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The Novel Phosphodiesterase 9A Inhibitor BI 409306 Increases Cyclic Guanosine Monophosphate Levels in the Brain, Promotes Synaptic Plasticity, and Enhances Memory Function in Rodents[S]

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ABSTRACT

N-methyl-D-aspartate (NMDA) receptor–dependent long-term potentiation (LTP) is an established cellular model underlying learning and memory, and involves intracellular signaling mediated by the second messenger cyclic guanosine monophosphate (cGMP). As phosphodiesterase (PDE)9A selectively hydrolyses cGMP in areas of the brain related to cognition, PDE9A inhibitors may improve cognitive function by enhancing NMDA receptor-dependent LTP. This study aimed to pharmacologically characterize BI 409306, a novel PDE9A inhibitor, using in vitro assays and in vivo determination of cGMP levels in the brain. Further, the effects of BI 409306 on synaptic plasticity evaluated by LTP in ex vivo hippocampal slices and on cognitive performance in rodents were also investigated. In vitro assays demonstrated that BI 409306 is a potent and selective inhibitor of human and rat PDE9A with mean concentrations at half-maximal inhibition (IC_{50}) of 65 and 168 nM. BI 409306 increased cGMP levels in rat prefrontal cortex and cerebrospinal fluid and attenuated a reduction in mouse striatum cGMP induced by the NMDA-receptor antagonist MK-801. In ex vivo rat brain slices, BI 409306 enhanced LTP induced by both weak and strong tetanic stimulation. Treatment of mice with BI 409306 reversed MK-801-induced working memory deficits in a T-maze spontaneous-alternation task and improved long-term memory in an object recognition task. These findings suggest that BI 409306 is a potent and selective inhibitor of PDE9A. BI 409306 shows target engagement by increasing cGMP levels in brain, facilitates synaptic plasticity as demonstrated by enhancement of hippocampal LTP, and improves episodic and working memory function in rodents.

SIGNIFICANCE STATEMENT

This preclinical study demonstrates that BI 409306 is a potent and selective PDE9A inhibitor in rodents. Treatment with BI 409306 increased brain cGMP levels, promoted long-term potentiation, and improved episodic and working memory performance in rodents. These findings support a role for PDE9A in synaptic plasticity and cognition. The potential benefits of BI 409306 are currently being investigated in clinical trials.

Introduction

Cognitive function, memory, and learning are closely associated with glutamatergic neurotransmission, with the postsynaptic *N*-methyl-D-aspartate (NMDA) receptor playing an essential role (Lee and Silva, 2009; Morris, 2013; Volianskis et al., 2015). The NMDA receptor signaling pathway involves several cascades and second messengers, including cyclic guanosine monophosphate (cGMP). As a downstream effector of NMDA receptor signaling, intracellular cGMP contributes to the strengthening of synaptic plasticity through long-term potentiation (LTP), which is regarded as a physiologic model for cellular processes underlying synaptic stabilization and memory formation in animals and human (Reneerkens et al., 2009; Dorner-Ciossek et al., 2017). Presynaptic and postsynaptic cGMP-dependent pathways have been implicated in early LTP (Arancio et al., 1995; Taqatqeh et al., 2009), and postsynaptic cGMP signaling can contribute to late LTP (Lu et al., 1999).

Degradation of cGMP is mediated by phosphodiesterase (PDE) enzymes, of which PDE9A selectively hydrolyses cGMP with highest affinity of all PDEs (Fisher et al., 1998). Furthermore, inhibition of PDE9A in brain tissue leads to an increase in cGMP, suggesting a role in regulation of basal levels of cGMP in the brain (Kleiman et al., 2012). As PDE9A

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is expressed in neurons of brain regions related to cognition, such as the neocortex and hippocampus (Andreeva et al., 2001), this enzyme isoform is well placed to regulate NMDA receptor signaling pathways associated with synaptic plasticity and cognitive function (Reneerkens et al., 2009; Kroker et al., 2012; Dorner-Ciossek et al., 2017). In support of this hypothesis, preclinical studies have shown that inhibition of PDE9A can increase cGMP levels in the rat brain, enhance LTP, and improve memory function in rodent cognition tasks (van der Staay et al., 2008; Hutson et al., 2011; Kleiman et al., 2012; Kroker et al., 2012, 2014). In addition, a dose-dependent increase in cGMP levels in cerebrospinal fluid (CSF) of healthy volunteers has been demonstrated following a single oral administration of the PDE9A inhibitor BI 409306 (Boland et al., 2017).

BI 409306 is a novel, potent, and selective PDE9A inhibitor in clinical development (Moschetti et al., 2016; Boland et al., 2017; Brown et al., 2019; Frölich et al., 2019). The current study aimed to assess the in vitro potency and selectivity of BI 409306 and to evaluate its functional target engagement in vivo through measurements of cGMP levels in brain tissue and CSF. Furthermore, the effects of BI 406309 on synaptic stabilization and plasticity were assessed through electrophysiological recordings of long-term potentiation in rat hippocampal slices. The efficacy of BI 409306 on cognition was also evaluated in tasks assessing working and episodic memory in mice.

Materials and Methods

Drugs

The structure of BI 409306 is shown in Fig. 1. BI 409306 was synthesized at Boehringer Ingelheim Italia, Chemistry Research Center (Milan, Italy). MK-801 as hydrogen maleate or hydrochloride salt was obtained from Sigma Aldrich (Saint-Quentin Fallavier, France, and Taufkirchen, Germany, respectively).

Determination of In Vitro Potency and Selectivity of BI 409306

Cell extracts containing PDE isoforms were produced using a baculovirus expression system (Bac-To-Bac; Invitrogen, Carlsbad, CA). The open reading frames of PDEs were cloned into the plasmid vector pFastbac (human PDE2A, 3A, 4B, 5A, 7A, and rat PDE9A) or pDEST8 (human PDE1A, 1C, 9A, 10A) and transformed into the *Escherichia coli* strain DH10Bac (Invitrogen). Bacmid DNA was isolated and purified to obtain baculoviruses coding for each PDE isoform. The baculovirus was amplified by three rounds of transfection into SF9 insect cells. Approximately 72 hours after transfection, once a cytotoxic effect was observed, SF9 cells were centrifuged and frozen in liquid nitrogen, then resuspended in phosphate-buffered saline containing 1% Triton X-100 with protease inhibitors. After 45 minutes of incubation on ice, cell debris was removed by centrifugation at 13,000 rpm for 30 minutes, and the protein content of the cytoplasmic cell extract containing the recombinant enzymes was determined using a bicinchoninic acid assay kit (Pierce Biotechnology, Rockford, IL).

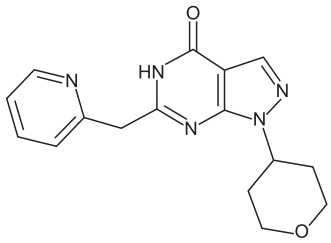

Fig. 1. Structure of BI 409306.

The inhibition of human PDE1A, 1C, 2A, 3A, 4B, 5A, 7A, 9A, and 10A and rat PDE9A by BI 409306 was assessed on the basis of the rate of hydrolysis of cGMP and cyclic adenosine monophosphate (cAMP) in the TRKQ7100 PDE [^{3}H]cGMP and TRKQ7090 PDE [^{3}H] cAMP scintillation proximity assays (SPA) (GE Healthcare Europe GmbH, Freiburg, Germany). The amount of cell extract containing PDE used in the SPA was adjusted for activity without inhibitors at approximately 1000–2000 counts per minute after 1 hour of incubation at room temperature. This corresponded to total protein concentrations of 480 pg/μl for PDE1A, 2000 pg/μl for PDE1C, 3000 pg/μl for PDE3A, 624 pg/μl for PDE5A, 160 pg/μl for human PDE9A, 300 pg/μl for PDE2A, 4B, 7A, and 10A, and 60 pg/μl for rat PDE9A. The reaction mixture contained 20 μl of serial dilutions of BI 409396 in an assay buffer containing 0.4% dimethyl sulfoxide and 10 μl of assay buffer containing the diluted PDE protein of interest. Assay buffer contained (in millimolars): Tris (pH 7.4 adjusted with hydrochloric acid) 50, $MgCl_2$ 8.3, EGTA (pH 7.4 adjusted with HCl) 1.7, 0.1% bovine serum albumin, 0.05% Tween-20. For the PDE1A and 1C assays, 200 nM calmodulin and 12 mM $CaCl_2$ were also added. Afterward, 10 μl of [^{3}H]cGMP or [^{3}H]cAMP (80 nM, 1 mCi/l in assay buffer) were added, and the reaction mixture was incubated for 1 hour at room temperature before the enzymes were inactivated by addition of 10 μl EX00000734 (50 μM) in assay buffer and 25 μl of yttrium silicate SPA beads (17.9 mg/ml) in H_2O. The conversion of [^{3}H]cGMP or [^{3}H]cAMP to [^{3}H]GMP or [^{3}H]AMP, respectively, was determined using a Topcount NXT scintillation counter (Perkin Elmer, Waltham, MA). All counts obtained in the SPA were subtracted from the counts for the negative control, which contained SF9 cytosolic extract from uninfected cells. Counts were then converted into percentage activity, where the count for a positive control assay, run in the absence of inhibitor, was set at 100%. Percentage activity was used to calculate the BI 409306 concentration at half-maximal inhibition (IC_{50}) using GraphPad Prism software.

The selectivity of BI 409306 against PDE6 and other non-PDE targets was evaluated using receptor binding assays according to standard procedure at MDS Pharma Services (Taipei, Taiwan).

Animals and Ethical Considerations

All animal procedures were performed in accordance with institutional guidelines and European legislation on the use and care of laboratory animals (CEE 86/609 at time of study) and were approved by the ethical committee of the responsible regional council. All efforts were made to minimize animal suffering and to use the minimum number of animals to produce reliable results.

ABBREVIATIONS: BAY 73-6691, 1-(2-chlorophenyl)-6-[(2*R*)-3,3,3-trifluoro-2-methylpropyl]-1,5-dihydro-4*H*-pyrazolo[3,4-d]pyrimidine-4-one; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CSF, cerebrospinal fluid; fEPSPs, field excitatory postsynaptic potentials; IC_{50}, concentration at half-maximal inhibition; LC-MS/MS, liquid chromatography coupled to mass spectrometry; LTP, long-term potentiation; MK-801, (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine; NMDA, *N*-methyl-D-aspartate; PDE, phosphodiesterase; PF-04447943, 1,5-dihydro-6-[(3*S*,4*S*)-4-methyl-1-(2-pyrimidinylmethyl)-3-pyrrolidinyl]-1-(tetrahydro-2*H*-pyran-4-yl)-4*H*-pyrazolo[3,4-d]pyrimidin-4-one; SPA, scintillation proximity assays.

Pharmacokinetic Studies

Male Wistar rats (Crl:WI[Han]; 200–250 g; Charles River, Sulzfeld, Germany) or male NMRI mice (Crl:NMRI; 20–25 g; Charles River) were orally administered BI 409306 1.5 mg/kg. Blood samples were collected at 0.5, 1, 2, and 4 hours and placed in EDTA tubes on ice. Plasma was separated by centrifugation and stored at −70°C. In a separate study, Wistar rats were orally administered BI 409306 1.5 mg/kg. After 30 minutes, rats were anesthetized with isoflurane and CSF collected using stereotaxic guidance. The CSF samples were stored at −70°C. Plasma and CSF concentrations of BI 409306 were determined using liquid chromatography coupled to mass spectrometry (LC-MS/MS).

Microdialysis for Determination of cGMP in the Rat Prefrontal Cortex

Determination of cGMP levels in the prefrontal cortex of male Sprague-Dawley rats (CD-COBS; 200–250 g; Charles River, Calco, Italy) was performed using transcerebral microdialysis as previously described (Marte et al., 2008). Briefly, microdialysis probes were transversely implanted under chloral hydrate anesthesia (400 mg/kg; permitted at the time of the study) using the following coordinates: anteroposterior +9.7, dorsoventral +8.4 from the interaural line (Paxinos and Watson, 1986). Following surgery, rats were allowed to recover for 24 hours. During experiments, the microdialysis probes were infused with artificial cerebrospinal fluid containing (in millimolars): NaCl 145, KCl 3, $MgCl_2$ 1, $CaCl_2$ 1.26, pH 7.4, at a flow rate of 5 μl/min Consecutive samples were collected from freely moving rats every 20 minutes for a total of 240 minutes.

Rats received an intraperitoneal injection (2.5 ml) of either BI 409306 (1.5, 7.5, or 1 mg/kg) or vehicle. The following were used to prepare 10 ml of vehicle (pH 4.5–4.6): 9 ml of NaCl 0.9%, 0.67 ml of HCl 0.1 M, 0.33 ml of NaOH 0.2 M. Injections were administered after the collection of the first three basal samples (0–20, 20–40, 40–60 minutes) to estimate baseline cGMP levels. Rats were subsequently euthanized by anesthetic overdose and the probe position verified by optical examination of the fiber tract. Dialysate cGMP content was assayed using a ^{125}I-cGMP dual range radioimmunoassay kit (Izotop, Budapest, Hungary) and an acetylation protocol (standard curve range 2–128 fmol) and a modified bound/unbound separation protocol (Wang et al., 2004).

Determination of cGMP in Rat CSF

Male Wistar rats (Crl:WI[Han]; 250–320 g; Charles River) 8–10 weeks of age were orally administered BI 409306 (0.3, 1, 3, or 10 mg/kg) or vehicle (sodium chloride solution, pH 2.8). After 30 minutes, rats were anesthetized with isoflurane, and CSF samples (50–100 μl) were collected through the cisterna magna and centrifuged at 10,000 rpm at 4°C for 5 minutes. The concentration of cGMP in rat CSF was determined by high-performance LC-MS/MS on an analytical hydrophilic interaction column with gradient elution. [$^{15}N_5$]cGMP was used as an internal standard. The lower limit of quantification was 0.800 nmol/l using 20 μl of CSF.

Determination of cGMP in Mouse Striatal Tissue

Adult male CD-1 mice (RjOrl:SWISS; 25–28 g; Janvier Laboratories, Le Genest-St-Isle, France) were intraperitoneally administered BI 409306 (0.15 or 0.5 mg/kg) or vehicle control with and without subcutaneous MK-801 (0.125 mg/kg). After 30 minutes, animals were euthanized by focused microwave irradiation, and samples of striatal tissue were collected, homogenized, and centrifuged as previously described (Schmidt et al., 2008; Kroker et al., 2014). Supernatant concentration of cGMP was determined by cGMP-ELISA using a commercially available kit (Enzo Life Sciences Inc., Farmingdale, NY) according to the manufacturer's protocol.

Electrophysiological Recording of Hippocampal LTP

This study was performed at Forschungsinstitut Angewandte Neurowissenschaften GmbH (Magdeburg, Germany).

Hippocampal Slice Preparation. Male Wistar rats (HsdCpb: Wu; Harlan Winkelmann GmbH, Borchen, Germany) 7 to 8 weeks of age were euthanized by a blow to the neck followed by decapitation. The brain was extracted and incised between the hemispheres. The hippocampus was removed, transferred to a cold metal block and mounted onto a tissue chopper. Transverse hippocampal slices of 400 μM thickness were prepared at a cutting angle of approximately 70° to the blade to preserve the laminar structure of the hippocampus.

After preparation, slices were maintained in an interface-type perfusion chamber at a depth of 1–3 mm beneath the surface of the experimental solution at a constant temperature of 33°C for at least 1 hour before recordings were made. Experimental solution was gassed with carbogen (95% O_2/5% CO_2) in a prechamber under mildly hyperbaric conditions (approx. 1 atm) and circulated at a flow rate of 2.5 ml per minute. The experimental solution contained (in millimolar): NaCl 124, KCl 4.9, $MgSO_4$ 1.3, anhydrous $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.6, and glucose 10. The osmolarity and pH of the solution were corrected to 330 mOsm and pH 7.4, respectively.

Recording. Hippocampal LTP recordings were performed as previously described (Rönicke et al., 2009). Briefly, recordings of field excitatory postsynaptic potentials (fEPSPs) were obtained from recording electrodes placed in the apical dendritic layer of the stratum radiatum of the CA1 region using a direct current amplifier (Model 1700 Differential AC Amplifier; AM-Systems, Sequim, WA). Recording electrodes were pulled from borosilicate glass (1–5-MΩ, 3–20-μm tip diameter) and were filled with experimental solution. Field potentials were filtered using a low pass filter (5kHz).

The Schaffer collateral commissural fibers were locally stimulated (1–5 V) using a varnished steel spring wire monopolar stimulation electrode with 20–150-Ω impedance (Sprint Metal, Reichshof, Germany) and a constant current biphasic stimulus generator (Model 2100 Isolated Pulse Stimulator; AM-Systems). Test stimuli were adjusted to elicit a population spike of approximately 30% of maximum amplitude.

Following 60 minutes of baseline recordings, LTP was induced using a weak (four sets of paired pulses applied at 200-millisecond intervals) or a strong (10 four-pulse bursts applied at 200-millisecond intervals repeated once after 10 minutes) tetanic stimulation paradigm. The duration of each pulse was 0.2 milliseconds, and the interpulse interval within pairs or bursts was 10 milliseconds.

BI 409306 (0.1, 0.3, or 1 μM) or vehicle control (0.01% dimethyl sulfoxide) was bath-applied from 30 minutes prior to the weak tetanic stimulus until the end of the experiment or from 60 minutes prior to the strong tetanic stimulus until 10 minutes after the final stimulus, to evaluate potential long-lasting effects of BI 409306 on protein synthesis–dependent late-phase LTP induced by the strong tetanic stimulus.

Cognitive Assessments

The T-maze and object recognition tasks were employed to assess the effect of BI 409306 on working and episodic memory, respectively.

T-Maze Continuous Alternation Task in Mice. The study was performed using male CD-1 mice (RjOrl:SWISS; Janvier Laboratories) 4 to 5 weeks of age at Neurofit S.A.S. (Illkirch, France). Mice were group-housed on a reversed light-dark cycle with food and water available ad libitum. The continuous alternation task was performed using a nontransparent T-maze as previously described (de Bruin et al., 2010). Briefly, the experimental protocol consisted of a single session comprising one forced-choice followed by 14 free-choice trials. During the forced-choice trial, one of the arms (either left or right) was blocked, and mice were allowed to explore the open arm. Mice were then allowed to enter either arm in the 14 free-choice trials. If a mouse entered the same arm on two subsequent trials, an error response was recorded. Each session ended once 14 free-choice trials were completed

or 10 minutes had elapsed, whichever occurred first. Working memory performance was assessed by the percentage of successful alternation over the 14 free-choice trials.

Mice received oral BI 409306 (0.007, 0.02, 0.05, 0.1, 0.5, or 2.5 mg/kg) or vehicle (0.5% Tylose) 60 minutes prior to the T-maze test. MK-801 (0.1 mg/kg) or vehicle (saline) was administered subcutaneously 30 minutes before the assessment.

Object Recognition Task in Mice. Male C57BL/6J mice (C57BL/6JRj; Janvier Laboratories) 3 to 4 months of age were group-housed under standard light conditions with food and water available ad libitum. The object recognition task was performed as described previously (Dodart et al., 1997). Briefly, during trial 1, mice explored an open field in containing two similar and unfamiliar objects. During trial 2, conducted 24 hours later, mice explored an open field containing a familiar object encountered during trial 1 and a novel object. Exploration time in both trials was 10 minutes, and time spent exploring each object was recorded. Episodic memory performance was assessed by the difference between the time spent exploring the novel object compared with the familiar object.

Mice were administered BI 409306 (0.015 or 0.15 mg/kg) or vehicle (saline) via intraperitoneal injection 30 minutes prior to trial 1.

Statistical Analyses

Microdialysis data were analyzed using nonparametric Kruskal-Wallis test followed by Steel multiple comparison test using KyPlot 3.0 (Kyenslab Inc., Tokyo, Japan). Levels of cGMP in rat CSF and mouse striatal tissue were analyzed using one-way analysis of variance (ANOVA) and with post-hoc Dunnett tests using GraphPad Prism (GraphPad, San Diego, CA). Recording and analysis of fEPSPs was performed using custom-made software (PWIN; Leibniz Institute for Neurobiology, Magdeburg, Germany). Averaging of fEPSP slopes was performed using locally written macros, and LTP data were analyzed using two-way repeated-measures ANOVA. Performance in cognitive tasks was compared using one-way ANOVA with post-hoc Fisher's least significant difference test for the T-maze task, and post-hoc Bonferroni's test for the object recognition task, using GraphPad Prism.

Results

Potency and Selectivity of BI 409306 In Vitro. BI 409306 demonstrated both potent and selective inhibition of human PDE9A activity, with a calculated mean (S.D.) IC_{50} of 65 (11) nM (Supplementary Fig. 1; Table 1). The IC_{50} for inhibition of rat PDE9A was 168 nM (Table 1). IC_{50} values of 1.45 and 1.17 μM were calculated for PDE1A and PDE1C, respectively, and no inhibitory activity was observed for PDE2A, 3A, 4B, 5A, 6AB, 7A, and 10A with BI 409306 up to a concentration of 10 μM (Table 1). There was no significant activity of BI 409306 at 10 μM against 95 non-PDE targets (Supplementary Table 1).

Pharmacokinetics. Following oral dosing with BI 409306 1.5 mg/kg, rat pharmacokinetic studies indicated that maximal plasma concentration of BI 409306 was reached after approximately 30 minutes, with mean (S.E.M.) C_{max} of 892 (47) nM (Table 2). In mice, maximal plasma concentration of BI 409306 was reached after approximately 60 minutes, with mean (S.E.M.) C_{max} of 2227 (288) nM. The concentration of BI 409306 in CSF, a surrogate for free brain concentration, was approximately 20% of the plasma concentration.

Effect of BI 409306 on cGMP in Rat Prefrontal Cortex and CSF. Following microdialysis, the mean (S.E.M.) basal extracellular cGMP concentration in the rat prefrontal cortex was 4.08 (0.51) fmol/100 μl. At 20 minutes post-intraperitoneal injection of BI 409306 1.5–15 mg/kg, a significant dose-dependent increase in extracellular cGMP levels was observed ($X^2 = 13.95$; $P < 0.01$; df = 3; $N = 5$ animals per group), which peaked after 20 minutes and gradually returned to control values approximately 100 minutes after the injection (Fig. 2A). At 20 minutes, the mean (S.E.M.) percentage increase in extracellular cGMP levels relative to baseline was 187.9% (49.2) for BI 409306 7.5 mg/kg and 97.6 (49.2) for BI 409306 15 mg/kg ($P < 0.05$). With BI 409306 15 mg/kg, significant increases over control values were also observed 40 and 180 minutes after BI 409306 administration. The 1.5 mg/kg dose did not significantly increase extracellular cGMP over baseline at any time point.

Furthermore, cGMP levels in rat CSF increased in a dose-dependent manner following BI 409306 administration, as determined by high-performance LC-MS/MS. Compared with vehicle control, significant increases in cGMP were induced by BI 409306 3 and 10 mg/kg ($N = 2$–6 animals per group; $P < 0.001$) but not by the lower doses of 0.3 and 1 mg/kg (Fig. 2B). Mean CSF concentration of BI 409306 30 minutes after oral administration of BI 409306 0.3 mg/kg in these rats was 67 nM. On the basis of the potency of BI 409306 against the rat PDE9 enzyme, a 50% increase in CSF cGMP was achieved at a CSF BI 409306 exposure of approximately 40% of the IC_{50} against PDE9. This is roughly in line with data from a clinical study measuring cGMP increase in CSF after oral treatment with BI 409306 in healthy male volunteers (Boland et al., 2017).

Effect of BI 409306 on MK-801-Induced Reduction in cGMP Levels in Mouse Striatal Tissue. The amount of cGMP detected per mg of mouse striatum was significantly reduced following the administration of the NMDA receptor antagonist MK-801 ($N = 8$ animals per group; $P < 0.01$; Fig. 3). This reduction was significantly attenuated by coadministration of BI 409306 0.5 mg/kg, with a mean increase in cGMP levels of 41% compared with MK-801 alone ($N = 8$ animals per group; $P < 0.01$). After coadministration of BI 409306 0.15 mg/kg, cGMP levels were increased by 31% compared with MK-801 alone, but this difference did not reach significance.

TABLE 1

Potency and selectivity of BI 409306 for inhibition of human PDE1A, 1C, 2A, 3A, 4B, 5A, 6AB, 7A, 9A, and 10A, and rat PDE9A determined using an SPA to measure hydrolysis of either cGMP or cAMP

	PDE Isoform										
	1A	1C	2A	3A	4B	5A	6AB	7A	9A (Human)	9A (Rat)	10A
cGMP/cAMP	cGMP	cGMP	cGMP	cAMP	cAMP	cGMP	cGMP	cAMP	cGMP	cGMP	cAMP
IC_{50}, μM, mean (S.D.)[a]	1.45	1.17 (0.27)[b]	>10	>10	>10	>10	>10	>10	0.07 (0.01)[c]	0.17	>10

[a] $N = 2$ dilutions from one experiment unless otherwise stated.
[b] $N = 6$ dilutions from three experiments.
[c] $N = 8$ dilutions from four experiments.

TABLE 2
Plasma concentrations of BI 409306 in rats and mice 0.5, 1, 2, and 4 hours after oral administration of BI 409306 1.5 mg/kg

	Time (h)			
	0.5	1.0	2.0	4.0
Mean[a] (S.E.M.) plasma concentration [nM]				
Rat	892 (47)	714 (146)	683 (129)	50 (15)
Mouse	2097 (215)	2227 (288)	1042 (262)	183 (62)

[a]N = 3 animals for each experiment.

Effect of BI 409306 on Hippocampal LTP Ex Vivo. When BI 409306 was applied 30 minutes before a weak tetanic stimulation, it significantly enhanced LTP at 1 μM compared with vehicle control (vehicle control, N = 12 slices from six animals; BI 409306, N = 9 slices from five animals; P = 0.021), but not at 0.1 or 0.3 μM (Fig. 4A). When applied 60 minutes before a strong stimulus, BI 409306 significantly enhanced LTP compared with vehicle control at 0.3 μM (vehicle control, N = 10 slices from five animals; BI 409306, N = 10 slices from five animals; P = 0.048), but not at 1 or 0.1 μM (Fig. 4B).

Effect of BI 409306 on Working and Episodic Memory. In the T-maze task, administration of MK-801 0.1 mg/kg, led to a significant reduction in spontaneous alternation (i.e., the percentage of trials in which correct alternation was observed) from a mean (S.D.) of 71.4% (2.1) observed with vehicle to 44.3% (2.1), approximately chance level (50% correct alternation), with MK-801 ($P < 0.001$). Oral treatment of mice with BI 409306 at all doses tested (0.007–2.5 mg/kg) led to an attenuation or reversal of the MK-801-induced reduction in spontaneous alternation compared with the MK-801-treated group (N = 10 animals per group; $P < 0.05$; Fig. 5A). On the basis of the mean plasma concentration of BI 409306 measured in mice 1 hour after administration of BI 409306 1.5 mg/kg, the extrapolated plasma exposure levels of mice in the T-maze at most effective doses (0.02–0.5 mg/kg intraperitoneal) would be in the range of 67–1675 nM. These extrapolated plasma exposures correspond to BI 409306 concentrations in CSF of 13–335 nM, which is approximately 0.1- to 2-fold of the PDE9A IC_{50} on the basis of potency against the rat enzyme. Since the IC_{50} value for inhibition of PDE1 isoforms by BI 409306 is >1 μM, the memory-improving efficacy of BI 409306 can be attributed solely to the inhibition of PDE9.

In the object recognition task, during trial 1 exploration times for the two objects did not differ between mice treated with vehicle or BI 409306 at either 0.015 or 0.15 mg/kg (data not shown). At trial 2, which was performed 24 hours after trial 1, animals treated with BI 409306 0.15 mg/kg spent significantly more time exploring the novel object compared with the familiar object ($P < 0.001$), whereas there was no significant difference between time spent exploring the novel and the familiar object in mice treated with vehicle or BI 409306 0.015 mg/kg (Fig. 5B).

Discussion

This study assessed the in vitro potency and selectivity of BI 409306 and characterized its effects on synaptic plasticity and cognitive performance in rodents. Enzymatic and receptor binding assays demonstrated that BI 409306 is a potent and selective inhibitor of human PDE9A (IC_{50} = 65 nM), with weak affinity for PDE1 (approx. 20-fold selectivity) and >100-fold selectivity for PDE9A over other PDE enzymes as well as a range of non-PDE targets. Systemic administration of BI 409306 led to an increase in cGMP levels in the rat prefrontal cortex and CSF, and to a reversal of cGMP reduction induced by the NMDA receptor antagonist MK-801 in mouse striatal tissue. These observations suggest that BI 409306 binds to and inhibits PDE9A in the brain in vivo, showing functional target engagement. Furthermore, this study demonstrated for the first time that NMDA receptor blockade leads to a decrease

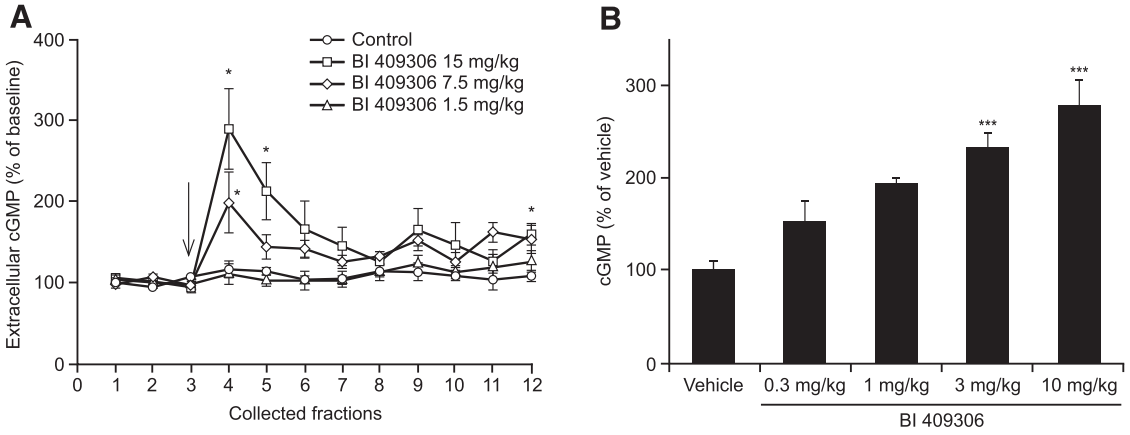

Fig. 2. The effect of BI 409306 on cGMP levels in (A) rat prefrontal cortex and (B) CSF. (A) BI 409306 1.5, 7.5, or 15 mg/kg was administered intraperitoneally (arrow). Microdialysis fractions were collected every 20 minutes. Data are expressed as mean (±S.E.M.) percentages of the mean basal level of extracellular cGMP (defined as 100%), which was determined by averaging the content of the first three samples collected before BI 409306 administration. N = 5 animals per group. (B) BI 409306 0.3, 1, 3, or 10 mg/kg was administered orally, and CSF was collected 30 minutes later. Data are expressed as mean (±S.E.M.) percentage of cGMP levels after administration of vehicle control (defined as 100%). N = 2–6 animals per group. *$P < 0.05$, ***$P < 0.001$ compared with control group.

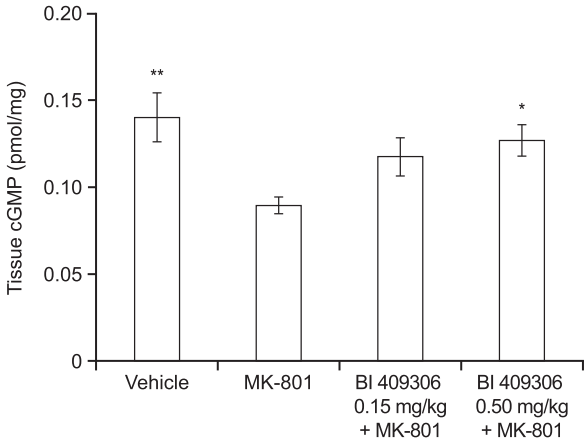

Fig. 3. The effect of BI 409306 on MK-801-induced reduction in cGMP levels in mouse striatal tissue. BI 409306 0.15 or 0.5 mg/kg was administered intraperitoneally. MK-801 0.125 mg/kg was administered subcutaneously, and tissue was collected 30 minutes later. Data are presented as mean (±S.E.M.). $N = 8$ animals per group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with MK-801 group.

in cGMP levels that could be reversed by PDE9A inhibition. This supports the hypothesis that PDE9A is located downstream of NMDA receptor activation and acts as a regulator of the cGMP-related postsynaptic signaling cascade, which is involved in synaptic plasticity. Indeed, application of BI 409306 facilitates synaptic plasticity in hippocampal slices as demonstrated by enhanced LTP induced by both weak and strong tetanic stimulation. This suggests an enhancement of both early and late phases of LTP, which are independent of and dependent on protein synthesis, respectively. Finally, mice treated with BI 409306 demonstrated improved performance on T-maze and object recognition tasks, which assess working and episodic memory, respectively.

In the first-in-human study, BI 409306 demonstrated good safety and tolerability following the administration of single rising doses up to 350 mg in healthy male participants, and BI 409306 was rapidly absorbed and eliminated (Moschetti et al., 2016). BI 409306 was also well tolerated over 14 days of daily treatment in healthy individuals (Moschetti et al., 2018) and in patients with schizophrenia (Brown et al., 2018). However, to fully assess the efficacy of BI 409306 in human patients, translatable biomarkers are required that provide similar objective measures of drug outcomes in patients, healthy volunteers, and animal models. In the present study, levels of cGMP in the rat prefrontal cortex reflected cGMP levels in rat CSF following administration of BI 409306. This is consistent with previous studies showing increases in cGMP levels following PDE9A inhibition in rodent CSF (Hutson et al., 2011; Kleiman et al., 2012) and in mouse hippocampus (Kroker et al., 2014). This suggests that cGMP levels in CSF might be a suitable biomarker for central inhibition of PDE9A in human patients. In support of this, a phase I proof-of-mechanism study of BI 409306 (25-, 50-, 100-, or 200-mg single dose) in healthy males demonstrated dose-dependent increases in CSF cGMP that reflected plasma and CSF drug exposure (Boland et al., 2017). Overall, these studies support the utility of CSF cGMP levels as a translatable biomarker of functional target engagement between animal and human studies.

Previous studies investigating the effects of PDE9A inhibitors on LTP had findings similar to those observed in this study. For example, the PDE9A inhibitor BAY 73-6691 enhanced early LTP induced by weak theta stimulation in rat hippocampal slices (van der Staay et al., 2008) and protein synthesis–dependent late LTP induced by spaced high-frequency (100-Hz) tetanic stimulation in mouse hippocampal slices (Kroker et al., 2012). PF-04447943, another PDE9A inhibitor, promoted LTP induced by a weak but not strong tetanic stimulation in ex vivo mouse hippocampus (Hutson et al., 2011). In the present study, BI 409306 enhanced LTP induced with both weak and strong tetanic stimulation, suggesting that BI 409306 can promote both early- and late-phase LTP. In addition, given that BI 409306 reversed the MK-801-induced decrease in brain cGMP levels, BI 409306 may enhance hippocampal LTP by increasing cGMP levels and promoting NMDA receptor signaling even under conditions of compromised NMDA receptor function or signaling, which has been suggested as playing a role in neuropsychiatric diseases such as schizophrenia (Lin et al., 2012). However, it should be noted that the enhancement of LTP induced with weak and strong tetanic stimulation was only observed at BI 409306 concentrations of 1 and 0.3 μM, respectively, which may indicate a bell-shaped dose-response curve, and thus presumably a narrow window of efficacy.

In previous studies assessing the effects of PDE9A inhibition on learning and memory, BAY73-6691 improved performance in object recognition and social recognition tasks, reversed an MK-801-induced working memory deficit, and reversed a memory deficit in a passive avoidance task induced by the muscarinic antagonist scopolamine (van der Staay et al., 2008). In addition, we have shown previously that BAY73-6691 attenuated deficits in an object location memory task in amyloid precursor protein-transgenic Tg2576 mice exhibiting Alzheimer's disease pathology (Kroker et al., 2014). Another compound, PF-04447943, also showed a reversal of working memory deficits induced by the NMDA receptor antagonist ketamine, and of deficits in episodic and spatial memory induced by the muscarinic antagonist scopolamine (Kleiman et al., 2012). In the present study, animals treated with BI 409306 showed significant reversal of an MK-801-induced deficit in spontaneous alternation, indicating improved working memory performance. In addition, BI 409306 also improved episodic memory performance, demonstrated by an object recognition task with a 24-hour delay between trials 1 and 2. At trial 2, animals treated with BI 409306 0.15 mg/kg spent significantly more time exploring the novel object compared with the familiar object. This indicates that animals treated with BI 409306 0.15 mg/kg remembered the object presented at trial 1, in contrast to animals treated with vehicle or 0.015 mg/kg. Notably, BI 409306 was administered only at memory acquisition phase, 24 hours prior to the trial in which improvements in memory retrieval were observed. This suggests that BI 409306 may promote consolidation of short- to long-term memory following the administration of a single dose as was also suggested by studies of other PDE inhibitors (Heckman et al., 2018). In existing literature, a distinction is drawn between mechanisms underlying short-term and long-term memory; the former has been suggested as reflecting the early phase of LTP, whereas the latter might reflect the long-lasting late phase of LTP (Reneerkens et al., 2009). Presynaptic cGMP signaling is thought to support early LTP and to activate the conversion of early LTP to late LTP through a cyclic

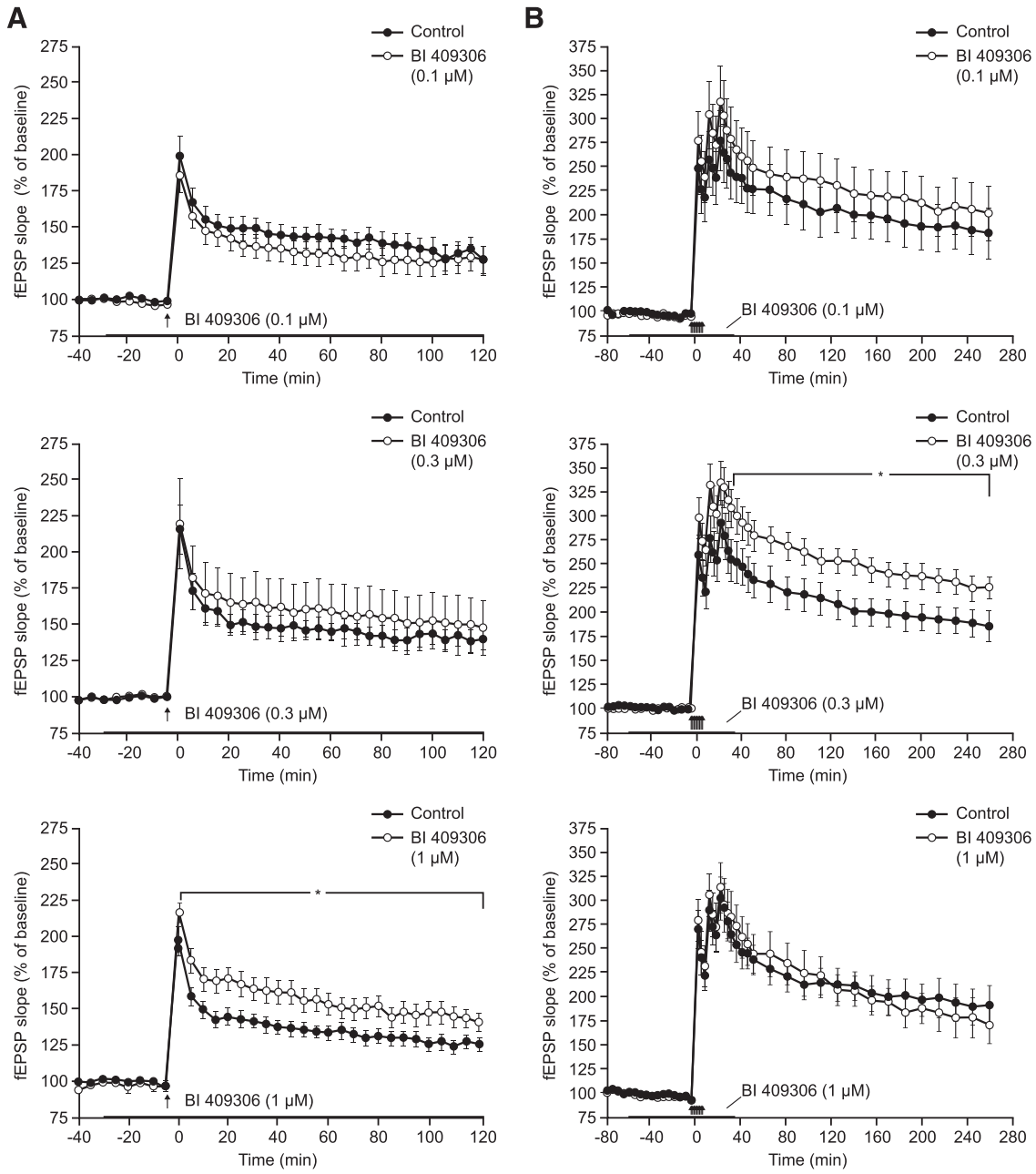

Fig. 4. The effect of BI 409306 on LTP in ex vivo rat hippocampus induced by (A) weak and (B) strong tetanic stimulation of Schaffer collateral-commissural fibers in the stratum radiatum of the CA1 region. BI 409306 0.1, 0.3, or 1 μM was bath-applied during the period indicated on the x-axis. Arrows indicate the onset of the weak or strong tetanic stimulus. Data are expressed as mean (±S.E.M.) percentage of average fEPSP slope at baseline (defined as 100%). N = 3–12 slices from three to six animals per group. *$P < 0.05$ for effect of treatment compared with control group.

adenosine monophosphate–dependent pathway (Ricciarelli and Fedele, 2018). Conversion of early LTP signaling to late LTP supported by cGMP might explain the improvement in episodic memory performance 24 hours after a single dose of BI 409306 in the present study. This might also indicate that BI 409306 improves long-term memory performance through cGMP-mediated promotion of synaptic plasticity, including structural changes such as enhanced development of dendritic spines and synapses, which has been demonstrated in cultured hippocampal neurons and in cortical pyramidal neurons in mice following PDE9A inhibition (Hutson et al., 2011; Lai et al., 2018).

The effects of BI 409306 on cognition have also been explored in a double-blind, randomized phase II study (NCT02281773) comparing BI 409306 at doses up to 100 mg and placebo in patients with schizophrenia. No significant differences were observed on a range of cognitive outcomes, and the primary endpoints were not met after 12 weeks of treatment (Brown et al., 2019). Two double-blind, randomized phase II studies (NCT02240693 and NCT02337907) have also been conducted in 128 and 329 patients with prodromal and mild Alzheimer's disease, respectively. These studies demonstrated no significant effect of BI 409306 compared with placebo on the primary efficacy endpoint of Neuropsychological Test Battery total z-score after 12 weeks of treatment (Frölich et al., 2019). Furthermore, in a phase I study that included measures of verbal learning and visuospatial memory

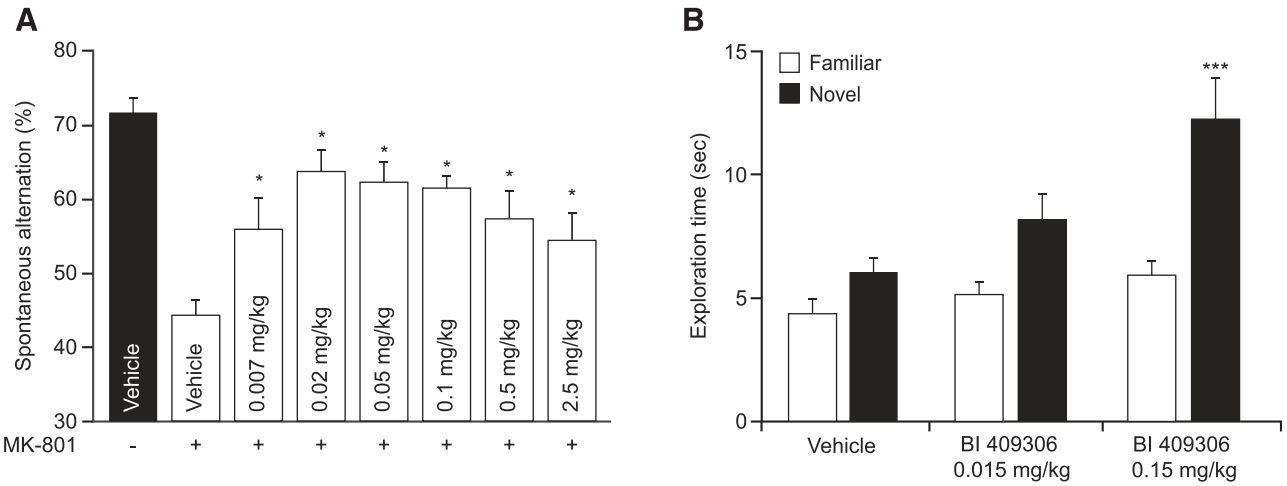

Fig. 5. The effect of BI 409306 on (A) working memory in the T-maze spontaneous alternation task and (B) 24-hour episodic memory in trial 2 of the object recognition task in mice. Data are expressed as mean (±S.E.M.). (A) BI 409306 0.007, 0.02, 0.05, 0.1, 0.5, or 2.5 mg/kg was administered orally 60 minutes before the task. $N = 10$ animals per group. $^{*}P < 0.05$ compared with the control MK-801 group. (B) BI 409306 0.015 or 0.15 mg/kg was administered intraperitoneally 30 minutes before trial 1. $N = 10$–11 animals per group. $^{***}P < 0.001$ compared with the familiar object.

as secondary endpoints, no significant treatment effects of BI 409306 on cognitive outcomes were observed in 38 patients with schizophrenia (Brown et al., 2018). Overall, the observed effects of BI 409306 on cognition in patients contrast with the effects in rodents reported in the present study. This may indicate that the effects of BI 409306 in patients require further investigation. For example, BI 409306 may have beneficial effects on cognition when administered at a higher dose, over a longer treatment period, or in patients with different stages of schizophrenia or Alzheimer's disease (Brown et al., 2019; Frölich et al., 2019). Alternatively, it may be that drugs enhancing synaptic plasticity are more effective in the treatment of other indications, or that the effects of such drugs observed in the rodent models used in the present study translate poorly to cognitive improvement in patients with schizophrenia and Alzheimer's disease.

In conclusion, these data suggest that PDE9A inhibition with BI 409306 increases cGMP levels in the brain, promotes both early and late LTP, and improves performance on cognitive tasks assessing working and episodic memory in rodents.

Acknowledgments

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Authorship Contributions

Participated in research design: Rosenbrock, Giovannini, Schänzle, Koros, Fuchs, Marti, Reymann, Schröder, Fedele, Dorner-Ciossek.

Conducted experiments: Rosenbrock, Schänzle, Fuchs, Marti, Schröder, Fedele, Dorner-Ciossek.

Contributed new reagents or analytic tools: Runge.

Performed data analysis: Rosenbrock, Giovannini, Schänzle, Koros, Runge, Fuchs, Marti, Schröder, Fedele, Dorner-Ciossek.

Wrote or contributed to the writing of the manuscript: All authors.

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The novel phosphodiesterase 9A inhibitor BI 409306 increases cyclic guanosine monophosphate levels in the brain, promotes synaptic plasticity and enhances memory function in rodents

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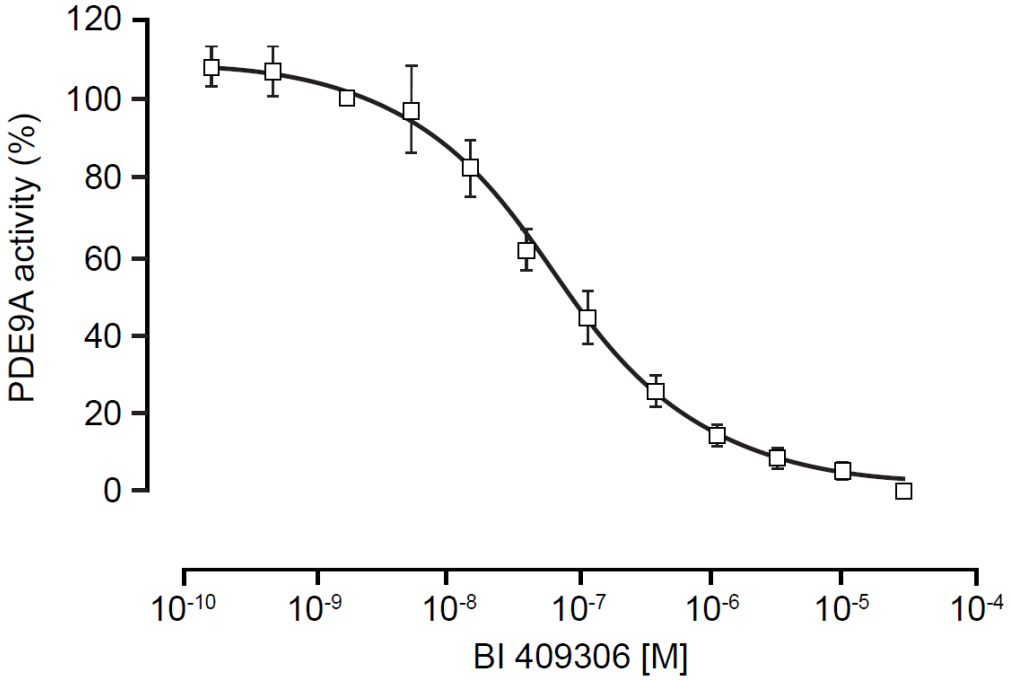
Supplementary Figure S1 Concentration-activity curve for inhibition of PDE9A by BI 409306 determined by a SPA



Data are expressed as mean (±SD). N=8 dilutions from 4 experiments.

PDE9A, phosphodiesterase 9A; SD, standard deviation; SPA, scintillation proximity assay

Supplementary Table S1 Potency and selectivity of BI 409306 for non-PDE targets

Target pathway	Target	Inhibition of binding[a] with BI 409306 10 µM
Acetylcholine	M_1 receptor	<50 %
Acetylcholine	M_2 receptor	<50 %
Acetylcholine	M_3 receptor	<50 %
Acetylcholine	M_4 receptor	<50 %
Acetylcholine	M_5 receptor	<50 %
Acetylcholine	nAChR	<50 %
Acetylcholine	α_1 nAChR (bungarotoxin)	<50 %
Acetylcholine	$\alpha_4\beta_2$ nAChR (cytisine)	<50 %
Acetylcholine	α_7 nAChR (bungarotoxin)	<50 %
Acetylcholine	α_7 nAChR (methyllycaconitine)	<50 %
Acetylcholine	Choline transporter	<50 %
Adenosine	A_1 receptor	<50 %
Adenosine	A_{2a} receptor	<50 %
Adenosine	A_3 receptor	<50 %
Androgen	Androgen receptor (testosterone)	<50 %
Bradykinin	B_1 receptor	<50 %
Bradykinin	B_2 receptor	<50 %
Calcium channel	L-Type VGCC (benzothiazepine)	<50 %
Calcium channel	L-Type VGCC (dihydropyridine)	<50 %
Calcium channel	N-Type VGCC	<50 %
Cannabinoid	CB_1 receptor	<50 %
Cannabinoid	CB_2 receptor	<50 %
Dopamine	D_1 receptor	<50 %
Dopamine	D_2 receptor long splice variant	<50 %
Dopamine	D_2 receptor short splice variant	<50 %
Dopamine	D_3 receptor	<50 %
Dopamine	$D_{4.2}$ receptor variant	<50 %
Dopamine	$D_{4.4}$ receptor variant	<50 %
Dopamine	$D_{4.7}$ receptor variant	<50 %
Dopamine	D_5 receptor	<50 %
Dopamine	Dopamine transporter	<50 %
Endothelin	ET_a receptor	<50 %
Endothelin	ET_b receptor	<50 %
Estrogen	ER_α receptor	<50 %
GABA	$GABA_A$ receptor (flunitrazepam)	<50 %
GABA	$GABA_A$ receptor (muscimol)	<50 %
GABA	Hippocampal $GABA_A$ receptor (Ro-15-1788)	<50 %
GABA	Cerebellar $GABA_A$ receptor (Ro-15-1788)	<50 %
GABA	$GABA_A$ receptor (TBOB)	<50 %
GABA	$GABA_A$ receptor (TBPS)	<50 %
GABA	$GABA_B$ receptor (non-selective)	<50 %
GABA	$GABA_B$1a receptor subunit	<50 %
GABA	$GABA_B$1b receptor subunit	<50 %
GABA	GABA transporter	<50 %
Glucocorticoid	Glucocorticoid receptor	<50 %
Glutamate	Kainate receptor	<50 %
Glutamate	NMDA receptor (agonism)	<50 %

Glutamate	NMDA receptor (glycine)	<50 %
Glutamate	NMDA receptor (phencyclidine)	<50 %
Glutamate	NMDA receptor (polyamine)	<50 %
Glycine	Strychnine-sensitive receptor	<50 %
Glycine	Glycine transporter	<50 %
G-protein coupled receptor	GPR103 receptor	<50 %
Histamine	H_1 receptor	<50 %
Histamine	H_2 receptor	<50 %
Histamine	H_3 receptor	<50 %
Imidazoline	I_2 receptor (central)	<50 %
Leukotriene	$CysLT_1$ receptor	<50 %
Melatonin	MT_1 receptor	<50 %
Monoamine	Monoamine transporter	<50 %
Neuropeptide Y	Y_1 receptor	<50 %
Neuropeptide Y	Y_2 receptor	<50 %
Norepinephrine	α_{1a} receptor	<50 %
Norepinephrine	α_{1b} receptor	<50 %
Norepinephrine	α_{1d} receptor	<50 %
Norepinephrine	α_{2a} receptor	<50 %
Norepinephrine	β_1 receptor	<50 %
Norepinephrine	β_2 receptor	<50 %
Norepinephrine	Norepinephrine transporter	<50 %
Opioid	δ receptor	<50 %
Opioid	κ receptor	<50 %
Opioid	μ receptor	<50 %
Phorbol ester	Phorbol ester	<50 %
Platelet activating factor	Platelet activating factor	<50 %
Potassium channel	K_{ATP} channel	<50 %
Potassium channel	hERG channel	<50 %
Prostanoid	EP4 receptor	<50 %
Purinergic	P_{2x} receptor	<50 %
Purinergic	P_{2y} receptor	<50 %
Phosphodiesterase	Phosphodiesterase (rolipram)	<50 %
Serotonin	5-HT_{1a} receptor	<50 %
Serotonin	5-HT_{1b} receptor	<50 %
Serotonin	5-HT_{2a} receptor	<50 %
Serotonin	5-HT_{2b} receptor	<50 %
Serotonin	5-HT_{2c} receptor	<50 %
Serotonin	5-HT_3 receptor	<50 %
Serotonin	5-HT_4 receptor	<50 %
Serotonin	5-HT_{5a} receptor	<50 %
Serotonin	5-HT_6 receptor	<50 %
Serotonin	Serotonin transporter	<50 %
Sigma	σ_1 receptor	<50 %
Sigma	σ_2 receptor	<50 %
Sodium channel	Receptor site 2	<50 %
Tachykinin	NK_1 receptor	<50 %
Thyroid hormone	Thyroid hormone	<50 %

[a]Inhibition of binding of the endogenous ligand, or the drug or ligand shown in brackets.

5-HT, 5-hydroxytryptamine; A, adenosine; B, bradykinin; CB, cannabinoid; CysLT, cysteinyl leukotriene; D, dopamine; EP, prostaglandin E; ER, estrogen receptor; ET, endothelin;

GABA, γ-aminobutyric acid; GPR103, G-protein coupled receptor 103; H, histamine; hERG, human ether-a-go-go-related gene; I, imidazoline; K_{ATP}, ATP-sensitive potassium channel; M, muscarinic; MT, melatonin; nAChR, nicotinic acetylcholine receptor; NK, neurokinin; NMDA, N-methyl-D-aspartate; P, purine; PDE, phosphodiesterase; VGCC, voltage-gated calcium channel