Received: 8 January 2021 | Revised: 28 July 2021 | Accepted: 3 August 2021

DOI: 10.1111/bph.15663

RESEARCH ARTICLE

Taspine is a natural product that suppresses P2X4 receptor activity via phosphoinositide 3-kinase inhibition

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Funding information
Malaysian Government PhD Studentship; Malaysian Government Studentship; British Heart Foundation, Grant/Award Number: PG/16/69/32194

Background and Purpose: P2X4 is a ligand-gated cation channel activated by extracellular ATP involved in neuropathic pain, inflammation and arterial tone.

Experimental Approach: Natural products were screened against human or mouse P2X4 activity using fura-2 loaded 1321N1 cells for measurement of intracellular Ca^{2+} responses. Whole-cell currents were measured by patch clamp. Human primary macrophage chemokine release was used to assess effect of taspine on inflammatory cell function. An enzymatic assay was performed to assess the effect of taspine on recombinant PI3-kinase.

Key Results: A natural product screen identified taspine as an inhibitor of human P2X4 activity. Taspine inhibits human and mouse P2X4-mediated Ca^{2+} influx in 1321N1 cells expressing receptors but lacked activity at human P2X2, P2X3, P2X2/3 and P2X7 receptors. Taspine inhibited the maximal response at human and mouse P2X4 but effective on ATP potency. Taspine has a slow onset rate (~15 min for half-maximal inhibition), irreversible over 30 min of washout. Taspine inhibits P2X4-mediated Ca^{2+} signalling in mouse BV-2 microglia cells and human primary macrophage. Taspine inhibited P2X4-mediated CXCL5 secretion in human primary macrophage. Taspine reversed ivermectin-induced potentiation of P2X4 currents in 1321N1 stably expressing cells. The PI3-kinase inhibitor LY294002 mimicked the properties of taspine on P2X4-mediated Ca^{2+} influx and whole-cell currents. Taspine directly inhibited the enzymatic activity of recombinant PI3-kinase in a competitive manner.

Conclusion and Implications: Taspine is a novel natural product P2X4 receptor inhibitor, mediating its effect through PI3-kinase inhibition rather than receptor antagonism. Taspine can inhibit the pro-inflammatory signalling by P2X4 in human primary macrophage.

KEYWORDS
ion channel, natural product, P2X receptor, purinergic

Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-bisphosphate.

1 | INTRODUCTION

P2X receptors are a family of ligand-gated non-selective cation channels activated by extracellular **adenosine 5′-triphosphate (ATP).** P2X receptor activation causes membrane depolarisation and increased cytoplasmic Ca^{2+}. The human genome encodes seven P2X receptor subtypes capable of forming homomeric and heteromeric trimer in a subtype-dependent fashion. **P2X4** has attracted wide attention as a target for pharmacological modulation (Stokes et al., 2017). It is a modestly desensitising receptor (Fountain & North, 2006) and has an established role in a number of physiological processes. In the cardiovascular system, P2X4 is involved in cardiac contractility (Hu et al., 2002; Yang et al., 2014) and flow-dependent vasodilation and remodelling of arteries (Yamamoto et al., 2000; Yamamoto et al., 2006). In the pulmonary system, P2X4 activity regulates surfactant secretion in type II epithelial cells of alveoli (Miklavc et al., 2011). Activation of P2X4 is associated with inflammation involving peripheral and central inflammatory cell types (Layhadi et al., 2018; Layhadi & Fountain, 2019; Ulmann et al., 2008; Wareham et al., 2009). Up-regulation of P2X4 in spinal microglia contributes to neuropathic pain in preclinical models (Biber et al., 2011; Tsuda et al., 2003; Williams et al., 2019).

Despite P2X4 being an attractive drug target there remains a paucity of small molecules that can selectively modulate its activity. Some antidepressants, such as **paroxetine**, duloxetine and **carbamazepine** derivatives, can inhibit P2X4 receptor activity at micromolar concentrations (Nagata et al., 2009; Tian et al., 2014; Yamashita et al., 2016), although it is disputed whether this is a result of direct receptor antagonism (Toulme et al., 2010). **5-(3-Bromophenyl)-1,3-dihydro-2H-benzo[3,2-e]-1,4-diazepin-2-one (5-BDBD)**, a widely claimed selective antagonist, has an IC_{50} value of 0.5 μM (Donnelly-Roberts et al., 2008) and blocks P2X4 currents in CHO cells. 5-BDBD has modest effects at rat **P2X1** and **P2X3 receptors** (Coddou et al., 2019). More recently compounds including PSB12054, PSB12062 (Hernandez-Olmos et al., 2012), **BX430** (Ase et al., 2015) and **NP-1815-PX** (Matsumura et al., 2016) have been identified as P2X4 inhibitors. Natural products have also been a valuable source of P2X4 modulators. The best known is **ivermectin**, a bacteria-derived macrocyclic lactone that acts as a positive allosteric modulator of P2X4 receptors (Priel & Silberberg, 2004), but it is also active at other channels (Chen & Kubo, 2018). More recently, protopanaxadiol ginsenosides from the plant *Panax ginseng* have been identified as positive allosteric modulators of P2X4 (Dhuna et al., 2019). Despite recent advances in the identification of molecules that modulate P2X4 receptor activity (Stokes et al., 2017), a mechanistic description of how such molecules work is often limited.

We have identified that taspine, an alkaloid extract of the plant *Croton lechleri*, inhibits P2X4 receptor activity. Extracts of *Croton lechleri* cortex are used in traditional medicine by Amazonian tribes for wound healing (Perdue et al., 1979). The anti-inflammatory and cicatrizant properties of *Croton lechleri* extract are principally attributed to taspine (Vaisberg et al., 1989). Here, we describe the inhibitory properties of taspine on P2X4 receptor activity and its mechanism of action.

What is already known

- Taspine is a natural product with anti-inflammatory activity.

What does this study add

- Taspine suppresses P2X4 receptor activity via PI3-kinase inhibition.
- Taspine inhibits pro-inflammatory signalling via inhibition of P2X4 receptors in macrophage.

What is the clinical significance

- Inhibition of P2X4 receptor activity with taspine has anti-inflammatory activity.

2 | METHODS

2.1 | Cell lines

1321N1 cells (ECACC Cat# 86030402, RRID:CVCL_0110) were grown in DMEM containing 10% (v/v) FBS, 50 $U \cdot ml^{-1}$ penicillin and 50 $\mu g \cdot ml^{-1}$ streptomycin. Clonal 1321N1 stable cells stably expressing human P2X4, mouse P2X4 or human P2X7 receptors were generated by lentiviral transduction. Human P2X4 (Accession Q99571), mouse P2X4 (Accession Q9JJX6) or human P2X7 (Accession Q99572) were cloned into a pLVX-IRES-mCherry vector (Clontech). Lentiviral particles were propagated in HEK293T cells. Transduced 1321N1 cells were sorted for high expression of mCherry fluorescence using a BC FACS Aria II and clones expanded. BV-2 mouse microglia cells (BCRJ Cat# 0356, RRID:CVCL_0182) were cultured in RPMI 1640 medium containing 10% (v/v) FBS, 50 $U \cdot ml^{-1}$ penicillin and 50 $\mu g \cdot ml^{-1}$ streptomycin. Human **P2X2**, **P2X3** and P2X2/3 1321N1 stable cells were gifted through collaboration with Afferent Pharmaceuticals and described previously (Richards et al., 2019). BV-2 cells were exposed to 10 $\mu g \cdot ml^{-1}$ LPS for 24 h before experimentation. All cell lines were maintained in a humidified environment at 37°C and 5% CO_2/95% air.

2.2 | Human primary macrophage

The use of human blood samples in this research was approved by the Faculty of Medicine & Health Sciences Research Ethics Committee,

University of East Anglia. Monocyte-derived macrophage were prepared as described previously (Layhadi et al., 2018). Briefly, peripheral venous blood was collected from healthy human volunteers through the National Health Service Blood and Transplant (Addenbrooke's Hospital, Cambridge University Hospital, Cambridge, U.K.). Blood was layered on top of Histopaque-1077 (Sigma-Aldrich, Haverhill, U.K.) and centrifuged at 1000× *g* for 25 min. Buffy coat layers were collected and peripheral blood mononuclear cells were counted. Cells were adhered onto T75 flasks (Corning, UK) for 2 h and cultured in RPMI 1640 with 2 mM L-glutamine, 2.5% (v/v) heat-inactivated autologous serum, 50 U·ml^{-1} penicillin and 50 μg·ml^{-1} streptomycin at 37°C for 6 days with 10 ng·ml^{-1} GM-CSF (PeproTech).

2.3 | Intracellular Ca^{2+} measurements

Cells were seeded at 2.5 × 10^3 cells per well in 96-well culture plates and maintained in cell culture conditions overnight. Culture medium was replaced with HEPES-buffered physiological saline (HBPS) containing (mM): NaCl, 130; KCl, 5; HEPES, 10; D-glucose, 8; $MgCl_2$, 1.2; $CaCl_2$, 1.5; pH 7.4, NaOH. Cells were loaded with Fura-2-AM calcium indicator by incubation for 1 h at 37°C in darkness with HBPS containing 2 μM Fura-2-AM (TEF labs) and 0.01% (v/v) pluronic F-127. Fura-2 measurements were made using a Flexstation III instrument (Molecular Devices) and 340/380 nm excitation and 520 nm emission wavelengths with 2 s sampling. One second sampling was used for P2X3 stable cells. Calcium responses are given as *F ratio*, which is the ratio of 520 nm fluorescence intensity at 340/380 nm excitation. Cells were incubated with antagonists or vehicle control for 30 min prior to the start of experiments. For experiments performed in the absence of extracellular Ca^{2+} the physiological saline contained (mM): NaCl, 130; KCl, 5; HEPES, 10; D-glucose, 8; $MgCl_2$, 2.7; EGTA, 2; pH 7.4, NaOH.

2.4 | Patch-clamp electrophysiology

Whole-cell patch-clamp electrophysiology was performed using the port-a-patch planar patch instrument (Nanion) fitted with an eight-valve gravity-fed perfusion panel. Cells in culture were dissociated using TrypLE (Invitrogen) and resuspended at 1 × 10^6 cells·ml^{-1} in extracellular recording solution containing (mM): NaCl, 140; KCl, 4; $MgCl_2$, 1; $CaCl_2$, 2; D-glucose, 5; HEPES, 10; pH 7.4. 3–5 MΩ NPC-1 chips were filled with internal recording solution contained (mM): NaCl, 10; CsF, 140; EGTA, 2; HEPES, 10; pH 7.4; 10 mM BAPTA replaced EGTA in internal recording solutions for experiments to test the dependency of intracellular Ca^{2+} on drug effects. Seals of ≥1 GΩ were formed and allowed to stabilise for 2 min before breakthrough to the whole-cell configuration. Following whole-cell access, slow capacitance was measured using the auto C-slow function (Patchmaster software, HEKA) and cells were held at −80 mV for 2–3 min prior to the initial agonist application. ATP and other agonists were applied for 2 s at 4 min intervals. Taspine, LY294402 or vehicle controls were applied between ATP applications. Data were sampled at 1 KHz and low-pass filtered at 10 kHz (HEKA EPC 10 amplifier).

2.5 | CXCL5 secretion assay

Human CXCL5 chemokine was quantified in supernatants of ATP-stimulated human primary macrophage cultures by ELISA (Biolegend, Cambridge, UK). Cells were cultured in 96-well plates and treated with taspine or PSB-12062 for 30 min prior to ATP challenge.

2.6 | In vitro phosphoinositide 3-kinase activity assay

The enzymatic activity of human recombinant PI3-kinase δ (PI3Kδ, Promega) was quantified using an ADP-Glo lipid kinase system (Promega) and 25 μl reaction volumes. Reactions contained 400 μg·μl^{-1} recombinant PI3Kδ and 50 μg·ml^{-1} phosphoinositol-4,5-biphosphate: phosphatidylserine. Drugs or vehicle control were incubated with the reaction mix for 30 min prior to initiating the kinase reaction by the addition of ATP. All reactions were performed a 25°C. The amount of ADP produced through kinase activity was quantified by luciferase bioluminescence following its conversion to ATP using the ADP-Glo lipid kinase system. Luciferase bioluminescence was measured using a Flexstation III instrument (Molecular Devices) and 500 ms integration time. The relationship between substrate concentration and reaction velocity was fitted using by the Michaelis–Menten equation:

$$v = \frac{V_{max}[S]}{K_M + [S]}$$

where *v* is the reaction velocity, V_{max} is the maximum velocity, [S] is the concentration of substrate and K_M is the Michaelis constant. Lineweaver–Burk plots were generated from the linear first-order phases of Michaelis–Menten curves and used to determine K_M and V_{max} values.

2.7 | Cytotoxicity assay

LDH content of cell supernatants was quantified using colorimetric LDH assay kit (Abcam, Cambridge). 2.5 × 10^3 cells per well were seeded into 96-well well plates and cultured overnight. Culture medium was replaced with HBPS and experiments performed at 37°C. LDH release was detected by absorbance at 450 nm using a Flexstation III instrument (Molecular Devices).

2.8 | Experimental design, data analysis and statistics

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). Studies were designed to groups of equal size, using randomisation and blinded analysis where technically or practically feasible. All data analysis was performed using OriginPro software (OriginLab). Data distribution was tested using a Shapiro–Wilk test for normality. Population means were compared with an ANOVA for parametric datasets and Mann–Whitney test for non-parametric datasets if F in ANOVA achieved the necessary level of statistical significance. Where ANOVA is used followed by post test, a post test was only performed if F achieved $P < 0.05$ and there was no significant variance inhomogeneity. Data are expressed as mean ± SEM and n is the number of technical repeats or the number of biological repeats (donors) for primary macrophage work. Statistical analysis was performed using independent values from technical or biological repeats. The threshold for statistical significance was $P < 0.05$ throughout. Statistical analysis was only performed on datasets where $n \geq 5$. Data point outliers were included in all analysis and presentation of data. Agonist and antagonist concentration–response curves were fitted using a modified Hill equation as below:

$$Y = \text{Start} + (\text{End} - \text{Start})\frac{X^n}{k^n + X^n}$$

where k = Michaelis constant and n number of cooperative sites. The data sets were then compared with an F test. Pairwise comparison of EC_{50} values generated in curve shift experiments was conducted using a paired sample t test. For intracellular Ca^{2+} measurements, statistical comparison is made between the peak Ca^{2+} response in 1321N1 cells and between Ca^{2+} response AUC when using BV-2 cells and human primary macrophage. Data normalisation to control values has been applied to control for inter-experimental variation where applicable. For transformed datasets (e.g. data express as % control), test data points are normalised to control values within the same technical repeat.

2.9 | Materials

The initial chemical screen against ATP-evoked Ca^{2+} responses in the 1321N1 human P2X4 stable cell line was performed using the National Cancer Institute (NIH, USA) Natural Product Set III. The purity of taspine in this library was >90%. Data in this study were generated using commercially available taspine (>97% purity; Santa Cruz Biotechnology). PSB-12062, BX-430, **LY294002**, **ivermectin**, ATP, α,β-methylene ATP, **bzATP**, **staurosporine**, **thapsigargin** and all basic salts were purchased from Sigma Aldrich.

2.10 | 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 2019/20 (Alexander et al., 2017).

3 | RESULTS

3.1 | Taspine inhibits recombinant human and mouse P2X4 receptor activity

Taspine (Figure 1a), a natural product alkaloid found in the sap of the *Croton lechleri* tree (Gupta et al., 2008; Kelly & Xie, 1998), was identified as a hit compound in a human P2X4 receptor activity screen of 117 natural products (Natural Product Set III; National Cancer Institute, NIH, USA). Taspine inhibited Ca^{2+} responses evoked by a maximal ATP concentration in 1321N1 cells stably expressing the human P2X4 receptor (Figure 1b). The half-maximal concentration of inhibition by taspine was 1.6 ± 0.4 μM ($n = 5$) (Figure 1c). To confirm the effect of taspine was due to inhibition of P2X4-dependent Ca^{2+} entry in these assays and not due to adverse effects on Ca^{2+} mobilisation from intracellular stores, we performed control experiments in the absence of extracellular Ca^{2+} and tested the effect of taspine on store mobilisation using thapsigargin. In the absence of extracellular Ca^{2+}, ATP evoked no change in intracellular Ca^{2+} in 1321N1 human P2X4 cells confirming the contribution of Ca^{2+} influx to the observed response (Figure S1). In addition, mobilisation of intracellular Ca^{2+} stores by thapsigargin was not affected by taspine (Figure S1). A comparison of known P2X4 antagonists (Figure 1c) revealed that the activity of taspine was significantly less potent that PSB12062 (Hernandez-Olmos et al., 2012) or BX430 (Ase et al., 2015), equally potent as 5-BDBD (Abdelrahman et al., 2017) and significantly more potent than **PPADS** (Jones et al., 2000). Taspine inhibited Ca^{2+} responses in 1321N1 stably expressing the mouse P2X4 receptor activated by a maximal ATP concentration (Figure 1d). The potency of taspine at mouse P2X4 (Figure 1e) was not significantly different from the potency at human P2X4. ATP-evoked Ca^{2+} influx was not observed in untransfected 1321N1 cells. Taspine inhibited human P2X4 activity in a non-competitive fashion (Figure 2a). Taspine at 10 μM (IC_{80}; Figure 1c) caused an approximate 80% reduction in response maxima, whilst the ATP EC_{50} remained unchanged (Table 1). These effects were mirrored for mouse P2X4 (Table 1). To further explore the mechanism of action of taspine, we examined the rate of onset and reversibility of taspine inhibition in Ca^{2+} assays. These experiments revealed that taspine had a slow onset of inhibition, reaching half maximal inhibition after approximately 15 min incubation and maximal inhibition at 30 min (Figure 2b). In reversibility experiments, ATP responses recovered by approximately 95% following 30 min

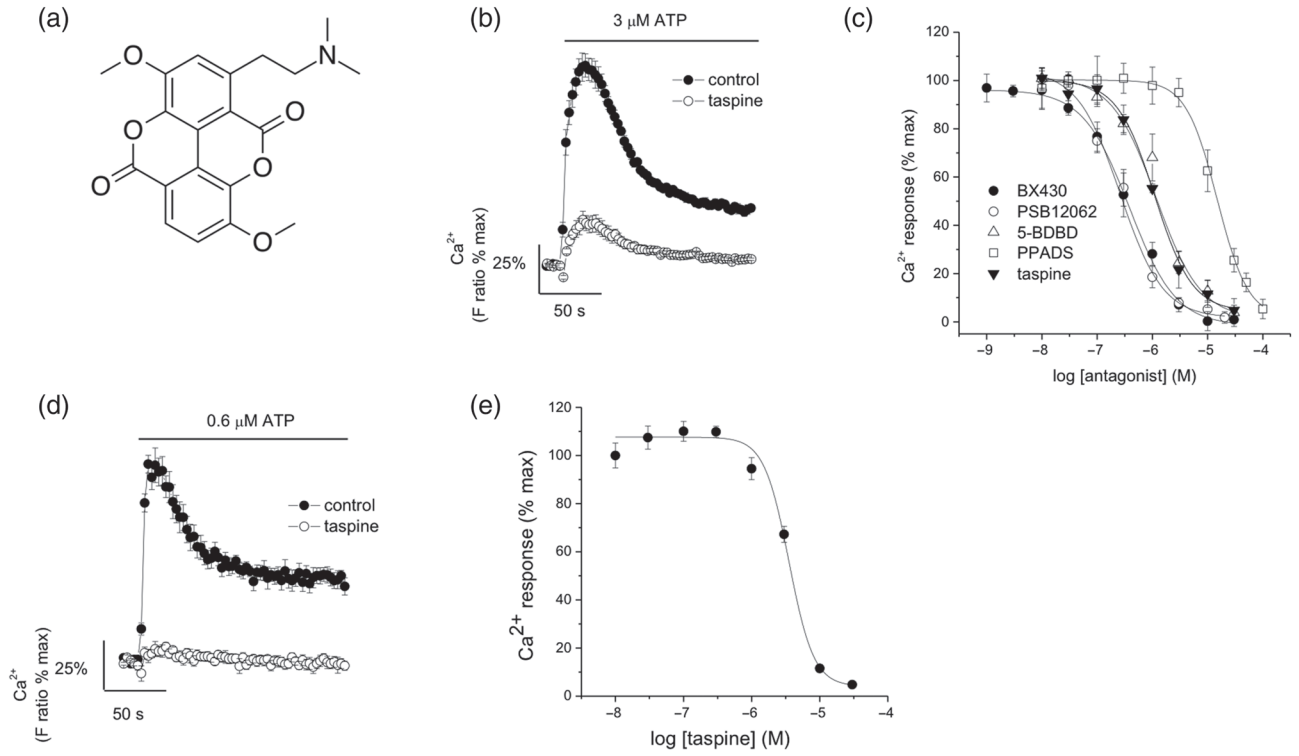

FIGURE 1 Taspine inhibition of recombinant human and mouse P2X4 receptors. (a) Taspine chemical structure. (b) Intracellular Ca^{2+} response evoked in 1321N1 human P2X4 stable cells by 3 μM ATP with and without taspine (10 μM; 30 min; $n = 5$). (c) Concentration-inhibition curves comparing taspine potency with P2X4 antagonists at human P2X4 (3 μM ATP; $n = 5$). (d) Intracellular Ca^{2+} response evoked in 1321N1 mouse P2X4 stable cells by 0.6 μM ATP with and without taspine (10 μM; 30 min; $n = 5$). (e) Concentration-inhibition curves for taspine at mouse P2X4 (0.6 μM ATP; $n = 5$)

washout of vehicle control (Figure 2c). No significant recovery (<10%) in ATP response was observed following taspine washout for 30 min (Figure 2c). Taspine lacked cytotoxic effects in these assays as measured by LDH release (Campwala et al., 2014; Day et al., 2019), though the positive control staurosporine caused substantial cytoxicity (Figure 2d).

3.2 | Taspine selectivity for the P2X4 receptor

The selectivity of taspine for P2X4 over other human P2X receptor subtypes was investigated using 1321N1 stably expressing P2X2, P2X3, P2X2/3 or P2X7 receptors. Agonist EC_{50} values and response maxima for P2X2, P2X3, P2X2/3 or P2X7 receptors were unaffected by treatment with 10 μM taspine (≈IC_{80} human P2X4; 30 min) (Table 1). To further investigate selectivity and to negate any generalised effects on calcium responses, we took advantage of the endogenous **M_2 muscarinic receptors** expressed by 1321N1 cells (Kunysz et al., 1989). **Carbachol**-evoked Ca^{2+} responses were unaffected by taspine treatment (10 μM, 30 min) (Table 1). These data demonstrate taspine selectivity for inhibition of P2X4 activity over other P2X subtypes tested.

3.3 | Taspine inhibits P2X4 receptor-dependent signalling in inflammatory cells

To investigate the effectiveness of taspine in inhibiting the activity of native P2X4 receptors, we selected inflammatory cell types where a role for P2X4 has been documented previously. Unlike 1321N1 cells that are void of native **P2Y receptors** (Schachter et al., 1996). We have previously observed that the contribution of P2X receptors to ATP-evoked intracellular Ca^{2+} responses in inflammatory cells is often masked by a predominant P2Y response and the P2X4 positive allosteric modulator ivermectin is useful in unmasking P2X4-mediated responses in native cells (Layhadi et al., 2018; Layhadi & Fountain, 2017; Layhadi & Fountain, 2019). In BV-2 mouse microglia cells, pharmacological antagonism of P2X4 has no significant effect on ATP-evoked Ca^{2+} signalling (Dhuna et al., 2019), though a P2X4-dependent component can be revealed using ivermectin, a positive allosteric modulator (Priel & Silberberg, 2004). In agreement with previous studies (Dhuna et al., 2019), P2X4 antagonism with PSB12062 (10 μM, 30 min) had no effect on ATP-evoked Ca^{2+} signalling in BV-2 cells. Taspine also had no effect. However, ivermectin potentiated ATP-evoked Ca^{2+} signalling could be completely reversed by PSB12062 (Figure 3a,b) confirming that the effects of

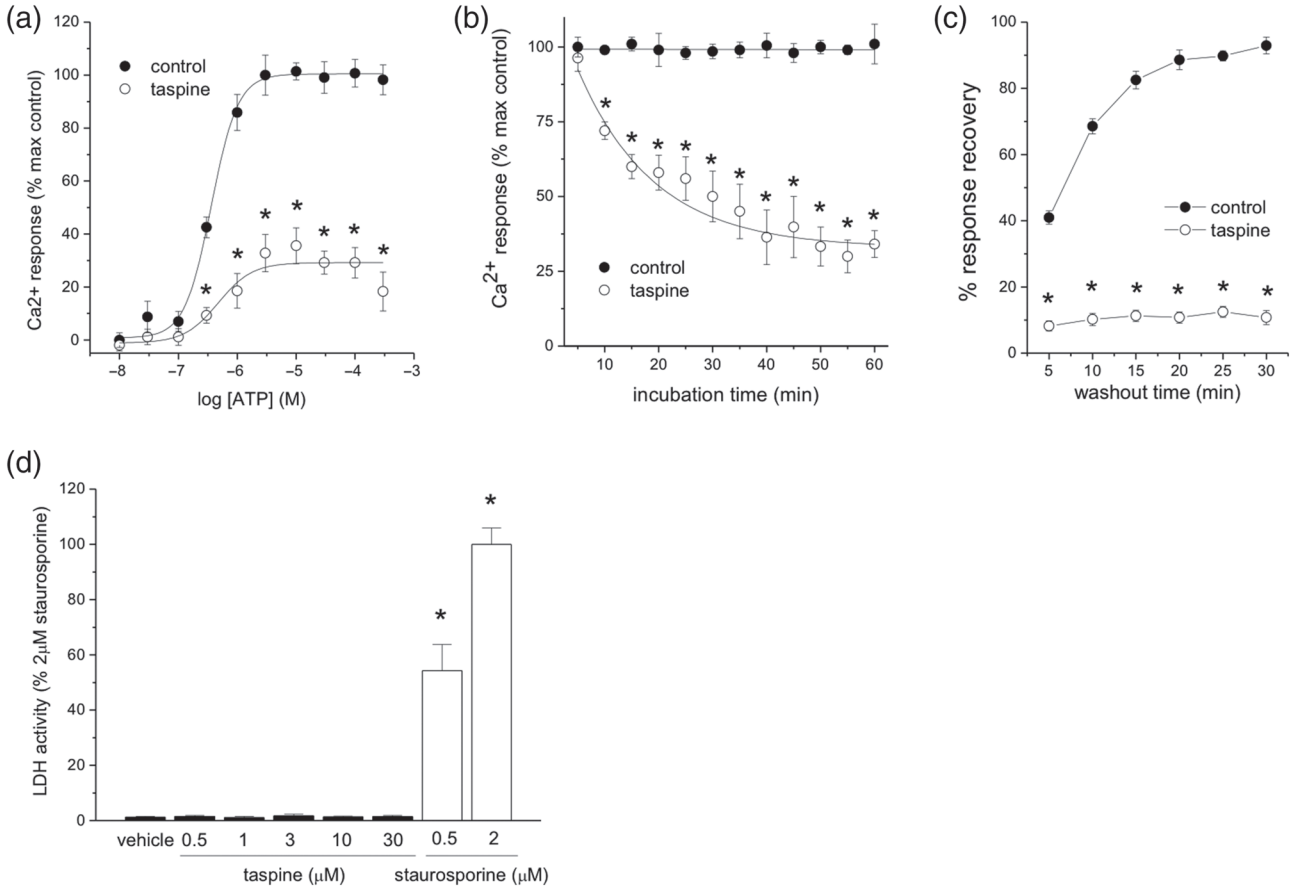

FIGURE 2 Taspine action at human P2X4. (a) ATP concentration–response relationship for intracellular Ca^{2+} responses in 1321N1 human P2X4 cells in the presence and absence (vehicle control) of taspine (10 μM; 30 min; $n = 5$; *$P < 0.05$ vs. control). (b) Onset of taspine effect. 1321N1 human P2X4 cells were incubated with vehicle (control) or 10 μM taspine for different times before Ca^{2+} responses were evoked with 3 μM ATP ($n = 5$; *$P < 0.05$ vs. control). (c) Irreversibility of taspine inhibition using 1321N1 human P2X4 cells. Data show the second Ca^{2+} response evoked by 3 μM ATP, in the presence or absence of taspine, relative (% recovery) to an initial response evoked by 3 μM ATP in the presence of vehicle. Responses are following different periods of washout ($n = 9$; *$P < 0.05$ vs. control). (d) Taspine lack of cytotoxicity. 1321N1 human P2X4 cells incubated with vehicle control, taspine, or staurosporine (positive control) for 3 h. Cytotoxicity measured by LDH release ($n = 6$; *$P < 0.05$ vs. control)

TABLE 1 Effect of taspine and LY294002 on receptor responses

Receptor	EC_{50} (control)	EC_{50} (test)	Response maxima (% control response)
Human P2X2	0.74 ± 0.2 μM (6); ATP	Taspine 0.72 ± 0.3 μM (6) ^{ns} LY 3.16 ± 0.4 μM (5)*	Taspine 98.2 ± 3.2% (6) ^{ns} LY 67.1 ± 5.3% (5)
Human P2X3	0.92 ± 0.3 μM (7); α,β-meATP	Taspine 0.89 ± 0.4 μM (7) ^{ns} LY 1.21 ± 0.8 μM (5) ^{ns}	Taspine 102.4 ± 4.2% (6) ^{ns} LY 95 ± 4.2% (5) ^{ns}
Human P2X2/3	1.8 ± 0.3 μM (6); α,β-meATP	Taspine 1.5 ± 0.3 μM (6) ^{ns} LY 7.29 ± 1.6 μM (5)*	Taspine 96.4 ± 3.4% (6) ^{ns} LY 54.2 ± 5.2% (5)*
Human P2X4	0.47 ± 0.1 μM (6); ATP	Taspine 0.48 ± 0.2 μM (6) ^{ns} LY 0.62 ± 0.2 μM (5) ^{ns}	LY 15.2 ± 4.5% (5)*
Mouse P2X4	0.36 ± 0.1 μM (6); ATP	Taspine 0.39 ± 0.1 μM (6) ^{ns} LY 0.64 ± 0.3 μM (5) ^{ns}	Taspine 9.5 ± 3.8% (6)* LY 12.4 ± 3.2% (5)*
Human P2X7	13.2 ± 0.2 (6); bzATP	Taspine 14.2 ± 0.2 (6) ^{ns} LY 16.8 ± 1.2 (5) ^{ns}	Taspine 106 ± 2.8% (6) ^{ns} LY 98 ± 3.6% (5) ^{ns}
Human M2 muscarinic	2.2 ± 0.2 (8); carbachol	Taspine 2.3 ± 0.4 (7) ^{ns}	Taspine 101 ± 4.8% (7) ^{ns}

Note: 10 μM taspine (≈IC_{80} at human P2X4) or 50 μM LY294002 (LY; (≈IC_{80} at human P2X4). *N* number in parentheses. *ns* not significantly different to control values.

$^{*}P < 0.05$ compared with control values.

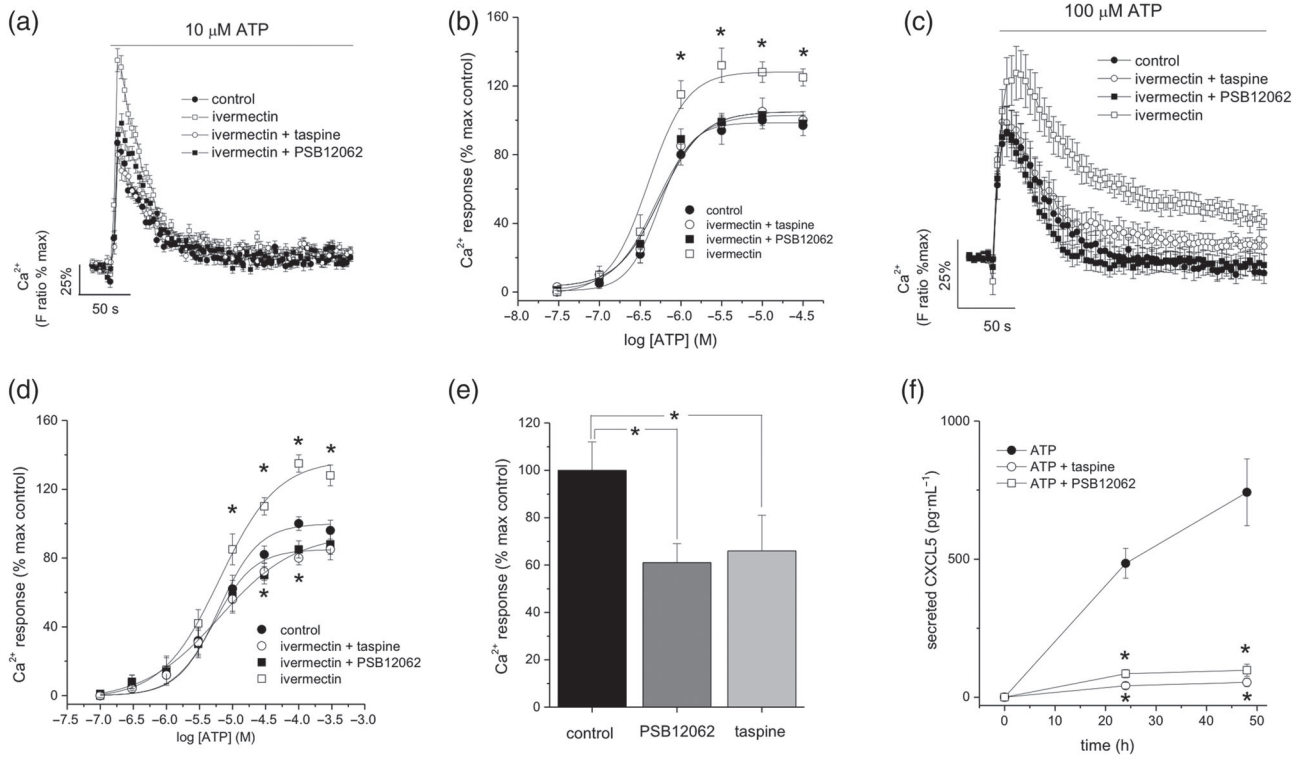

FIGURE 3 Taspine inhibits P2X4-dependent signalling in inflammatory cells. (a) Averaged ($n = 6$) ATP-evoked intracellular Ca^{2+} responses in mouse BV-2 microglia cells, showing potentiation by ivermectin (3 μM, 30 min). Ivermectin potentiation is reversed by PSB12062 (10 μM; 30 min) or taspine (10 μM; 30 min). (b) ATP concentration–response curve in BV-2 cells showing effect of ivermectin on response maxima and reversal by PSB12062 or taspine (10 μM; 30 min both; $n = 7$; $^*P < 0.05$ vs. control). (c) Averaged ($n = 7$) ATP-evoked intracellular Ca^{2+} responses in human primary macrophage showing potentiation by ivermectin (1 μM, 30 min). PSB12062 (10 μM; 30 min) or taspine (10 μM; 30 min) reverse response potentiation by taspine. (d) ATP concentration–response curve in human macrophage showing effect of ivermectin on response maxima and reversal by PSB12062 or taspine ($n = 7$; $^*P < 0.05$ vs. control). (e) Effect of PSB12062 (10 μM; 30 min) and taspine (10 μM; 30 min) on Ca^{2+} responses evoked by 100 μM ATP in human primary macrophage ($n = 6$; $^*P < 0.05$ vs. control). (f) CXCL5 secretion in human macrophage is stimulated by ATP. PSB12062 (10 μM) or taspine (10 μM) inhibits CXCL5 secretion in response to ATP ($n = 8$; $^*P < 0.05$ vs. ATP alone). PSB12062 or taspine was added 30 min before ATP challenge

ivermectin are dependent upon P2X4. Taspine mirrored the effects of PSB12062 and reversed potentiation by ivermectin (Figure 3a,b). Likewise, in human primary macrophage ATP-evoked Ca^{2+} responses were potentiated by ivermectin (Figure 3c,d) and the potentiation could be reversed by either PSB12062 or taspine (Figure 3c,d). In agreement with our previous study (Layhadi et al., 2018), inhibition of P2X4 with PSB12062 alone could inhibit Ca^{2+} responses evoked by maximal ATP concentrations in the absence of ivermectin (Figure 3e). This activity was mimicked by taspine (Figure 3e). Experiments were also performed to investigate the ability of taspine to inhibit ATP-evoked Ca^{2+} signalling in BV-2 and human primary macrophage when the contribution of P2Y receptors has been abolished with thapsigargin and the contribution of P2X receptors is revealed (Layhadi et al., 2018; Layhadi & Fountain, 2017) (Figure S2). In these experiments, inhibition of P2Y receptor evoked Ca^{2+} signalling by thapsigargin reveals a residual component that could be inhibited both by taspine and PSB12062 (Figure S2).

In a recent study, we demonstrated that ATP stimulated secretion of the pro-inflammatory mediator CXCL5 by human primary macrophage via activation of P2X4 (Layhadi et al., 2018). PSB12062 and taspine significantly reduced CXCL5 secretion in response to ATP (Figure 3f). These data demonstrate that taspine mirrors the effect of PSB12062 in suppressing P2X4-mediated signalling in inflammatory cells.

3.4 | Effect of taspine on P2X4 receptor currents

Robust and reproducible ATP-evoked inward currents could be recorded from 1321N1 human P2X4 stable cells in whole-cell configuration (Figure 4a). The internal and external salt solutions and agonist concentration employed minimise current rundown previously reported for human P2X4 (Fountain & North, 2006). Taspine application had no effect on the magnitude of currents (Figure 4b), though they could be reversibly blocked by BX430 (Figure 4c). However, potentiation of the current magnitude by ivermectin was completely reversed by taspine (Figure 4d). Potentiation by ivermectin was sustained over multiple ATP-evoked currents in control conditions (Figure 4e). In further experiments, 10 mM BAPTA replaced EGTA in the internal recording solution in an effort to determine the

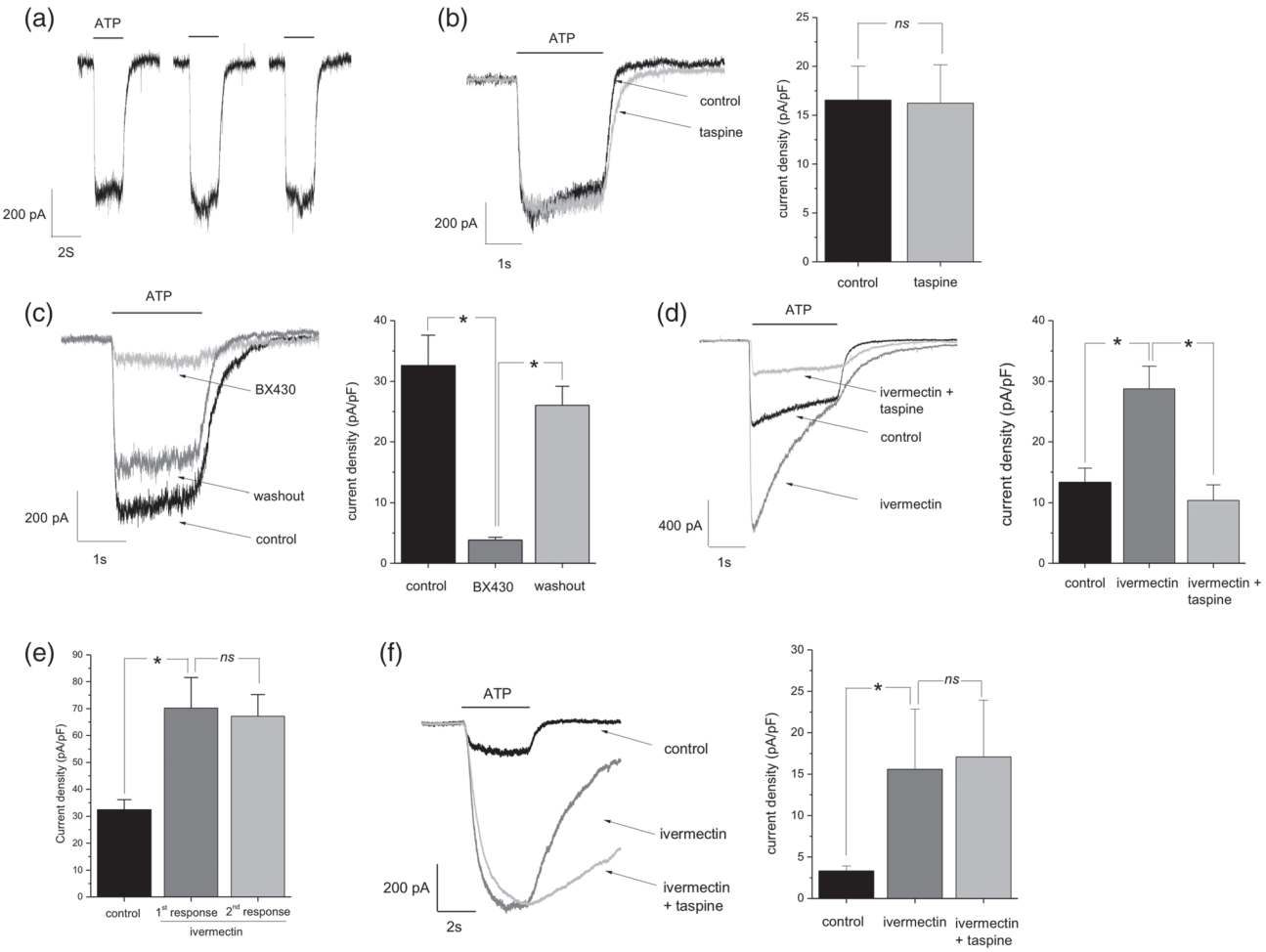

FIGURE 4 Taspine inhibition of human P2X4 currents. (a) Representative train of ATP-evoked inward currents in 1321N1 human P2X4 cells ($n = 5$); 3 μM ATP applied at 4 min intervals. (b) Representative traces (*left panel*) showing lack of taspine effect on P2X4 current density (*right panel*; $n = 5$; *ns*, not significant); 10 μM taspine applied for 4 min following the initial control response. (c) Representative traces (*left panel*) showing reversible inhibition by BX430 (*right panel*; 5 μM; $n = 5$, $^*P < 0.05$); 10 μM BX430 applied for 4 min following the initial control response. Washout is for 4 min. (d) Representative traces (*left panel*) showing current potentiation by ivermectin (3 μM, 4 min) and reversal of potentiation by taspine (10 μM, 4 min). Average current density data (*right panel*; $n = 5$; $^*P < 0.05$). (e) Averaged data showing sustained potentiation by ivermectin (3 μM, 4 min) following two ATP-evoked response at 4 min intervals ($n = 5$; $^*P < 0.05$; *ns*, not significant). (f) Experiments including 10 mM BAPTA in internal recording solution. Representative traces (*left panel*) showing current potentiation by ivermectin (3 μM, 4 min) and loss of taspine (10 μM, 4 min) inhibition. Average current density data (*right panel*; $n = 5$; $^*P < 0.05$; *ns*, not significant)

dependency of the taspine effect on intracellular Ca^{2+}. In these experiments, ivermectin could still potentiate P2X4 responses though the inhibitory action of taspine was lost (Figure 4f).

3.5 | The pharmacological properties of taspine are mimicked by LY294002

To further explore the mechanism of action, we sought to test the hypothesis that taspine suppresses P2X4 activity indirectly through inhibition of PI3-kinase. We hypothesised this could be a rational explanation for the following reasons. Firstly, the IC_{50} for taspine at mouse and human P2X4 orthologues is similar (Figure 1) and it acts in an apparent orthosteric fashion (Figure 2), possibly suggestive of a common target other than the receptor itself. Secondly, the onset of taspine action is very slow and irreversible (Figure 2), and these are unlikely characteristics of a direct receptor effect and suggestive of depletion of a cellular component or pathway that positively regulates P2X4. Thirdly, phosphoinositides positively regulate rat P2X4 (Bernier et al., 2008). Finally, taspine inhibits VEGF signalling in endothelial cells limiting phosphorylation of Akt and Erk1/2 (Zhao et al., 2008). As for taspine, the known PI3-kinase inhibitor LY294002 (Vlahos et al., 1994) inhibited ATP-evoked Ca^{2+} responses in 1321N1 human P2X4 cells (Figure 5a) (Table 1). The concentration of half-maximal inhibition was 25.8 ± 4.2 μM ($n = 6$) (Figure 5b). In patch-clamp electrophysiological studies, LY294002 displayed the same properties as taspine (Figure 4). LY294002 had no effect on P2X4 currents under control conditions (Figure 5c) but was able to fully reverse

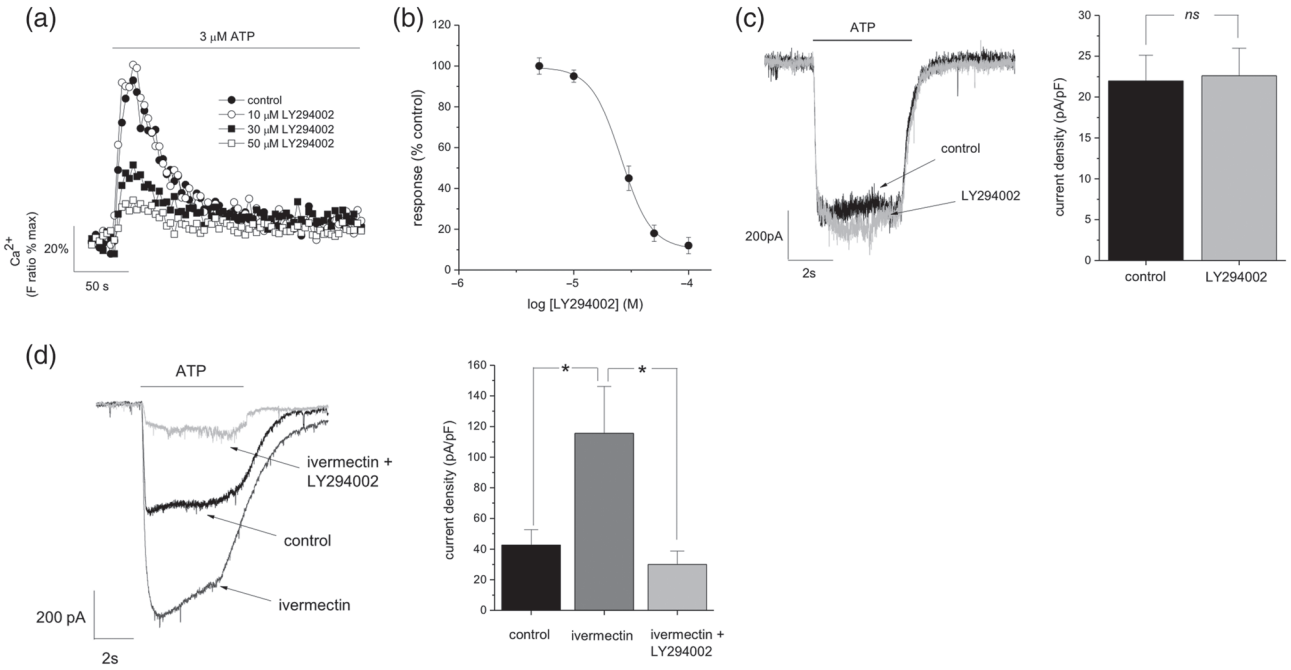

FIGURE 5 Effect of PI3-kinase inhibitor LY294002 on P2X4 receptor mediated Ca^{2+} responses and inward currents. (a) Representative Ca^{2+} responses in 1321N1 human P2X4 cells showing effect of LY294002 (10–50 μM, 30 min) ($n = 5$). (b) LY294002 concentration-inhibition curve against Ca^{2+} responses in 1321N1 human P2X4 cells evoked by 3 μM ATP ($N = 5$). (c) Representative traces (*left panel*) showing lack of LY294002 effect on P2X4 current density (*right panel*; $n = 5$; *ns*, not significant); 10 μM LY294002 applied for 4 min following the initial control response. (d) Representative traces (*left panel*) showing current potentiation by ivermectin (3 μM, 4 min) and reversal of potentiation by LY294002 (50 μM, 4 min). Average current density data (*right panel*; $n = 5$; $^*P < 0.05$)

potentiation of the current by ivermectin (Figure 5d). For completeness, we also tested the effect of LY294002 on the activity of other P2X receptors (Table 1). LY294002 showed no inhibitory effect on human P2X1, P2X3 or P2X7 but displayed activity at human P2X2, human P2X2/3 and mouse P2X4 in calcium assays (Table 1).

3.6 | Taspine inhibits PI3-kinase in a competitive manner

To investigate the mechanism of action of PI3-kinase inhibition by taspine, we compared the direct effects of LY294002 or taspine on the enzymatic kinetics of PI3-kinase using recombinant enzyme. In these assays, reaction velocities in all experiments followed Michaelis–Menten kinetics with increasing concentration of ATP substrate (Figure 6a,b). Neither LY294002 or taspine had any apparent effect on the maximum velocity (V_{max}) of PI3-kinase enzymatic kinetics (Figure 6a,b). Lineweaver–Burk plot analysis confirmed no effect of LY294002 on V_{max} (control, 467 ± 18 nmol·mg^{-1}·min^{-1}; 3 μM LY294002, 446 ± 32 nmol·mg^{-1}·min^{-1}; 10 μM LY294002, 462 ± 25 nmol·mg^{-1}·min^{-1}; 30 μM LY294002, 444 ± 22 nmol·mg^{-1}·min^{-1}), but a concentration-dependent significant increase in K_M (control, K_M 42 ± 8 μM ATP; 3 μM LY294002, K_M 66 ± 5 μM ATP; 10 μM LY294002, K_M 126 ± 12 μM ATP; 30 μM LY294002, K_M 294 ± 18 μM ATP; $n = 5$) (Figure 6c). These findings are consistent with LY294002 as a competitive inhibitor of PI3-kinase (Vlahos et al., 1994). Lineweaver–Burk plot analysis also confirmed no effect of taspine on V_{max} (control, 467 ± 18 nmol·mg^{-1}·min^{-1}; 3 μM taspine, 475 ± 28 nmol·mg^{-1}·min^{-1}; 10 μM taspine, 454 ± 34 nmol·mg^{-1}·min^{-1}; 30 μM taspine, 461 ± 30 nmol·mg^{-1}·min^{-1}; $n = 5$) (Figure 6d). However, taspine at 10 μM or greater caused a significant and concentration-dependent increase in K_M (control, K_M 42 ± 8 μM ATP; 3 μM taspine, K_M 51 ± 8 μM ATP; 10 μM taspine, K_M 102 ± 18 μM ATP; 30 μM LY294002, K_M 263 ± 27 μM ATP; $n = 5$) (Figure 6d). These data confirm that taspine inhibits PI3-kinase in a competitive manner.

4 | DISCUSSION

In this study, we demonstrate that taspine inhibits the P2X4 receptor over other P2X subtypes tested. Rather than a direct antagonism of the receptor, our data suggest that the effect of taspine on P2X4 is indirect and via inhibition of PI3-kinase and depletion of phosphoinositides. The identification of taspine as a PI3-kinase inhibitor is novel and our work supports a role of PI3-kinase and phosphoinositides in regulating P2X4 activity (Bernier et al., 2008, 2012). The activity of taspine is physiologically relevant as it can suppress P2X4-dependent activity in human primary macrophage including the secretion of the CXCL5 (Layhadi et al., 2018), supporting a pro-inflammatory role of PI3-kinase signalling (Guiducci et al., 2008; Koorella et al., 2014). This and our previous work

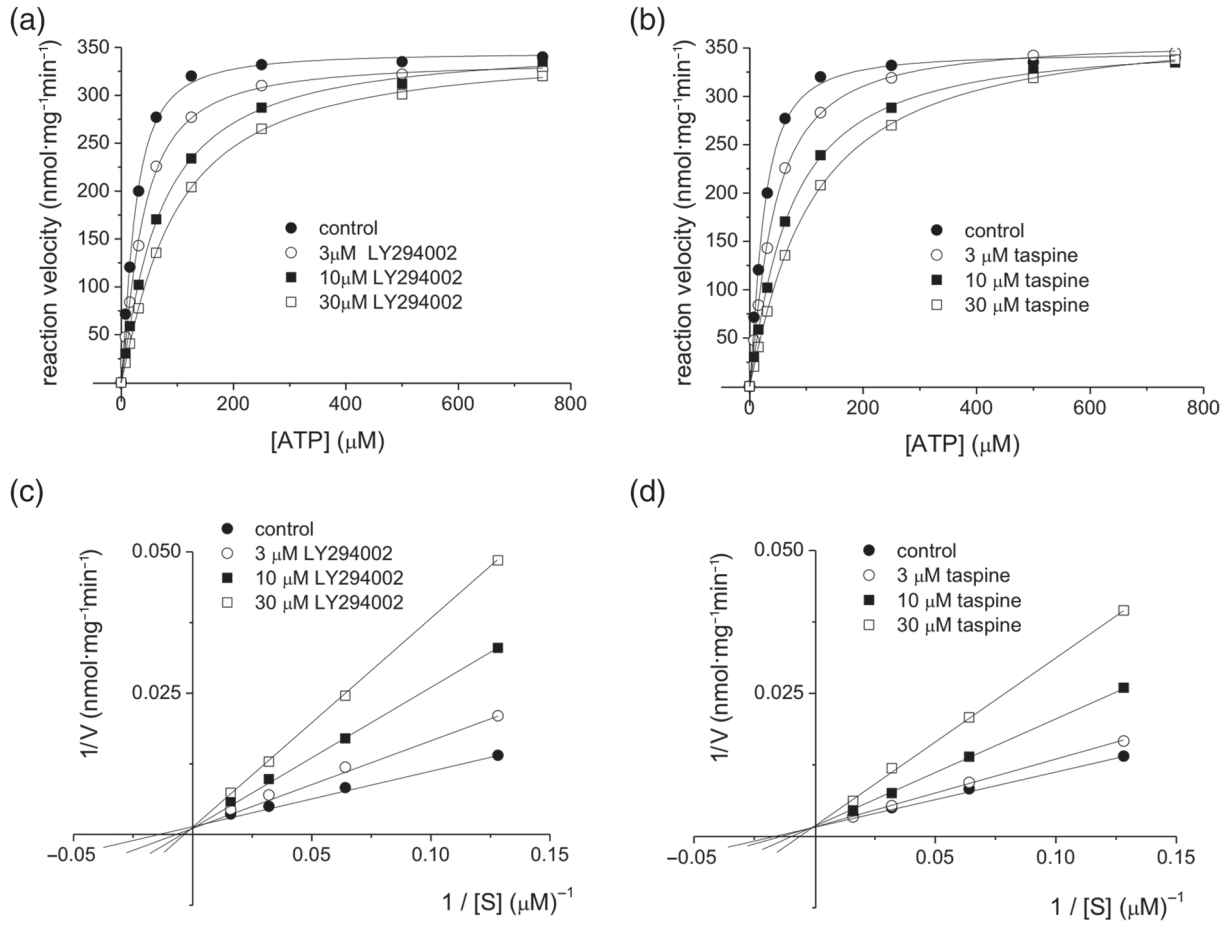

FIGURE 6 Effect of taspine on PI3-kinase enzymatic kinetics. (a, b) Michaelis–Menten curves showing reaction velocities (ADP production) for human recombinant PI3-kinase δ with varying ATP concentrations and phosphoinositol-4,5-biphosphate with phosphatidylserine as substrate. The concentration-dependent effects on the Michaelis–Menten curves of LY294002 (a) and taspine (b) are shown ($n = 5$). (c, d) Lineweaver–Burk plot analysis of initial enzymatic rates showing the concentration-dependent effect of LY294002 (c) and taspine (d) on V_{max} (y-intercept) and K_M (x-intercept) ($n = 5$). All control reactions are in the presence of vehicle alone

(Layhadi et al., 2018; Layhadi & Fountain, 2019) suggest that direct antagonism of P2X4 or inhibition of pathways that enhance P2X4 activity are both routes to anti-inflammatory effects. In addition, P2X4-mediated ATP-induced chemotaxis in microglia is inhibited by PI3-kinase inhibitors (Ohsawa et al., 2007). Taspine has previously been reported to possess anti-inflammatory properties (Vaisberg et al., 1989), though this is likely due to activity at multiple targets, possibly including P2X4.

Membrane permeability is a required property of taspine if it is to access and inhibit PI3-kinase. Taspine (calculated logP 2.7) and LY294002 (calculated logP 3.6) are both hydrophobic molecules and that are predicted to be membrane permeable. In addition, pharmacokinetic and tissue distribution of taspine after intravenous administration in mice supports cell penetrance and tissue accumulation (Lu et al., 2008). Other reported intracellular targets of taspine that support its cell penetrance include topoisomerases (Fayad et al., 2009). The slow onset and irreversibility of taspine action at P2X4 are consistent with phosphoinositide depletion (Zhao et al., 2008). The PI3-kinase pathway converts cellular **PIP_2** to **PIP_3** and can be activated by tyrosine kinase receptors and GPCRs. Its inhibition depletes cellular PIP_3 and downstream phosphoinositides that are dependent on PIP_3 for their synthesis (Jean & Kiger, 2014). Previous studies using wortmannin, a non-specific PI3-kinase inhibitor, have demonstrated that depletion of phosphoinositides reduces recombinant and native rodent P2X4 receptor activity, suggesting that the C-terminus of P2X4 harbours a binding domain for several biologically active phosphoinositides (Bernier et al., 2008). In contrast to this study, some previous studies investigating P2X3 and P2X2/3 (Mo et al., 2009) and P2X7 (Zhao et al., 2008) have observed either direct effects of phosphoinositide application or PI3-kinase inhibitors. In this study, we have focused on human P2X receptor subtypes and some differences observed here may be due to differences in the orthologue of P2X receptors investigated and the expression systems employed. A further interesting feature of taspine is ability of the fast Ca^{2+} chelator BAPTA to block its inhibitory activity in whole-cell patch-clamp experiments. Class II and class III PI3-kinase subtypes are known to be regulated by Ca^{2+} and bind lipids in a calcium-dependent fashion (Liu et al., 2006; Wen et al., 2008), in addition Ca^{2+} influx in

some nerves is known to activate PI3-kinase phosphorylation of Akt and GSK3 (Nicholson-Fish et al., 2016). The effects of BAPTA may therefore suggest taspine inhibits the activity of Ca^{2+}-dependent PI3-kinase though whether Ca^{2+} entry via P2X4 directly regulates PI3-kinase remains to be determined.

In agreement with our own data, Bernier et al. (2008) demonstrate that wortmannin alone has no effect on P2X4 currents, though the effectiveness of ivermectin as a positive allosteric modulator of P2X4 is reduced by wortmannin. This is an interesting observation and suggests ivermectin activity during electrophysiological recordings is inhibited following PI3-kinase inhibition with either wortmannin (Bernier et al., 2008), LY294002 or taspine. It is unlikely that these three chemically distinct molecules directly prohibit ivermectin binding to the P2X4 receptor (Gao et al., 2015; Latapiat et al., 2017). Ivermectin has direct effects on P2X4, reducing channel desensitisation and stabilising the channel in the open conformation (Priel & Silberberg, 2004). However, several studies have also suggested that ivermectin facilitates P2X4 activity by increasing the number of channels at the cell surface (Stokes, 2013; Toulme et al., 2006). As PI3-kinase regulates cellular trafficking (Lindmo & Stenmark, 2006), LY24002 and taspine may limit the trafficking activity of ivermectin and therefore reverse its effect on P2X4. Differences in trafficking pathways available for P2X4 (Royle et al., 2005) in intact cells (Ca^{2+} imaging experiments) versus dialysed cells (whole-cell patch clamp experiments) may also account for the difference effects of taspine and LY294002 observed in this study. Our data shows that the inhibitory action of taspine is restricted to P2X4, though the known PI3-kinase inhibitor LY294002 did cause significant inhibition of the human P2X2 and P2X2/3 response. The effect of LY294002 is consistent with the findings of Fujiwara and Kubo (2006) who observed acceleration of rat P2X2 desensitisation kinetics in the presence of LY294002. The effects of taspine and LY294002 are consistent for all P2X receptors tested except for human P2X2 and P2X2/3 receptors. This discrepancy is difficult to reconcile as both compounds inhibit PI3-kinase. Possible reasons include the observed less efficacious action of taspine on PI3-kinase compared with LY294002, or an off-target direct effect of LY294002 on the P2X2 receptor as observed for other kinase inhibitors and P2X receptors (Dayel et al., 2019).

Using human recombinant PI3-kinase, we demonstrated that taspine, like LY294002, directly inhibits the enzyme in a competitive manner. LY294002 is a known competitive PI3-kinase inhibitor (Vlahos et al., 1994), with recent structural information suggesting this mechanism is achieved through partial overlap of the binding site for LY294002 and the adenine group of ATP in the ATP/enzyme complex (Walker et al., 2000). Likewise, taspine must also exert its effect by competition with ATP binding in the enzyme, though the site is currently elusive. However, taspine shares a fused heterocyclic structure with wortmannin, another known competitive inhibitor of PI3-kinase (Walker et al., 2000).

In summary, taspine inhibits the activity of recombinant P2X4 and native P2X4 during physiologically relevant processes. We suggest that the apparent non-competitive inhibition of P2X4 by taspine is observed because of inhibiting a positive modulatory role of PI3-kinase, rather than any direct binding of taspine to P2X4 (Figure S3). We suggest the mechanism of taspine effect is via competitive inhibition of PI3-kinase. These data support a role of PI3-kinase in regulating P2X4 receptor function and the anti-inflammatory action of taspine.

ACKNOWLEDGEMENTS

This work was funded by a British Heart Foundation grant (PG/16/69/32194) awarded to S.J.F. and Malaysian Government Studentship awarded to I.B.N.

AUTHOR CONTRIBUTIONS

I.B.N., A.F.G., N.N., D.R., S.A. and S.J.F designed/performed experiments and analysed the data. M.S. and S.J.F. wrote and completed the manuscript. S.J.F. supervised the entire study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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 Natural Product Research and Design and Analysis.

DATA AVAILABILITY STATEMENT

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.

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How to cite this article: Nadzirin, I. B., Fortuny-Gomez, A., Ngum, N., Richards, D., Ali, S., Searcey, M., & Fountain, S. J. (2021). Taspine is a natural product that suppresses P2X4 receptor activity via phosphoinositide 3-kinase inhibition. *British Journal of Pharmacology*, 1–14. https://doi.org/10.1111/bph.15663