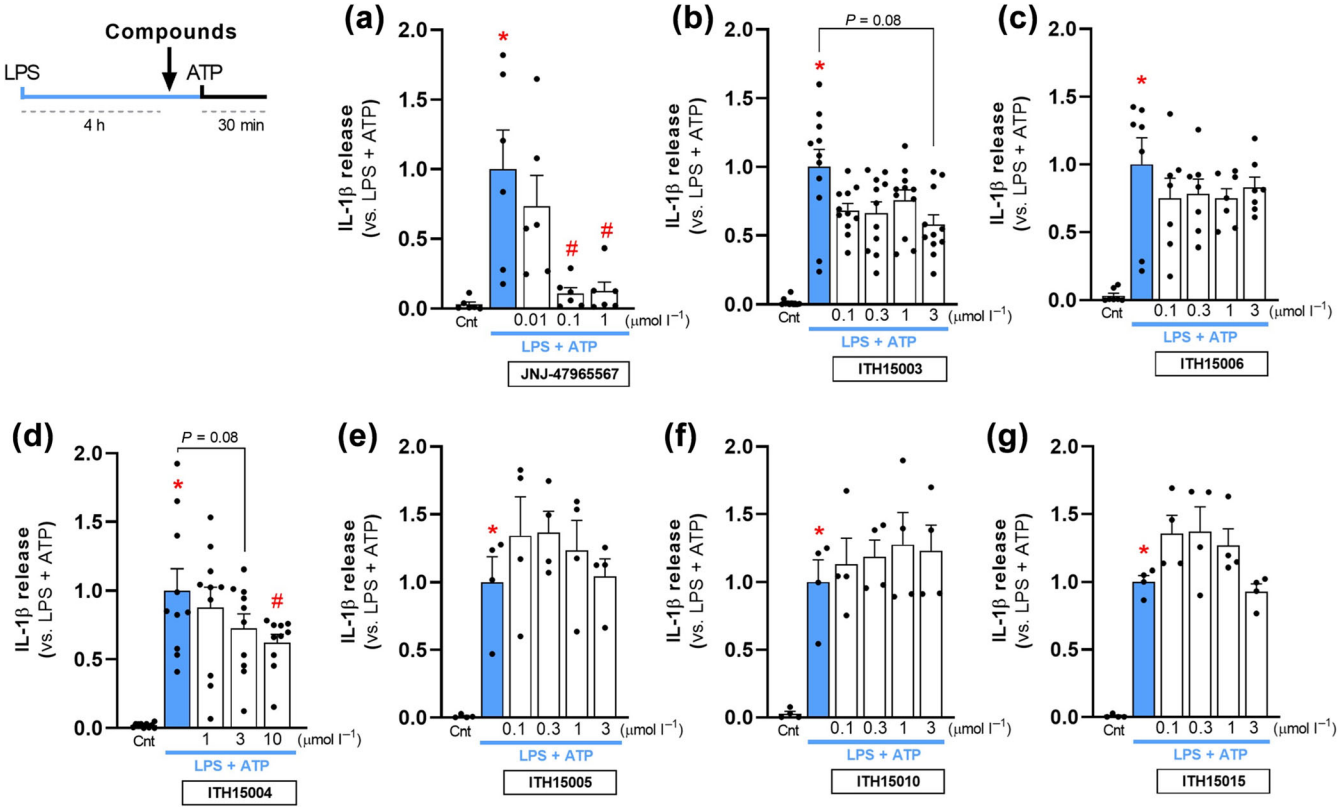

FIGURE 1 **Compound ITH15004 prevents P2X7 receptor-dependent IL-1β release in glial cells.** Murine mixed glial primary cultures were exposed to LPS (1 μg ml^{-1}) for 4 h followed by 30 min of ATP (5 mmol l^{-1}) in presence or absence of non-nucleotide purine derivatives. IL-1β release was evaluated by ELISA. (a) JNJ-47965567 (0.01, 0.1, 1 μmol l^{-1}, $n = 6$) served as a positive control for the prevention of IL-1β production in response to LPS + ATP. Non-nucleotide purine P2X7 antagonists were tested divided into sulfonyl group: (b) ITH15003 ($n = 11$) and (c) ITH15006 ($n = 7$), or ethanone group: (d) ITH15004 ($n = 10$), (e) ITH15005 ($n = 4$), (f) ITH15010 ($n = 4$) and (g) ITH15015 ($n = 4$). IL-1β release is expressed as relative levels in comparison to LPS + ATP stimuli in each independent experiment. Data show individual data distribution within bars depicting mean ± SEM. *$P \leq 0.05$ versus control, # $P \leq 0.05$ versus LPS + ATP by Kruskal–Wallis followed by Dunn's post hoc (Figure 1a,b,d) or one-way ANOVA followed by Dunnett's post hoc (Figure 1c). Figure 1e–g, because no effects were observed at any of the concentrations tested with $n = 4$, for ethical reasons no further animals were sacrificed to increase n and no statistical analysis was performed.

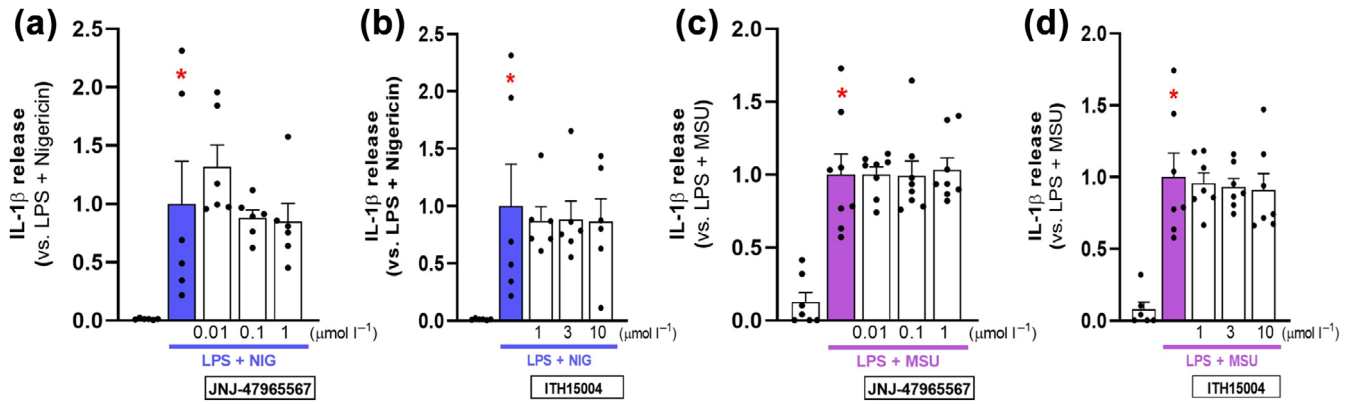

FIGURE 2 **Compound ITH15004 does not impede IL-1β release from different stimuli than ATP.** Selectivity of hit compound ITH15004 (1, 3, 10 μmol l^{-1}) and of JNJ-47965567 (0.01, 0.1, 1 μmol l^{-1}) was evaluated by analysing IL-1β production in response to 4 h LPS (1 μg ml^{-1}) stimulus followed by the non-P2X7 receptor-dependent signals (a, b) nigericin (20 μmol l^{-1}, 30 min) or (c, d) monosodium urate crystals (MSU; 200 μg ml^{-1}, 16 h), respectively. IL-1β release is expressed as relative levels in comparison to LPS + nigericin (Figure 2a,c, $n = 6$ per compound) or LPS + MSU (Figure 2c, $n = 8$ and Figure 2d, $n = 7$). Data show individual data distribution within bars depicting mean ± SEM. *$P \leq 0.05$ versus control by Kruskal–Wallis followed by Dunn's post hoc.

treated conditions ASC is randomly distributed in the cell cytoplasm, upon P2X7-NLRP3 stimuli with LPS + ATP, ASC aggregates at paranuclear position depicting a toroidal-shaped puncta (Figure 3). LPS + ATP stimuli significantly increased ASC speck positive cells in comparison to non-treated condition (Figure 3). JNJ-47965567 exhibited a strong blocking effect at concentrations of 0.1 and 1 μmol l^{-1}, significantly reducing the number of ASC speck positive cells and reaching almost basal levels at the highest concentration tested (Figure 3a). Also, ITH15004 at 3 and 10 μmol l^{-1} significantly decreased the number of ASC speck positive cells by 40% and 50%, respectively, compared to cells stimulated with LPS + ATP (Figure 3b). These results demonstrated the ability of ITH15004 to inhibit LPS + ATP-induced IL-1β release and ASC speck formation, as well as being consistent with ITH15004 blocking P2X7 receptors *in vitro*.

3.2 | Time-dependent implication of P2X7 receptor in neuroinflammatory response upon traumatic brain injury (TBI) *in vivo*

Despite neuroinflammation is a key factor in the severity and progression of TBI, with plenty of evidence pointing towards innate immune mechanisms on post-TBI sequelae, P2X7 receptor has not been extensively validated as a therapeutic target in this context. With the aim of exploring the role of P2X7 receptors from both genetic and pharmacological perspectives, we investigated the progression of TBI-induced neuroinflammation in *P2rx7*$^{-/-}$ mice as well as in WT animals treated with JNJ-47965567. First, we assessed the severity of the lesion performed after close-head injury model by analysing the weight loss and neurological damage of the animals. Neurological damage was evaluated through behavioural testing of WT and *P2rx7*$^{-/-}$ animals 1 and 24 h after TBI, and neurological severity score was calculated to evaluate neuromotor performance of the animals (Figure 4a). The neurological severity score significantly worsened 1 h after the lesion in WT and *P2rx7*$^{-/-}$ mice in comparison to their non-lesioned naïve counterparts. Whereas a tendency towards a reduction of the neurological severity score was observed in both genotypes 24 h after lesion, no significant improvement was determined, indicating that there is no effect of time on neurological damage in this model. No differences were also observed between the neurological severity score of WT and *P2rx7*$^{-/-}$ mice at 1 or 24 h after TBI. This effect was further supported by the analysis of the percentage of weight loss 24 h after TBI. We observed a 7.26 ± 2.06% reduction in WT mice and a 9.60 ± 4.47% in *Pr2x7*$^{-/-}$ mice (Figure 4b), indicating no genotype-related differences at the neurological damage level (Figure 4a,b). Next, we analysed the inflammatory profile in brain tissue from WT and *P2rx7*$^{-/-}$ animals 24 h after injury. Heat map depicting the main transcriptional alterations is shown in Figure 4c and detailed data are included in Figure S1A–E. In WT mice, TBI significantly increased relative mRNA levels of the cytokines *il1b*, *tnfa*, ***il6***, ***ccl2*** and *gfap* (glial fibrillary acidic protein gene) in the ipsilateral hemisphere compared to the contralateral hemisphere and to naïve animals, validating the model as previously determined (Lopez-Rodriguez et al., 2022). This inflammatory response was exacerbated in *P2rx7*$^{-/-}$ mice, where a significant increase of *il1b*, *tnfa*, *il6* and *ccl2* mRNA levels was observed in the ipsilateral hemisphere compared to the ipsilateral levels of WT injured mice. Moreover, naïve *P2rx7*$^{-/-}$ mice presented enhanced levels in the expression of these genes compared to their WT counterparts (Figures 4c and S1A–E). These results suggest that the genetic ablation of P2X7 receptors exerts a inhibitory effect on the progression of TBI as *P2rx7*$^{-/-}$ mice exhibit an exaggerated inflammatory response to brain damage.

The inflammatory response following TBI modulates both positive and detrimental effects depending on the timing and intensity of its activation. Inflammatory signals recruit immune cells and activate beneficial glial functions, but their excessive activation can lead to tissue

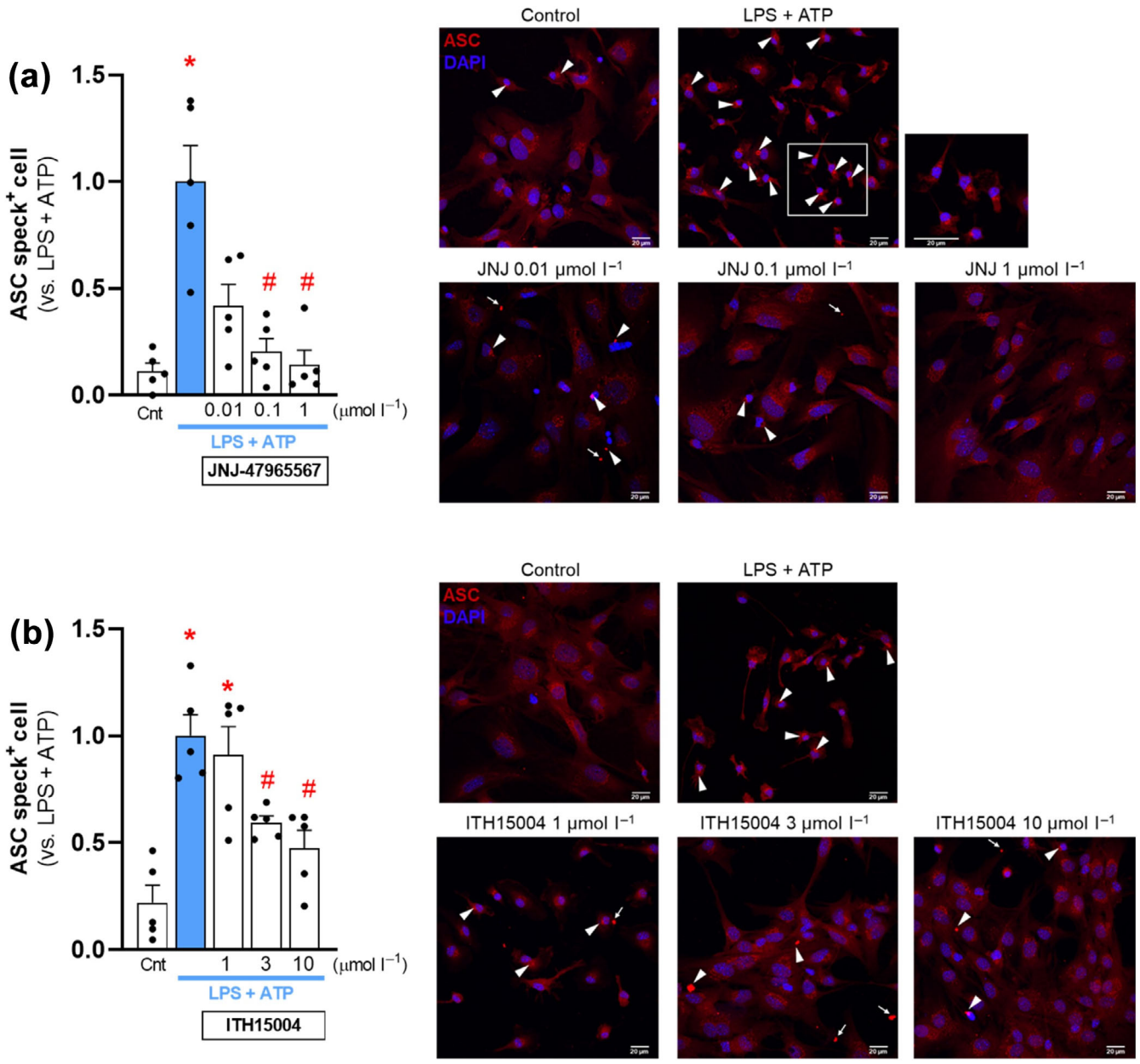

FIGURE 3 **Compound ITH15004 prevents the activation of NLRP3 inflammasome in response to LPS + ATP**. Modulation of P2X7 receptor-NLRP3 inflammasome activation by (a) JNJ-47965567 (0.01, 0.1, 1 μmol l^{-1}; $n = 5$) and (b) hit compound ITH15004 (1, 3,10 μmol l^{-1}; $n = 5$) was evaluated by immunofluorescence in response to 4 h LPS (1 μg ml^{-1}) followed by 30 min ATP (5 mmol l^{-1}). Figure 3a and b shows the quantification of apoptosis associated speck-like protein containing a CARD (ASC) speck$^+$ cells versus total cells relative to the ratio achieved in response to LPS + ATP in each independent experiment. Data show individual data distribution within bars depicting mean ± SEM. Representative images are shown besides each graph (630X magnification), with ASC (red) and DAPI (blue) labelling. White arrowheads depict ASC speck puncta at paranuclear position and white arrows depict extracellular puncta plausible to be released ASC specks. Scale bars represent 20 μm. *$P \leq 0.05$ versus control; # $P \leq 0.05$ versus LPS + ATP by Kruskal–Wallis followed by Dunns' post hoc (Figure 3a) or one-way ANOVA followed by Dunnett's post hoc (Figure 3b).

damage and chronic neuroinflammation. Defining a therapeutic window to modulate excessive neuroinflammation is a major challenge in the context of TBI. Thus, in a second approach, WT animals treated with either vehicle (Vh, 2% DMSO in 30% captisol in 0.9% NaCl, i.p.) or JNJ-47965567 (JNJ, 30 mg kg^{-1}, i.p.) 30 min post-TBI were analysed for weight loss and neurological damage 1 and 24 h post-injury. As observed earlier, neurological severity score was significantly increased from 1 h after TBI in animals in the vehicle (Vh) group compared to their naïve counterparts. Although a tendency towards improvement was observed at 24 h, no significant effect of time was determined in the Vh group (Figure 5a). Injured animals treated with JNJ-47965567 showed a significant improvement in neuromotor performance from 1 h post-TBI compared to Vh group, which was further observed after 24 h. Notably, the neurological severity score was not

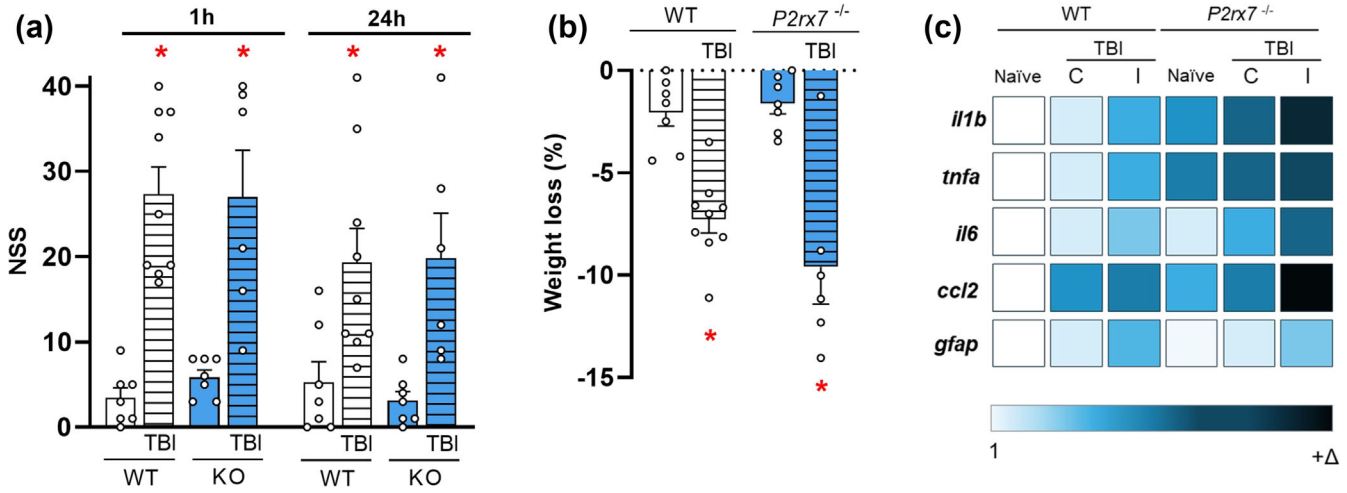

FIGURE 4 ***P2rx7*** **deficiency does not affect neurological damage but aggravates inflammatory response after traumatic brain injury (TBI).** (a) Evaluation of neuromotor function by neurological severity score (NSS) 1 and 24 h after TBI in WT (naïve $n = 7$, TBI $n = 9$) and $P2rx7^{-/-}$ (naïve $n = 7$, TBI $n = 6$) mice. *$P \leq 0.05$ versus naïve (same genotype and time) by three-way ANOVA followed by Bonferroni or Games–Howell post hoc. (b) Weight loss percentage 24 h after TBI in naïve and injured animals from both genotype groups. *$P \leq 0.05$ versus naïve (same genotype) by one-way ANOVA followed by Bonferroni post hoc. (c) Graphical summary of brain tissue mRNA changes from pro-inflammatory markers *il1b*, *tnfa*, *il6*, *ccl2* and *gfap* in each animal group studied relative to WT naïve levels. White depicts no change and intensification of blue represents higher increase in relative gene expression. C: contralateral; I: ipsilateral.

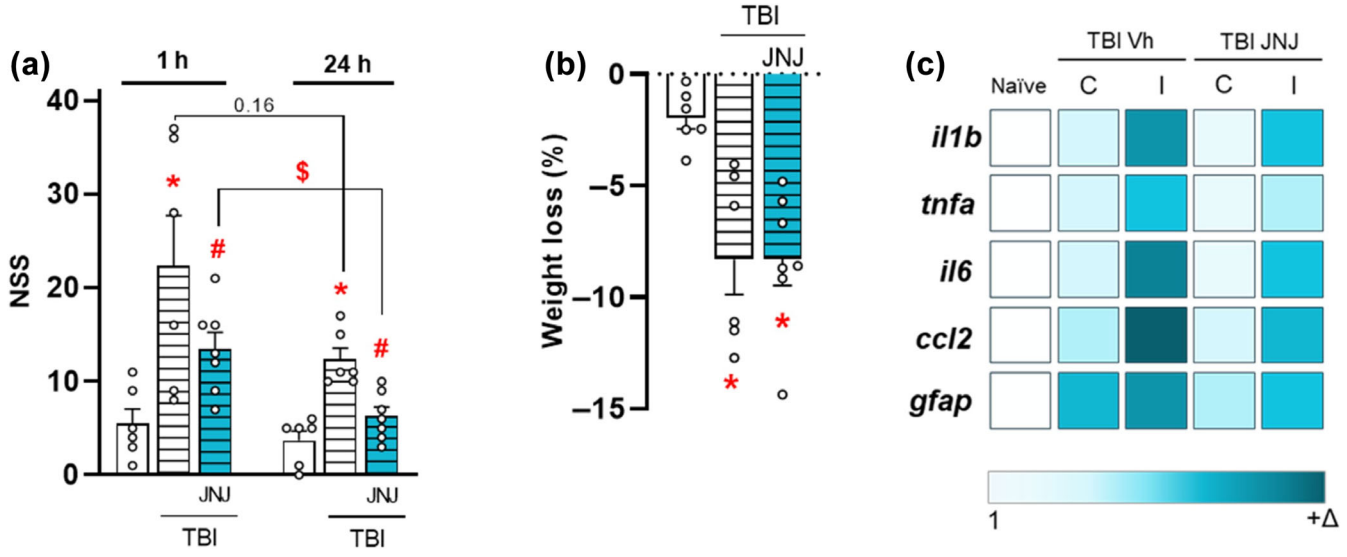

FIGURE 5 **Pharmacological P2X7 antagonism with JNJ-47965567 improves neurological damage and proinflammatory response after traumatic brain injury (TBI).** Mice were administered vehicle (Vh, 2% DMSO in 30% captisol in 0.9% NaCl; $n = 6$) or the selective P2X7 antagonist JNJ-47965567 (JNJ, 30 mg kg^{-1}, i.p.; $n = 7$) 30 min after TBI. Naïve not injured animals also received Vh ($n = 6$). (a) Evaluation of neuromotor function by neurological severity score (NSS) 1 h and 24 h after TBI. *$P \leq 0.05$ versus naïve (same time point); #$P \leq 0.05$ versus TBI Vh (same time point); \$$P \leq 0.05$ versus JNJ 1 h by three-way ANOVA followed by Bonferroni or Games–Howell test. (b) Weight loss percentage 24 h after trauma. *$P \leq 0.05$ versus naïve by one-way ANOVA followed by Bonferroni post hoc. (c) Graphical summary of brain tissue mRNA changes from pro-inflammatory markers *il1b*, *tnfa*, *il6*, *ccl2* and *gfap* in each animal group studied relative to naïve levels. White depicts no change and intensification of blue represents higher increase in relative gene expression. C: contralateral; I: ipsilateral.

significantly different between JNJ-treated and naïve groups at 24 h (Figure 5a). This overall improvement in neuromotor performance was not accompanied by a variation in the percentage of body weight loss 24 h after TBI (Figure 5b), indicating that both groups experienced comparable lesion severity, as assessed by weight loss. In terms of inflammatory response, mRNA expression levels 24 h after TBI of *il1b*, *tnfa*, *il6*, *ccl2* and *gfap* were reduced in the ipsilateral hemisphere of JNJ-treated animals compared to Vh group (Figure 5c), reaching a significant reduction in the expression of *ccl2*, *gfap* and *il6* (Figure S1F–J). Altogether, these results support that the pharmacological modulation of P2X7 receptor after TBI could be a successful strategy to control neurological injury and inflammation.

TABLE 1 Endpoints not firing any alerts for ITH15004 after *in silico* prediction with Derek Nexus v6.4.1.

5-alpha reductase inhibition	Mitochondrial dysfunction
Adrenal gland toxicity	Mutagenicity *in vivo*
Anaphylaxis	Nephrotoxicity
Androgen receptor modulation	Neurotoxicity
Bladder disorders	Non-specific genotoxicity *in vitro*
Bladder urothelial hyperplasia	Non-specific genotoxicity *in vivo*
Blood in urine	Occupational asthma
Bone marrow toxicity	Ocular toxicity
Bradycardia	Oestrogen receptor modulation
Cardiotoxicity	Oestrogenicity
Cerebral oedema	Peroxisome proliferation
Chloracne	Phospholipidosis
Cholinesterase inhibition	Photo-induced chromosome damage *in vitro*
Chromosome damage *in vitro*	Photo-induced non-specific genotoxicity *in vitro*
Chromosome damage *in vivo*	Photo-induced non-specific genotoxicity *in vivo*
Cumulative effect on white cell count and immunology	Photoallergenicity
Cyanide-type effects	Photocarcinogenicity
Developmental toxicity	Photomutagenicity *in vitro*
Glucocorticoid receptor agonism	Phototoxicity
HERG channel inhibition *in vitro*	Pulmonary toxicity
High acute toxicity	Respiratory sensitisation
Irritation (of the eye)	Skin irritation/corrosion
Irritation (of the gastrointestinal tract)	Splenotoxicity
Irritation (of the respiratory tract)	Teratogenicity
Kidney disorders	Testicular toxicity
Kidney function-related toxicity	Thyroid toxicity
Lachrymation	Uncoupler of oxidative phosphorylation
Methaemoglobinaemia	Urolithiasis

3.3 | Toxicological assessment of ITH15004 *in silico* and *in vitro*

To gain more detailed insights into the safety profile of ITH15004 before performing *in vivo* experiments, we conducted an *in silico* analysis to predict any potentially toxic effects of the compound. Derek Nexus was selected to perform a comprehensive toxicity assessment by comparing the structural features of ITH15004 with several toxicophore patterns (structural alerts) stored in Lhasa's Derek KB 2024 1.0 Knowledge Database. Among the 67 toxicity endpoints analysed by Derek Nexus for ITH15004, no alerts were triggered for 56 endpoints (Table 1). However, alpha-2-μ-Globulin nephropathy and hepatoxicity in mammals were classified as equivocal, while carcinogenicity in mammals was deemed plausible because of the presence of a polyhalogenated aromatic structure in the molecule. In contrast, carcinogenicity in bacteria was considered impossible. Additionally, the query compound did not match any structural alerts associated with activity in a bacterial reverse mutation assay (Ames test), leading to an inactive prediction for in vitro mutagenicity. Skin sensitisation was predicted to be plausible in mammals, whereas it was impossible in bacteria. Notably, no alerts were triggered for high acute toxicity, neurotoxicity or cerebral oedema, suggesting a lower risk for these specific toxic effects. In addition, we performed MTT viability assay in BV2 to determine whether ITH15004 or JNJ-47965567 exposure could affect cell viability. Cells were treated with ITH15004 or JNJ-47965567 alone for 24 h and no significant reduction in viability was observed compared to untreated cells (Figure S2). Confirming that there is no acute toxicity *in vitro* for both compounds at the concentrations tested.

3.4 | P2X7 antagonist ITH15004 improves neurological damage and brain inflammatory profile after TBI

The results of the *in vitro* evaluation of this family of non-nucleotide purine derivatives led to the selection of ITH15004 as the compound with the strongest inhibitory effect on P2X7 receptor-dependent inflammation. After the *in silico* and *in vitro* validation of a safe toxicological profile for ITH15004, our next objective was to evaluate its anti-inflammatory properties in a TBI mouse model. Mice were treated with ITH15004 at 30 min post-TBI, in a similar timing pattern as for JNJ-47965567, but at a lower dose (1 mg kg^{-1}, i.p.) based on previous evidence in literature (Beamer et al., 2022). Vh-administered group in this set of experiments received 30% captisol in 0.9% NaCl to compare the lesion damage and brain inflammation profile in naïve or injured animal groups. First, we found no differences in the body weight of Vh-treated and ITH-treated animals (Figure S3), supporting the notion that ITH15004 does not exert acute toxic effects and aligning with predictions from *in silico* and *in vitro* analyses of toxicity. Neurological severity score was significantly reduced 1 h after TBI in the animals administered with ITH15004 compared to Vh group. This effect was even more evident at 24 h (Figure 6a). In contrast, weight loss 24 h after TBI significantly increased in both Vh- and ITH15004-treated groups compared to naïve animals, with no differences observed between injured groups (Figure 6b). Regarding inflammatory markers, the increase in *il1b*, *tnfa*, *ccl2*, *gfap* and *il6* mRNA levels in the ipsilateral hemisphere of the injured Vh group was significantly reduced in the brain tissue from ITH15004-treated animals 24 h post-TBI (Figure 6c–e). These results support the impact of P2X7 activation on TBI, demonstrating the anti-inflammatory effect of P2X7 receptor blockade with ITH15004 and suggesting P2X7 receptors as a pharmacological target to suppress inflammation after TBI.

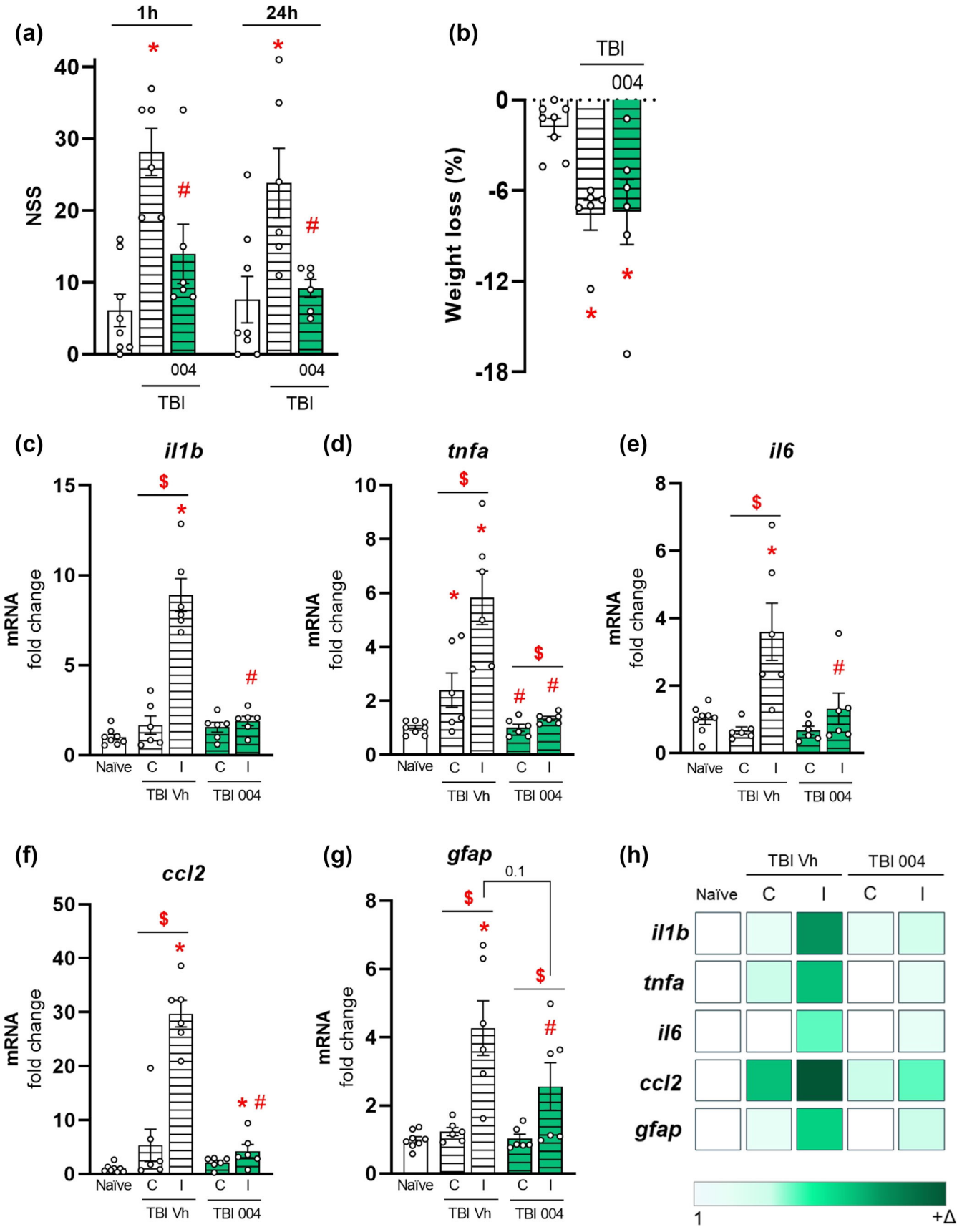

FIGURE 6 Legend on next page.

FIGURE 6 **P2X7 antagonist ITH15004 improves neuro-behavioural score and brain inflammatory profile after traumatic brain injury (TBI).** Mice were administered vehicle (Vh, 30% captisol in 0.9% NaCl, $n = 6$) or ITH15004 (1 mg kg^{-1}, i.p., $n = 6$) 30 min after TBI. Naïve not injured animals also received Vh ($n = 8$). (a) Evaluation of neuromotor function by neurological severity score (NSS) 1 h and 24 h after TBI. *$P \leq 0.05$ versus naïve (same time point); #$P \leq 0.05$ versus TBI Vh (same time point); \$$P \leq 0.05$ versus 004 1 h by three-way ANOVA followed by Bonferroni or Games–Howell test. (b) Weight loss percentage 24 h after trauma; *$P \leq 0.05$ versus naïve by two-way ANOVA followed by Bonferroni test. Inflammatory transcripts panel evaluated the change in brain mRNA levels of pro-inflammatory markers (c) *il1b*, (d) *tnfa*, (e) *il6*, (f) *ccl2* and (g) *gfap*. Naïve and contralateral or ipsilateral hemispheres were compared. mRNA levels relative to naïve levels are shown. *$P \leq 0.05$ versus naïve; #$P \leq 0.05$ versus TBI Vh (same side); \$$P \leq 0.05$ versus ipsilateral (same treatment) by three-way ANOVA followed by Bonferroni or Games–Howell test. (h) Graphical summary of brain tissue mRNA changes from pro-inflammatory markers in each animal group studied relative to naïve levels. White depicts no change and intensification of green represents higher increase in relative gene expression. C: contralateral; I: ipsilateral.

TABLE 2 Demographic details of studied cohort.

			N	Age (y.o., mean ± SD)	Sex
Control			9	44 ± 18	55.5% male
TBI	Severity	Mild TBI	13	58 ± 21	53.8% male
		Severe TBI	11	48 ± 17	81.1% male
	Outcome at 6 months	Favourable	13	56 ± 19	46.15% male
		Unfavourable	11	51 ± 1	81.1% male

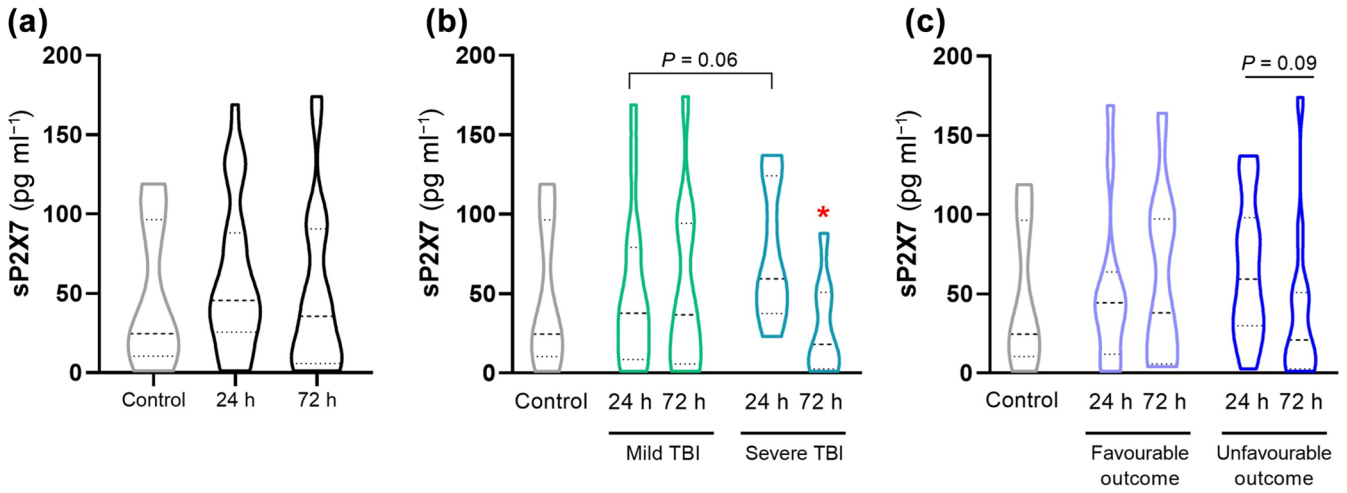

FIGURE 7 **Soluble (s)P2X7 receptor serum levels are reduced 72 h after severe traumatic brain injury (TBI).** sP2X7 receptor serum levels were determined by ELISA in samples from TBI-patient cohort ($n = 24$) and control counterparts ($n = 9$). Data are grouped as (a) sP2X7 receptor levels in healthy subjects versus 24 or 72 h post-TBI; (b) patient classification according to TBI severity lesion based of Glasgow Coma Scale (GCS) at hospitalisation, divided as mild (GSC score: 13–15) or moderate–severe (GSC score: 3–12); or (c) patient classification according to patient outcome 6 months after TBI, stratifying outcomes as favourable or unfavourable. *$P \leq 0.05$ versus 24 h (same severity) by two-way ANOVA followed by Bonferroni test.

3.5 | Soluble (s)P2X7 receptor serum levels are modulated after TBI in humans

A shed/soluble form of P2X7 receptor (sP2X7 receptor) has been detected in blood and their levels were associated with the severity of both acute and chronic conditions with underlying pro-inflammatory status such as COVID-19 (García-Villalba et al., 2022; Vultaggio-Poma et al., 2023) and obesity (Di Vincenzo et al., 2023), respectively. However, to date there are no data evaluating sP2X7 receptor variation after TBI. Therefore, sP2X7 receptor levels were analysed in serum of a cohort of 33 subjects, 24 TBI patients and nine healthy controls, whose data are presented in Table 2. Serum samples were collected 24 and 72 h after injury and no differences were observed between values of TBI patients and controls at any time point (Figure 7a). When the data were grouped according to TBI severity based on Glasgow Coma Scale (GCS) at admission (GCS score: 13–15 for mild TBI and 3–12 for moderate–severe TBI), no differences were detected between levels of any severity group at any time point compared to levels in healthy subjects. However, a tendency towards an increase was observed in sP2X7 receptor levels at 24 h post-TBI in severe TBI patients compared to mild TBI group at this same time point. Moreover, in patients with severe TBI, following an initial increase at 24 h, sP2X7 receptor levels significantly decreased at 72 h (Figure 7b). In a second approach, data were classified according to

the patients outcome 6 months after TBI, stratified with the GOSE (Glasgow Outcome Scale-Extended) in favourable or unfavourable outcome. In this case, no differences in sP2X7 receptor levels were observed between TBI and control groups at any time point. When we separately compared the levels of sP2X7 in each outcome group, no differences were identified between 24 and 72 h levels in favourable outcome patients, whereas a tendency towards a reduction was observed on sP2X7 receptor levels 72 h post-TBI compared to 24 h post-TBI levels in patients with an unfavourable outcome (Figure 7c). These results suggest that P2X7 receptor activation could be involved in modulating the inflammatory response and potentially influencing TBI severity, although further studies in an expanded patient cohort are needed to clarify its exact role.

4 | DISCUSSION

Increasing evidence suggests that P2X7 receptors plays a key role in neuroinflammatory responses following brain injuries (Aivar et al., 2023; Smith et al., 2023; Wilmes et al., 2022). In this study, we have gone deeper on understanding the importance of P2X7 receptor in the acute phase of traumatic brain injury (TBI) and its suitability as therapeutic target to dampen neuroinflammation. Here, we have selected two families of purine derivatives that block P2X7 receptors (Calzaferri et al., 2021) and have evaluated their ability to counteract NLRP3 inflammasome-dependent IL-1β release and ASC speck formation by glial cells exposed to LPS + ATP. Among the tested compounds, ITH15004 was identified as the best one, as its results in antagonising P2X7 receptor were consistent with its inability to block IL-1β release when LPS was followed by stimuli other than ATP. *In silico* toxicity assessment of ITH15004 showed a promising safety profile. Moreover, our findings revealed that TBI triggers an inflammatory response in WT animals, yet *P2rx7*$^{-/-}$ mice exhibited heightened inflammatory responses after TBI without significant effects on neurological damage. However, animals treated with JNJ-47965567 or ITH15004 30 min post-TBI showed better neurological outcomes and brain inflammatory responses, demonstrating the importance of timely interventions. In addition, we showed tendency towards an increase in circulating sP2X7 receptor in a patient cohort. These data demonstrate that P2X7 receptors plays a pivotal role in the acute phase of TBI, reinforcing the idea that NLRP3 inflammasome is crucial in post-TBI inflammation and highlighting the impact of P2X7 receptor activation on injury severity.

In the first part of this study, we investigated six non-nucleotide purine derivative compounds that had demonstrated a significant reduction of IL-1β production by primed primary bone marrow-derived macrophages (BMDM) (Calzaferri et al., 2021). We observed that many of the compounds tested in primary glial cultures, however, lost the effect previously observed in BMDM. Only ITH15004 and ITH15003 compounds showed a significant effect or a trend towards reducing IL-1β release, while the remaining compounds showed no effect (Figure 1). This discrepancy may be explained by the different in cell composition in glial cultures compared to BMDM cultures. Primary glial cultures are composed of different cell types, primarily astrocytes and microglia, and a lower proportion of oligodendrocytes, whereas BMDM cultures are composed exclusively by macrophages. The differential proportion of cell types in glial cultures, along with their differential expression and response to P2X7 receptor activation (Sidoryk-Węgrzynowicz & Strużyńska, 2021; Zhao et al., 2021), could have affected the results from P2X7 blockade.

Several pharmacological tools have been tested for their ability to inhibit P2X7 receptor in animal models of neurodegenerative diseases (Bianchi et al., 2023; Carmo et al., 2014) and acute brain lesions, including TBI (Liu et al., 2017; Nadal-Nicolás et al., 2016). These studies have highlighted several limitations of current P2X7 antagonists that need to be improved to overcome the challenges in advancing these therapies to later clinical trial stages. We compared the "hit" compound ITH15004 with JNJ-47965567 because of its well-known efficacy blocking P2X7 receptor and NLRP3 inflammasome activation (Bhattacharya et al., 2013). We investigated the P2X7 receptor-mediated action of the compound ITH15004 in our cell model (Figure 2), previously evaluated by voltage-clamp recordings in oocytes (Calzaferri et al., 2021), and demonstrated a similar function to JNJ-47965567. Both compounds reduced the release of IL-1β induced by ATP but not by nigericin or MSU. These experiments were consistent with ITH15004 acting on P2X7 receptors, because it is well-known that ATP-induced IL-1β release depends on P2X7 receptor activations (Di Virgilio et al., 2017). However, further experiments would be needed to more robustly carried out to determine that the compound is selective for and acts exclusively via P2X7 receptors. In consequence, both compounds also exhibit similar effect modulating NLRP3 inflammasome activation and so reduced ASC specks assembly, although JNJ-47965567 was more potent and effective than ITH15004 at this in the *in vitro* scenario. One possible explanation is that, although both compounds likely act as allosteric antagonists by binding to a highly lipophilic pocket located between adjacent subunits in the extracellular domain of the P2X7 receptor (Bin Dayel et al., 2019; Calzaferri et al., 2021), they may not interact with the same amino acid residues. As a result, the binding affinity and inhibitory efficacy of each compound could differ. In the TBI animal model, we selected the dose of 30 mg kg^{-1} for JNJ-47965567 based the results from the recent literature on CNS pathology models (Ruiz-Ruiz et al., 2020) and confirmed that this dose was effective in our TBI model at improving neuro-behavioural parameters of treated animals 24 h post-TBI. A similar reduction in the neurological severity score scale and in several inflammatory genes were achieved using 1 mg kg^{-1} of the compound ITH15004. We hypothesise that ITH15004 is more bioavailable than JNJ-47965567, as it has better ability to cross the BBB in parallel to less sensibility to efflux pumps such as the P-glycoprotein efflux pump (Pgp), as it was measured in a previous work (Calzaferri et al., 2021). To our knowledge, this is the first study that have tested the *in vivo* effectiveness of ITH15004 via intraperitoneal administration.

The *in silico* toxicity evaluation of ITH15004 showed a promising safety profile. Because of the intended route of administration,

potential skin sensitisation in mammals was not considered to be a significant concern. However, analysis indicates a possible carcinogenic risk and suggests there is some evidence to support this hypothesis. Derek Nexus 6.4.0 includes 83 active alerts for carcinogenicity (both genotoxic and non-genotoxic) and is widely used in drug safety assessments (Matthews et al., 2008). Nonetheless, no evidence of genotoxicity was detected and carcinogenicity in bacteria was predicted to be unlikely. However, it is important to consider several factors beyond the scope of *in silico* predictions. First, ITH15004 is intended as a single-dose treatment that does not typically induce carcinogenesis. This is because cancer development is a multi-step process that requires multiple genetic and cellular events over time (Calabrese & Blain, 1999). Second, *in vivo* studies have shown that the effective doses are not high, reducing the likelihood of immediate DNA damage typically seen at higher doses. Additionally, differences in metabolism and biological processes between animal models and humans may result in species-specific risks that translate to human toxicity (Borgert et al., 2021). Nonetheless, given the plausible carcinogenicity prediction, a more in-depth investigation should be conducted to further evaluate the potential risks associated with ITH15004.

Previous data from our laboratory led us to hypothesise that NLRP3 inflammasome plays an important role after cerebral ischaemia that must be tightly controlled. While it needs to be activated soon upon the ischaemia onset to control the damage, this activation must be controlled within 1–2 h after the ischaemic onset to prevent excessive inflammation because of BBB disruption (Palomino-Antolin et al., 2022). Moreover, these results had been also confirmed in the TBI model used in this study (Lopez-Rodriguez et al., 2022). Here we show that the results obtained using *P2rx7*$^{-/-}$ mice are consistent with our previous results and confirm the importance of this pathway in regulating inflammation in brain injury. We demonstrated that *Pr2x7*$^{-/-}$ mice subjected to TBI exhibit excessive inflammation compared to control WT mice (Figure 4). However, P2X7 blockade after TBI with JNJ-47965567 or ITH15004 reduced the inflammation almost to control levels. One explanation to this dual and antagonistic effect (pre- vs post-TBI) could be attributed to the timing of the compound application as mentioned above or the divergent roles of P2X7 receptor activation in different brain cells. Recently, a cell type-specific contribution of the P2X7 receptor to seizures and epilepsy has been demonstrated, showing that loss of P2X7 receptors from microglia has anticonvulsant and antiepileptic effects, whereas deletion of P2X7 receptors from neurons leads to increased excitability and a more severe seizure phenotype (Alves et al., 2024). In the future, this hypothesis may be tested in our TBI model by using cell-specific KO mice of P2X7 receptor. It is also important to note that one limitation of this study is the use of male animals and future experiments including female mice will be necessary to address potential sex-related differences in the inflammatory response and treatment efficacy.

The P2X7 receptor -NLRP3 pathway plays a crucial role in neuroinflammatory processes common to various acute and neurodegenerative diseases. Their sustained activation triggers proinflammatory signals and ATP release, creating a positive feedback loop that amplifies the inflammatory response (Cisneros-Mejorado et al., 2015). Evidence has shown that mediators of this pathway are elevated in stroke, Alzheimer's Disease (Aivar et al., 2023) and multiple sclerosis (Inoue & Shinohara, 2013), among others. In the same line, enhanced levels of sP2X7 receptor have been detected in circulation of acute and chronic inflammatory disease patients, whose levels correlate with those of C-reactive protein and disease severity (Di Vincenzo et al., 2023; García-Villalba et al., 2022; Vultaggio-Poma et al., 2023). Our results revealed an initial increase of sP2X7 receptors at 24 h that is accompanied by a significant decrease at 72 h in those patients with more severe TBI or with unfavourable outcomes (Figure 7). There is previous evidence of pro-inflammatory cytokine release, as it occurs in acute TBI phases, inducing the increase in P2X7 receptor expression at the cell membrane of glial cells (Narcisse et al., 2005), which might act as an increased pool of P2X7 receptors to be shed/released to the circulation and might explain those changes. However, understanding the mechanism of production and the pathophysiological function of sP2X7 receptor fraction requires further research in an expanded patient cohort.

In conclusion, our data provide new evidence for a key role of P2X7 receptor activation in the pathophysiology of TBI. We demonstrated that ITH15004 was the best among the compounds tested *in vitro* and that this blockade was consistent with ITH15004 acting via P2X7 receptor. Overall, our data indicate that P2X7 blockade with ITH15004 or JNJ-47965567, administered 30 min post-TBI, could serve as a promising therapeutic strategy to mitigate neurological damage by controlling neuroinflammation.

AUTHOR CONTRIBUTIONS

Javier Egea conceptualised and administered the work. Javier Egea and Inés Valencia supervised the research activity planning. Inés Valencia, Andrea Pastor-Martínez, Céline Decouty-Pérez, Ana Belen Lopez-Rodriguez, María Álvarez-Rubal, Eva Ramos, Javier Rodríguez de Cía and Alejandro Romero performed the experiments. Inés Valencia and Andrea Pastor-Martínez performed formal analysis of the results. Francesco Calzaferri and Cristóbal de los Ríos developed compound synthesis methodology. Jorge Zamorano-Fernández, Javier Giner-García, Alexis J. Palpán-Flores and Víctor Rodríguez-Domínguez provided patients samples. Javier Egea and Inés Valencia drafted the manuscript. All authors participated in reviewing and editing the last version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

All authors declare that they have no conflicts of interest related to this work.

DATA AVAILABILITY STATEMENT

Data available on request from the authors: The data that support the findings of this study are available from the corresponding author

upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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