

Phosphodiesterase 10A is a critical target for neuroprotection in a mouse model of ischemic stroke

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Abstract

Phosphodiesterase 10A (PDE10A) hydrolyzes adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP). It is highly expressed in the striatum. Recent evidence implied that PDE10A may be involved in the inflammatory processes following injury, such as ischemic stroke. Its role in ischemic injury was unknown. Herein, we exposed mice to 90 or 30 min middle cerebral artery occlusion, followed by the delivery of the highly selective PDE10A inhibitor TAK-063 (0.3 mg/kg or 3 mg/kg) immediately after reperfusion. Animals were sacrificed after 24 or 72 hours, respectively. Both TAK-063 doses enhanced neurological function, reduced infarct volume, increased neuronal survival, reduced brain edema, and increased blood-brain barrier integrity, alongside cerebral microcirculation improvements. Post-ischemic neuroprotection was associated with increased phosphorylation (i.e., activation) of pro-survival Akt, Erk-1/2 and GSK-3 α/β , decreased phosphorylation (i.e., activation) of pro-survival mTOR, increased HIF-1 α , MMP-9 and anti-apoptotic Bcl-xL abundance, and reduced pro-apoptotic Bax abundance. Interestingly, PDE10A inhibition reduced inflammatory cytokines/chemokines, including IFN- γ and TNF- α , analyzed by planar surface immunoassay. In addition, liquid chromatography-tandem mass spectrometry revealed 40 proteins were significantly altered by TAK-063. Our study established PDE10A as a target for ischemic stroke therapy.

Introduction

Phosphodiesterase 10A (PDE10A) is a dual-substrate specific enzyme and an essential regulator of cell signaling by hydrolyzing the second messengers cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) [1,2], which play crucial roles in neuronal activity, synaptic plasticity, neurogenesis and apoptosis in the central nervous system (CNS) [3,4]. In the CNS, PDE10A is highly expressed in medium spiny neurons (MSNs), which make up approximately 95% of all striatal neurons. Through degradation of cAMP and cGMP, PDE10A regulates MSN excitability [5]. Inhibition of PDE10A increases cyclic nucleotide levels and activates its downstream signal transduction pathways including cAMP/ protein kinase A (PKA) signaling [6,7,4]. The cAMP/PKA signaling pathway regulates many important cellular processes such as cellular proliferation, differentiation, and apoptosis.

Due to its distribution pattern in the striatum where it contributes to the control of movement and cognition, PDE10A has been suggested to be a target for the treatment of neurodegenerative disorders. Indeed, RNA-sequencing transcriptome analysis revealed reduced expression of *Adora2a* (adenosine receptor A2A), *Drd2* (dopamine receptor D2), and *Pde10a* in spontaneously recovered mice after ischemic stroke [8]. Very recently, Birjndi *et al*/ showed that pharmacological deactivation of PDE10A improved motor recovery after striatal but not cortical ischemic injury, which was associated with increased axonal neuroplasticity and elevated brain-derived neurotrophic factor (BDNF) levels [9]. In this study, the PDE10A inhibitor was administered in the post-acute stroke phase, starting five days post stroke. Structural neuroprotective effects of PDE10A inhibition were not studied.

In the striatum of transgenic mice developing Huntington's disease pathology, PDE10A protein and mRNA levels were found to be decreased prior to the onset of motor symptoms [10]. Again in mice with Huntington's disease pathology, pharmacological PDE10A inhibition reduced striatal and cortical cell loss, the formation of striatal neuronal intranuclear inclusions, and microglial activation [11]. In a mouse model of Parkinson's disease, PDE10A inhibition increased neuronal survival and decreased microglial activation via cAMP/PKA signaling [12].

TAK-063 [1-[2-fluoro-4-(1H-pyrazol-1-yl)phenyl]-5-methoxy-3-(1-phenyl-1H-pyrazol-5-yl)pyridazin-4(1H)-one] is a potent and highly selective inhibitor of PDE10A [13]. Preclinical and clinical data indicate that TAK-063 is safe and well tolerated in humans [14,15]. Harada et. al. demonstrated that PDE10A occupancy increases in the rat striatum following oral administration of TAK-063 in a dose dependent manner using non-radiolabeled T-773 as a tracer. [16]. In addition, TAK-063 doses of up to 3 mg/kg (orally administered) did not affect plasma prolactin and glucose levels in rats [17]. TAK-063 was shown to cross the blood-brain barrier [18].

Although PDE10A has been evaluated as treatment target in models of neurodegenerative diseases, its role in acute ischemic stroke has not yet been uncovered. Here, we investigated the neuroprotective effects of PDE10A inhibition by TAK-063 after transient focal cerebral ischemia in mice. To this end, low dose (0.3 mg/kg) or high dose (3 mg/kg) TAK-063 were orally administered immediately after ischemia-reperfusion injury induced by intraluminal middle cerebral artery occlusion (MCAO). Neurological deficits, infarct volume, brain swelling, blood brain barrier (BBB) integrity, disseminated neuronal injury were analyzed by histochemistry, as was cerebral microcirculation, an important denominator of stroke outcome, in the ischemic core and periphery by laser speckle imaging (LSI). Signal pathways and protein responses were evaluated by Western blotting, liquid chromatography - tandem mass spectrometry (LC-MS/MS) and planar surface immunoassay.

Materials And Methods

Ethics statement

Experiments were performed in accordance to National Institutes of Health (NIH) guidelines for the care and use of laboratory animals and approved by local government authorities (Istanbul Medipol University, Animal Research Ethics Committee). All animals were maintained under a constant 12-h light/dark cycle (lights on at 07:00 daily). Investigators were blinded for experimental groups at all stages of experiments and data analysis.

Experimental design and groups

The PDE10A inhibitor TAK-063 was purchased from MedKoo Biosciences (510331, North Carolina, USA) and suspended in a 1% (v/v) solution of dimethyl sulfoxide (DMSO) in tap water. Adult male C57BL/6j mice weighing 21–26 g were randomly assigned to one of three groups and administered with oral delivery of i) vehicle (100 µl water containing 1% DMSO), ii) 0.3 mg/kg TAK-063 (dissolved in 100 µl water containing 1% DMSO) or iii) 3 mg/kg TAK-063 (dissolved in 100 µl water containing 1% DMSO) at the reperfusion onset. In the first set; vehicle, low dose or high dose of TAK-063 was orally administered without any injury induction (n=4/ group). One hour after oral application of PDE10A inhibitor or vehicle, Western blotting analysis was carried out to assess the efficiency of PDE10A inhibition. In the second set, mice were subjected to 90 min of MCAO followed by 24 h reperfusion for the evaluation of neurological score, brain infarct volume, cerebral edema and serum IgG extravasation (n=7-8 per group). The third set of mice was subjected to 90 min of MCAO followed by 24 h reperfusion for the analysis of cerebral microcirculation by LSI (n=4-5 per group). The fourth set of mice was subjected to 30 min of MCAO and 72 h reperfusion for the analysis of disseminate neuronal injury in the striatum, cytokine/chemokine expression profiles, intracellular signal pathway analysis, and proteomic analysis (n=7-8 per group).

Middle cerebral artery occlusion (MCAO)

Mice were anesthetized with 1% isoflurane (30% O₂, reminder N₂O), and rectal temperature was controlled between 36.5 and 37.0 °C using a feedback-controlled heating system. During the experiments, cerebral blood flow (CBF) was monitored via laser Doppler flowmetry (LDF) using a flexible 0.5 mm fiber optic probe (Perimed, Sweden) which was attached with tissue adhesive to the intact skull overlying the MCA territory (2 mm posterior and 6 mm lateral from the bregma). Focal cerebral ischemia was induced using an intraluminal filament technique [11]. Briefly, after a midline neck incision, the left common and external carotid arteries were isolated and ligated. A microvascular clip (FE691; Aesculap, Tuttlingen, Germany) was temporarily placed on the internal carotid artery. A 7-0 silicon-coated nylon monofilament (701934PK5Re, Doccol, Massachusetts, USA) was inserted through a small incision into the common carotid artery and advanced 9 mm distal to the carotid bifurcation for MCAO. Reperfusion was initiated 30 or 90 min after onset of ischemia by gentle monofilament removal. Thereafter, mice were placed back into their home cages.

In mice subjected to 90 min MCAO, neurological deficits were evaluated 24 h after MCAO using the following 5-point scoring: 0 = normal function, 1 = flexion of torso and of the contralateral forelimb upon lifting of the animal by the tail, 2 = circling to the contralateral side but normal posture at rest, 3 = reclination to the contralateral side at rest, and 4 = absence of spontaneous motor activity. At 72 h (for 30 min MCAO) or 24 h (for 90 min MCAO) after reperfusion, mice were sacrificed under deep anesthesia (4% isoflurane with 30% O₂, remainder N₂O). Brains were removed, frozen on dry ice, and cut on a cryostat

into coronal 18- μ m sections, which were subsequently used for the analysis of disseminate neuronal injury, infarct volume and brain swelling and serum IgG extravasation. For 30 min MCAO, tissue samples obtained from the ipsilateral to the stroke were pooled for Western blots.

Analysis of infarct volume and brain swelling

For the evaluation of infarct volume and brain swelling, coronal brain sections were collected at four equidistant brain levels, 2 mm apart, from mice subjected to 90 min MCAO, which were stained with cresyl violet according to a standard protocol [19]. Within the sections, the border between infarcted and non-infarcted tissues was outlined using an image analysis system (Image J; National Institute of Health, Maryland, USA), and the infarct area was assessed by subtracting the area of the non-infarcted ipsilateral hemisphere from that of the contralateral side. Infarct volume was calculated by integration of these infarct areas. Edema was calculated as the volume difference between the ischemic and the non-ischemic hemisphere and expressed as mm³.

Analysis of serum IgG extravasation

With gentle stirring, brain sections from the bregma 0.0 mm level of mice subjected to 30 min MCAO followed by 72 h reperfusion were rinsed for 10 min at room temperature in 0.1 M PBS to remove intravascular IgG, and were fixed in 4% PFA [7]. Following the blocking of endogenous peroxidase with methanol/ 0.3% H₂O₂ and immersion in 0.1 M PBS containing 5% bovine serum albumin (BSA) and normal swine serum (1:1000), sections were incubated for 1 h in biotinylated horse anti-mouse IgG (BA-1300-2.2, Vectastain Elite; Vector Labs, California, USA), and stained with an avidin peroxidase kit (PK-7800; Vectastain Elite; Vector Labs) and DAB Substrate Kit (SK-4100; Vector Labs). For reasons of data comparability, all sections were processed in parallel. Sections were scanned and the integrated density of ipsilesional and contralesional hemisphere was quantified using the NIH ImageJ software. Afterward, density of ipsilesional tissue compared to contralesional tissue to determine BBB leakage. [19].

Laser Speckle Imaging (LSI)

To analyze the effect of low and high dose of TAK-063 on cerebral microcirculation, LSI was performed as described previously [20,19]. Briefly, C57BL/6 mice were subjected to 90 min MCAO as described above followed by the oral delivery of vehicle, low dose TAK-063 or high dose TAK-063 at the onset of reperfusion. Thereafter, cerebral microcirculation was recorded by Pericam PSI System (Perimed) for 90 min. To analyze CBF changes in the ischemic core and periphery, regions of interest (ROI) covering 1.0 mm x 5.5 mm (in lateral and rostrocaudal direction, respectively) were defined 1.5 and 2.5 mm lateral and 0.5 mm posterior to the bregma, in which mean CBF was calculated using a blood perfusion imaging

software (PIMSoft; Perimed) [19]. Regional CBF was recorded throughout the 90 min observation period. From the measurements obtained, relative CBF changes (in %) at the end of the observation period compared to the beginning of the observation period were calculated.

Analysis of neuronal survival

Neuronal survival was evaluated as previously described [21]. Coronal brain sections were fixed in 4% paraformaldehyde (PFA)/ 0.1 M phosphate buffered saline (PBS), washed and immersed for 1 h in 0.1 M PBS containing 0.3% Triton X-100 (PBS-T)/ 10% normal goat serum. Sections were incubated overnight at 4 °C with Cy3-conjugated monoclonal mouse anti-NeuN (MAB377C3; Merck, New Jersey, USA). The next day, sections were incubated with 4',6-diamidino-2-phenylindole (DAPI). Sections were analyzed using a confocal laser scanning Zeiss LSM 780 microscope (Carl Zeiss, Jena, Germany). Nine different ROI in the striatum, each measuring 62,500 μm^2 , were evaluated. Mean numbers of NeuN cells were calculated in the ischemic and contralesional striatum. By dividing the results obtained from ischemic striatum by the results of non-ischemic striatum and multiplying by 100, the percentage of surviving neurons in the ischemic striatum was determined.

Analysis of neuronal injury

For the evaluation of DNA fragmentation, coronal brain sections at the level of the striatum from mice subjected to 30 min MCAO were fixed with 4% paraformaldehyde (PFA)/ 0.1 M phosphate buffered saline (PBS) and were labeled using a TUNEL assay kit (In Situ Cell Death Detection Kit; Roche, Switzerland). Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) [21]. Stainings were analyzed by quantifying TUNEL (+) cells (which in 30 min MCAO are equivalent to neurons) in twelve adjacent ROI in the striatum, each measuring 62,500 μm^2 , under a confocal laser scanning Zeiss LSM 780 microscope (Carl Zeiss).

Western blot

For Western blot analysis, harvested tissue samples from the ischemic striatum belonging to the same group were pooled, homogenized, sonicated, and treated with protease/ phosphatase inhibitor cocktail (5872, Cell Signaling, Massachusetts, USA). Total protein content was evaluated using Qubit 3.0 Fluorometer (Q33216, Invitrogen, Life Technologies Corporation, California, USA) according to the manufacturer's protocol. Equal amounts of protein (20 μg) were size-fractionated using 4-20% Mini-PROTEAN TGX (4561096, Bio-Rad, Life Sciences Research, California, USA) gel electrophoresis and then transferred to a PVDF membrane using the Trans-Blot Turbo Transfer System (1704155, Bio-Rad, Life Sciences Research). Thereafter, membranes were blocked in 5% nonfat milk in 50 mMol Tris-buffered

saline (TBS) containing 0.1% Tween (TBS-T; blocking solution) for 1 h at room temperature, washed in 50 mM TBS-T, and incubated overnight with monoclonal mouse PDE10A (sc-515023; Santa Cruz Biotechnology), polyclonal rabbit anti-phospho-Akt (9275, Cell Signaling), monoclonal rabbit anti-phospho-p44/42 MAPK (Erk1/2) (4370; Cell Signaling), polyclonal rabbit anti-phospho-PTEN (9551; Cell Signaling), polyclonal rabbit anti-phospho-GSK-3 α/β (9331; Cell Signaling), polyclonal rabbit anti-phospho-mTOR (2971, Cell Signaling), polyclonal rabbit anti-MMP-9 (Ab38898, Abcam, Cambridge, UK), monoclonal rabbit anti-HIF-1 α (36169; Cell Signaling), polyclonal rabbit anti-Bax (2772; Cell Signaling) or monoclonal rabbit anti-Bcl-xL (2764; Cell Signaling) antibody. On the next day, membranes were washed with 50 mM TBS-T and incubated with horseradish peroxidase-conjugated goat-anti-rabbit (sc-2004; Santa Cruz Biotechnology, Heidelberg, Germany) or goat anti-mouse (sc-2005; Santa Cruz Biotechnology) antibody (diluted 1:2500) for 1 h at room temperature. Blots were performed at least three times. Protein loading was controlled by stripping and re-probing with polyclonal rabbit anti- β -actin antibody (4967, Cell Signaling Technology). Blots were developed using Clarity Western ECL Substrate kit (1705060, Bio-Rad; Life Sciences Research) and visualized using the ChemiDoc MP System (1708280, Bio-Rad; Life Sciences Research). Protein levels were analyzed densitometrically using an image analysis system (Image J; National Institute of Health), corrected with values determined on β -actin blots and expressed as relative values compared with vehicle treated group.

Cytokine array

Tissue sections of the ipsilesional striatum were harvested, homogenized, sonicated, and treated with protease/ phosphatase inhibitor cocktail (5872, Cell Signaling). The expression of cytokines was analyzed by the Proteome Profiler Mouse Cytokine Array Panel A Kit (ARY006, R&D Systems, Boston, USA) from a total of 9 animals (n=3 for vehicle, n=3 for 0.3 mg/kg TAK-063, and n=3 for 3 mg/kg TAK-063), according to the manufacturer's recommendation. Array panels were visualized using the ChemiDoc MP System (1708280, Bio-Rad). Protein levels were analyzed densitometrically using an image analysis system (Image J), corrected with values determined on positive controls and expressed as relative values compared with vehicle treated group.

Sample preparation for liquid chromatography tandem-mass spectrometry (LC-MS/MS)

Seventy-two hours after the onset of reperfusion, animals were sacrificed (n= 4-5 per group). Thereafter, brains were immediately removed, frozen on dry ice and stored at -80 °C. The brain tissues were taken from ipsilesional striatum and were homogenized in 50 mM ammonium bicarbonate and lysed by heating at 95 °C in protein extraction reagent kit (UPX Universal; Expedon, Heidelberg, Germany). Samples were incubated for an hour at 4 °C. After incubation step, samples were centrifuged at 14,000 G for 10 min. Then supernatants were collected. Protein concentrations were determined via Qubit 3.0 Fluorometer (Q33216, Invitrogen, Life Technologies) according to the manufacturer's protocol. FASP (Filter Aided

Sample Preparation) Protein Digestion Kit (ab270519, Abcam) was used for generating tryptic peptides according to the manufacturer's protocol [22]. A total of 50 µg protein samples were filtered using 6 M urea in a 30 kDa cut-off spin column. After this step, samples were alkylated with 10 mM iodoacetamide in the dark for 20 min at room temperature. Then samples were incubated overnight with MS grade trypsin protease (ratio 1:100, 90057, Thermo Scientific) at 37 °C. The following day, peptides were eluted from the columns and lyophilized. At the end of the lyophilization process, the peptides were suspended in 0.1% formic acid (1002642510, Merck) and diluted to 100 ng/µl before injecting to the LC-MS/MS system (ACQUITY UPLC M-Class coupled to a SYNAPT G2-Si high-definition mass spectrometer (Waters, Massachusetts, USA)).

LC-MS/MS analysis and data processing

LC-MS/MS and protein identification were performed with small modifications according to previously published protocols [23,24]. The samples were loaded onto the ACQUITY UPLC M-Class coupled to a SYNAPT G2-Si high-definition mass spectrometer (Waters). To equilibrate the columns, 97% of mobile phase (including 0.1% formic acid in UHPLC grade water) was used and column was heated to 55 °C. Ninety min gradient elution from the trap column ACQUITY UPLC M-Class Symmetry C18 trap column (180 µm x 20 mm; 186007496, Waters) to the analytic column (ACQUITY UPLC M-Class HSS T3 Column, 100Å, 1.8 µm, 75 µm X 250 mm,; 186007474, Waters) at 0.400 µl/min flow rate with a gradient from 4% to 40% hypergrade acetonitrile (100029, Merck) containing 0.1% formic acid (v/v) was used for the peptide separation. Positive ion modes of MS and MS/MS scans with 0.7 sec cycle time were performed sequentially. Ten volts was set as low collision energy and 30 V as high CE. Ion mobility separation (IMS) was used for the ion separation. A wave velocity was ramped from 1000 m/s to 55 m/s over the full IMS cycle. The release time for mobility trapping was set as 500 µs, trap height was set to 15 V. IMS wave delay was 1000 µs for the mobility separation after trap release [25]. Without any precursor ion preselection, all the ions within 50-1900 m/z range were fragmented in resolution mode. Additionally, 100 fmol/µl Glu-1-fibrinopeptide B was infused as lockmass reference with a 60 s interval. Progenesis-QI for proteomics software (Waters) was used for the identification and quantification of the peptides. Whole proteins were identified by at least 2 unique peptide sequences and then, the expression ratio of proteins was calculated.

Statistical analysis

Statistical analysis was performed using SPSS (version 15, SPSS Inc., Chicago USA) software. Data were evaluated by one-way ANOVA followed by LSD tests. Data are presented as mean ± S.D. values. Throughout the study, p values < 0.05 were considered as statistically significant.

Results

Low dose (0.3mg/kg) and high dose (3mg/kg) TAK-063 reduced striatal PDE10A protein abundance.

Both low (0.3 mg/kg) and high (3 mg/kg) doses of orally administered TAK-063 reduced PDE10A protein abundance in the mouse striatum within one hour (Fig. 1a), providing evidence that PDE10A was efficiently blocked by the inhibitor. PDE10A protein level was reduced to 75 ± 13.7 and 48.8 ± 3.6 respectively, in mice receiving 0.3 mg/kg and 3 mg/kg TAK-063, as compared to vehicle treated mice.

Effects of TAK-063 on neurological deficit score, infarct size, cerebral edema and blood-brain barrier permeability.

To ensure reproducible brain injury among the experimental groups, cerebral blood flow (CBF) was monitored and analyzed in real-time via laser Doppler flow (LDF) measurement using a flexible probe attached to the animals' skulls above the core of the MCA region (Fig. 1b, 3a). We noted that TAK-063 doses used in this study resulted in a slightly higher cerebral blood flow during reperfusion compared to the control group animals, however, this increase was not significant. Twenty-four hours after 90 min ischemia, four-point neurological scoring was performed for the evaluation of neurological deficits. Infarct volume and brain edema were evaluated by creyls violet staining. In this model of combined subcortical-cortical infarction, low and high dose TAK-063 significantly reduced stroke-related neurological deficits, infarct volume and brain edema (Fig. 1c-e). Besides, BBB permeability assessed by serum IgG extravasation was decreased by low dose, but not high dose TAK-063 (Fig. 1f).

PDE10A inhibition increases regional microcirculation in ischemic core region.

To analyze the hemodynamic effects induced by orally administered TAK-063, we further evaluated regional cerebral microcirculation in the ischemic core (Fig. 2b) and ischemic periphery (Fig. 2c) by Laser Speckle Imaging (LSI). For this aim, mice were subjected to 90 min MCAO which is followed by secondary hypoperfusion that develops within 90 min after reperfusion onset [26]. Low dose TAK-063 which was administered at the beginning of the reperfusion significantly increased regional microcirculation in the ischemic core region starting 30 min after reperfusion onset, while high dose TAK-063 increased microcirculation in a more delayed fashion starting 100 min after reperfusion onset. Effects of TAK-063 on cerebral microcirculation in the ischemic periphery did not achieve significance. Hence, the microcirculatory effects of low dose TAK-063 were more pronounced than those of high dose TAK-063.

Effect of PDE10A inhibition on disseminate neuronal injury.

We next examined the effects of PDE10A inhibition in a model of mild focal cerebral ischemia induced by 30 min MCAO, which induces disseminate neuronal injury in the striatum developing over one to three

days [27]. In this model, low dose and high dose TAK-063 increased neuronal survival and decreased the number of DNA-fragmented (that is, irreversibly injured) cells in the ischemic striatum (Fig. 3b-c). Hence, PDE10A inhibition efficiently provided protection against a wide range of ischemic injury severities, preventing combined subcortical-cortical infarction and disseminate neuronal death at the same time.

PDE10A inhibition with TAK-063 regulates survival related proteins.

Western blot analysis was used to evaluate the effect of PDE10A inhibition with TAK-063 on cellular survival and apoptosis related protein levels in the ischemic striatum. Seventy-two hours after 30 min MCAO, PDE10A protein abundance was still reduced in the ischemic striatum (Fig. 4a). Both doses of TAK-063 increased the level of phosphorylated (that is, activated) Akt (Thr308) (Fig. 4b) and phosphorylated (that is, activated) GSK3 α/β (Fig. 4c) and decreased the level of phosphorylated (that is, inactive) mTOR (mammalian target of rapamycin) (Fig. 4d). Low dose TAK-063 also significantly increased the level of phosphorylated (that is, activated) ERK-1/2 (Fig. 4e) and decreased the level of phosphorylated (that is, inactive) PTEN (Fig. 4f). These data indicated that TAK-063 elicited a robust response of pro-survival proteins that contributed to post-ischemic neuroprotection induced by PDE10A inhibition.

In line with the stabilization of post-ischemic reperfusion and the stabilization of BBB integrity, the abundance of hypoxia inducible factor-1 α (HIF-1 α) (Fig. 4g) and matrix metalloproteinase-9 (MMP-9) (Fig. 4h) were significantly decreased by TAK-063. Moreover, we also evaluated the expression of pro-apoptotic Bax and anti-apoptotic Bcl-xL in the ipsilesional striatum. Both doses of TAK-063 significantly decreased Bax protein abundance and increased anti-apoptotic Bcl-xL protein abundance (Fig. 4i-j).

TAK-063 altered the cytokine/chemokine expression profile after focal cerebral ischemia.

The stabilization of BBB integrity by TAK-063 subsequently prompted us to evaluate the levels of cytokines and chemokines in the ischemic brain. The relative expression levels of 40 different cytokines and chemokines (CXCL13/BLC/BCA-1, C5a, G-CSF, GM-CSF, CCL1/I-309, CCL11/Eotaxin, ICAM-1, IFN- γ , IL-1 α /IL-1F1, IL-1 β /IL-1F2, IL-1 α /IL-1F3, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 p70, IL-13, IL-16, IL-17, IL-23, IL-27, CXCL10/IP-10, CXCL11/I-TAC, CXCL1/KC, M-CSF, CCL2/JE/MCP-1, CCL12/MCP-5, CXCL9/MIG, CCL3/MIP-1 α , CCL4/MIP-1 β , CXCL2/MIP-2, CCL5/RANTES, CXCL12/SDF-1, CCL17/TARC, TIMP-1, TNF- α , and TREM-1) in the ipsilesional striatum were evaluated using an antibody array (Fig. 5a). These analyses revealed a broad anti-inflammatory response. As such, the expression of G-CSF, I-309, INF- γ , IL-1 α , IL-1 β , IL-1 α , IL-2, IL-3, IL-7, IL-13, IL-16, IL-17, IL-23, IL-27, IP-10, I-TAC, KC, M-CSF, JE, MCP-5, MIG, MIP-1 α , MIP-1 β , MIP-2, RANTES, TNF- α , and

TREM-1 was decreased by TAK-063 (Fig. 5b). BLC, C5/C5a, IL-4, SDF-1, TARC, and TIMP-1 were not influenced by TAK-063 (Fig. 5b).

TAK-063 mediated PDE10A inhibition altered protein profile in the ipsilesional striatum.

To understand the changes in the protein profile after TAK-063 administration, ischemic tissues were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). A total of 1721 proteins were identified in vehicle and TAK-063 treated groups. Forty different proteins were significantly altered by low dose/high dose TAK-063 treatment (Fig. 6). These proteins are: 26S proteasome non-ATPase regulatory subunit 2 (PSMD2), 40S ribosomal protein S18 (RPS18-PS5), acyl-CoA thioesterase 7 (ACOT7), ADP/ATP translocase 1 (ADT1), ankyrin repeat and sterile alpha motif domain-containing protein 1B (ANKS1B), centromere-associated protein E (CENPE), cytoplasmic FMR1-interacting protein 1 (CYFP1), dedicator of cytokinesis protein 4 (DOCK4), dihydrolipoyl dehydrogenase, mitochondrial (DLDH), DNA polymerase epsilon catalytic subunit A (DPOE1), DNA polymerase zeta catalytic subunit (REV3L), dynactin subunit 4 (DCTN4), electron transfer flavoprotein subunit beta (ETFB), extracellular matrix protein 2 (ECM2), filamin-A (FLNA), flotillin (FLOT2), hydroxyacylglutathione hydrolase, mitochondrial (HAGH), importin subunit alpha-4 (IMA4), LanC-like protein 2 (LANCL2), long-chain-fatty-acid-CoA ligase ACSBG1 (ACBG1), microtubule-associated protein RP/EB family member 2 (MAPRE2), NADH-ubiquinone oxidoreductase 18 kDa subunit (NDUFS4), ornithine aminotransferase, mitochondrial (OAT), phosphatidylinositol 4-phosphate 5-kinase type-1 gamma (PIP5K1C), phosphofurin acidic cluster sorting protein 1 (PACS1), proteasome subunit beta type-1 (PSMB1), protein S100-B (S100B), pyridoxal phosphate homeostasis protein (PLPHP), Ras-related protein Rab-8B (RAB8B), Rho GTPase-activating protein 23 (ARHGAP23), Rho-associated protein kinase 2 (ROCK2), secretory carrier-associated membrane protein (SCAMP1), serpin B6 (SERPINB6), sodium leak channel non-selective protein (NALCN), solute carrier family 25 member 11 (SLC25A11), solute carrier family 25 member 12 (SLC25A12), T-complex protein 1 subunit epsilon (TCPE), tyrosine-protein phosphatase non-receptor type substrate 1 (SHPS1), tyrosine-tRNA ligase, cytoplasmic (SYYC), voltage-dependent anion-selective channel protein 3 (VDAC3).

Identified proteins were clustered into groups based on their molecular function and biological process using the PANTHER (protein annotation through evolutionary relationship) classification system (<http://www.pantherdb.org/>). Molecular function classification included proteins with binding, catalytic and transporter activity. PANTHER classification based on biological process included five predominant groups; cellular process, metabolic process, localization, biological regulation, and response to stimulus. In addition to this, six signaling pathways were identified as cytoskeletal regulation by Rho GTPase, dopamine receptor mediated signaling pathway, Huntington disease, integrin signaling pathway, Parkinson's disease, and Ubiquitin proteasome pathway.

Discussion

Phosphodiesterases (PDEs) are subdivided into 11 families and encoded by 21 genes resulting in more than 100 functionally distinct enzymes produced by alternative splicing [28,29]. Among them, the dual substrate enzyme PDE10A, which is highly abundant in the mammalian striatum, acts as an essential regulator of the signaling pathway by hydrolyzing the second messengers, cGMP and cAMP [1,2]. Therefore, it is suggested that PDE10A may play an essential role in the modulation of striatal activities including motor and cognitive processes [6,30,9]. Recent studies suggested that PDE10A can be an important target, especially in neurodegenerative diseases due to its modulatory roles in the CNS. To this end, we investigated the role of PDE10A in ischemic injury development.

To our knowledge, this is the first study showing neuroprotective effects of PDE10A inhibition in an ischemic stroke model. We therefore investigated molecular pathways mediating neuroprotection. PDE10A was effectively blocked by the PDE10A inhibitor, as shown by the fact that TAK-063 dose-dependently reduced striatal PDE10A abundance. Here, we showed that TAK-063 mediated PDE10A inhibition decreased neurological deficits after focal cerebral ischemia. It is well-established that the MCAO model used in this study causes an infarct size that is directly related to the degree of brain edema and BBB leakage [31,24,19]. Therefore, we evaluated infarct volume, brain swelling and blood-brain barrier (BBB) permeability in mice submitted to 90 min MCAO, which is a model of combined subcortical-cortical infarction, and disseminate neuronal injury in the striatum of mice exposed to 30 min MCAO. To the best of our knowledge this is the first study demonstrating neuroprotective efficacy of PDE10A inhibition in ischemic brain injury. Both TAK-063 doses reduced the infarct volume and brain edema after 90 min MCAO. In addition, 0.3 mg/kg TAK-063 increased BBB integrity. After 30 min MCAO, both TAK-063 doses reduced disseminate neuronal injury. That PDE10A inhibition similarly protected against combined subcortical-cortical infarction and disseminate neuronal injury emphasizes the critical role of PDE10A in ischemic injury development.

Focal cerebral ischemia induced by MCAO dramatically decreases CBF in the ischemic core region. Cessation of glucose and oxygen delivery and depletion of ATP in the ischemic core results rapid necrotic cell death of neurons and glial cells [32,33]. However, the area surrounding the ischemic core and the ischemic periphery, exhibit more moderate CBF reduction allowing neurons to survive for a limited time [32]. In the present study, we first examined CBF by LDF which provides instantaneous and continuous measurements of microcirculation over small area of brain tissue [34,35]. CBF was slightly but not significantly increased by TAK-063 treatment. Besides, CBF was monitored during MCAO operations and in the first 90 min of reperfusion via LSI which is a more powerful and accurate tool to analyze microcirculation in real-time in a large brain region. It should be noted that both TAK-063 treatments increased regional CBF in the ischemic core region, although higher dose induced significantly increased CBF levels later than the lower dose. This delayed increase in blood flow in the higher dose might be due

to the accumulation of cAMP/cGMP levels in response to PDE10A inhibition. Further studies are required to uncover the mechanism of effect of TAK-063 in the cerebral blood flow regulation.

To investigate the mechanism underlying TAK-063-induced recovery after focal cerebral ischemia, we analyzed signal transduction pathways which were related to neuronal survival, apoptosis and cellular energy homeostasis via Western blot. We observed that PDE10A protein expression was still reduced in a dose dependent manner even 72 hours after oral administration of TAK-063 in mice. It was observed that low dose of TAK-063 reduced PTEN phosphorylation, which is a negative regulator of phosphatidylinositol-3 kinase (PI3K) and increased Akt phosphorylation after focal cerebral ischemia. It is well known that PI3K/ Akt signaling controls neuronal survival, cell proliferation, apoptosis, glucose metabolism and inflammation [36-38]. Besides, activation of Akt leads to inhibition of apoptosis by phosphorylation of downstream molecules such as glycogen synthase kinase-3 (GSK3) which is active only when dephosphorylated [39]. Our results suggest that TAK-063 increases the phosphorylation status of GSK3 in a dose-dependent manner. Notably, expression of the mammalian target of rapamycin (mTOR) which is an essential regulator of cell metabolism, cell growth, proliferation and survival, is decreased in a dose dependent manner after ischemia [40]. TAK-063 increased phosphorylation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) which plays an essential role in the regulation of cell proliferation and apoptosis.

Accumulating evidence have demonstrated that hypoxia-inducible factor-1 α (HIF-1 α) which is a hypoxia-responsive gene, plays an essential role after ischemia/ reperfusion injury [41-43]. Inhibition of PDE10A significantly decreases HIF-1 α protein expression in the ipsilesional tissue. Focal cerebral ischemia causes the disruption of BBB which protects the brain from harmful substances, toxins and inflammation [44]. Furthermore, it is well known that protein expression of matrix metalloproteinases (MMPs), specifically MMP-9 levels increases after ischemia which is highly related to BBB leakage [45]. The results obtained in this study showed that TAK-063 profoundly diminished MMP9 protein expression after ischemic stroke. Anti-apoptotic Bcl-xL and pro-apoptotic Bcl-2-associated X-protein (Bax) play critical roles in the regulation of signal transduction pathways in apoptosis [46]. Following ischemia, oral delivery of TAK-063, especially low dose, decreased pro-apoptotic Bax protein expression. In addition, anti-apoptotic Bcl-xL protein expression was increased by TAK-063 in a dose-dependent manner after ischemia.

It should be noted that cell death after focal cerebral ischemia triggers the activation of cytokines and chemokine production which contribute to the progression of the ischemic injury. Animal studies and clinical studies demonstrated that increased pro-inflammatory cytokine production and decreased anti-inflammatory cytokine production increase brain damage in experimental models of ischemic stroke [47].

In the present study, we investigated the expression profile of cytokines and chemokines 72 h after 30 min of MCAO. Expression of pro-inflammatory cytokines/chemokines I-309, INF- γ , IL-1 α , IL-1 β , IL-2, IL-3, IL-7, IL-23, IP-10, KC, JE, MCP-5, MIP-1 α , MIP-1 β , TNF- α , and TREM-1 were decreased in a dose dependent manner after ischemia. However, low dose of TAK-063 decreased pro-inflammatory cytokines/chemokines IL-17, I-TAC, MIG, MIP-2, and RANTES protein expression more than high dose of TAK-063. Moreover, PDE10A inhibition increased expression of anti-inflammatory cytokines/chemokines IL-1ra, IL-4, IL-13, and TIMP-1. Interestingly, expression of G-CSF, IL-27, and M-CSF which are known as anti-inflammatory cytokines/chemokines were decreased. In addition, pro-/ anti-inflammatory cytokines/chemokines BLC, C5/ C5a, sICAM-1, IL-16, SDF-1, and TARC were not influenced by TAK-063.

LC-MS/MS based proteomics serve an emerging tool to understanding of the molecular and cellular mechanisms of neurodegenerative disorders including Parkinson's disease, Alzheimer's disease, and cerebral ischemia [48-50,24]. In the present study, we examined the protein profile changes in the ischemic brain induced by PDE10A inhibition. For this purpose, ipsilesional tissues from mice subjected to 30 min MCAO followed by 72 h reperfusion were analyzed by LC-MS/MS. For the first time, proteins that had significantly been altered by low dose or high dose TAK-063 have been identified. PSMD2, ADT1, ANKS1B, CYFP1, DLDH, DPOE1, DOCK4, REV3L, FLNA, HAGH, IMA4, LANCL2, NDUFS4, PIP5K1C, RAB8B, ARHGAP23, ROCK2, SCAMP1, NALCN, SLC25A11, SLC25A12, TCPE, and VDAC3 proteins were increased and ACOT7, DCTN4, ETFB, FLOT2, ACBG1, MAPRE2, OAT, PACS1, PSMB1, S100B, PLPHP, SERPINB6, SHPS1, and SYYC were decreased by TAK-063 treatments. Clustering of proteins into groups based on their molecular function and biological process using PANTHER (<http://www.pantherdb.org/>) revealed that the proteins identified included proteins with binding, catalytic and transporter activity. PANTHER classification based on biological process revealed five predominant groups (cellular process, metabolic process, localization, biological regulation, and response to stimulus). In addition to this, six signaling pathways were identified as cytoskeletal regulation by Rho GTPase, dopamine receptor mediated signaling pathway, Huntington disease, integrin signaling pathway, Parkinson's disease, and Ubiquitin proteasome pathway, demonstrating a profound effect of PDE10A on neuronal survival-associated cell signaling.

In conclusion, this study indicated that both low dose (0.3 mg/kg) and high dose (3 mg/kg) of TAK-063 reduced neurological deficits infarct volume, brain swelling, BBB leakage, and neuronal injury in the ischemic brain, which was associated with re-regulation of anti-apoptotic and pro-apoptotic proteins, re-regulation of the PI3K/Akt signal transduction pathway, decreased pro-inflammatory cytokine/chemokine responses and increased anti-inflammatory cytokine/chemokine responses. Our study identifies PDE10A as a potent target for neuroprotective therapies that in view of the restorative effect of PDE10A inhibition [9] deserves further scrutiny.

Declarations

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Conflicts of Interest: The authors declare that they have no conflict of interest.

Availability of data and material: The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Code availability: Not applicable.

Authors' Contributions: This work was carried out in collaboration between all authors. MCB, ABC and SA carried out experimental work, analyzed data and helped to write the manuscript. EO and TK performed LC-MS/MS experiments and analyzed data. NA and BC carried out Western blot and immunofluorescence studies. MCB, TRD, UK, DMH and EK defined the research theme and revised the manuscript critically.

Ethics approval: Experiments were performed in accordance to National Institutes of Health (NIH) guidelines for the care and use of laboratory animals and approved by local government authorities (Istanbul Medipol University, Animal Research Ethics Committee).

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Figures

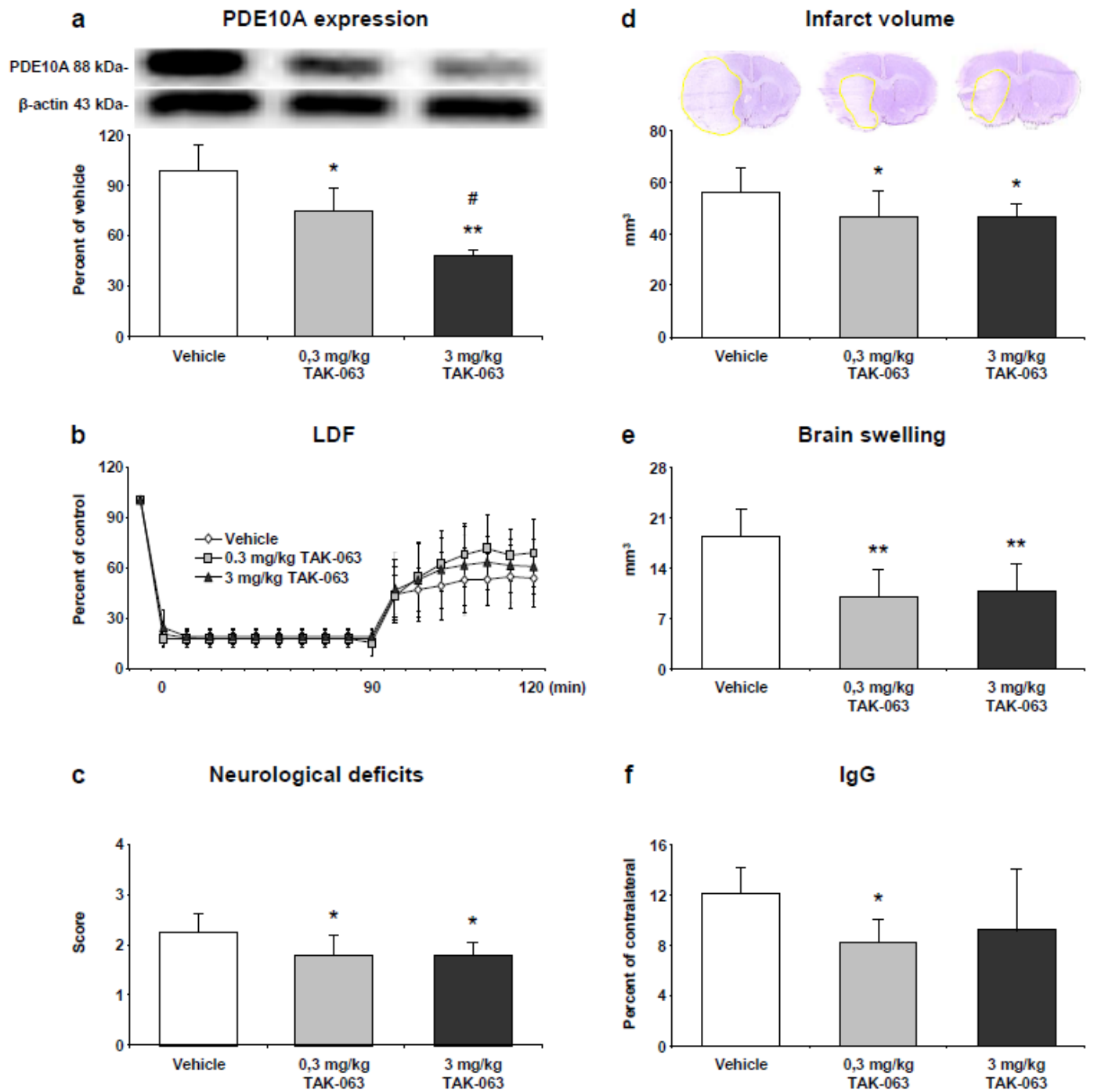


Figure 1

Effects of PDE10A inhibition by TAK-063 on PDE10A expression, neurological deficits, infarct volume, brain swelling and blood-brain barrier leakage. Both low (0.3 mg/kg) and high (3 mg/kg) doses of TAK-063 significantly reduced PDE10A protein expression in the striatum (a). Cerebral blood flow (CBF) was analyzed via Laser Doppler flowmetry (LDF) during MCAO experiments (b). In addition, low dose or high dose of TAK-063 decreased neurological deficits (c), infarct volume (d), and brain swelling (e). Only low dose of TAK-063 significantly reduced blood-brain barrier (BBB) permeability (f) evaluated by serum IgG

extravasation in mice submitted to 90 min of intraluminal MCAO, which induces brain infarcts of the striatum and overlying cortex. Data are represented as mean + S.D. (n = 7-8 mice/group). **p < 0.01/ *p < 0.05 compared with vehicle, #p < 0.05 compared with 0.3 mg/kg TAK-063 treated group.

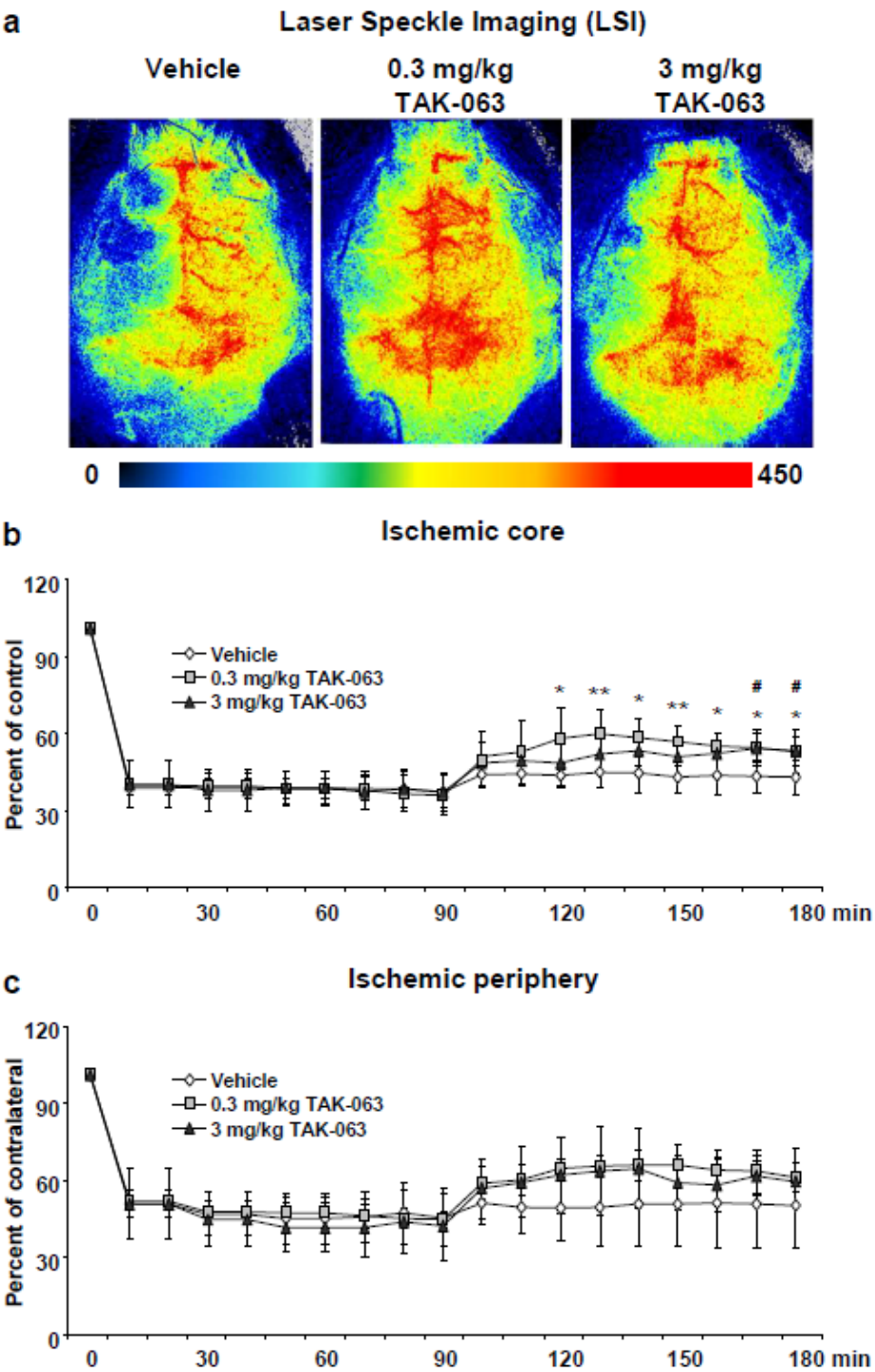


Figure 2

Effects of TAK-063 mediated PDE10A inhibition on post-ischemic regional cerebral blood flow. Representative images of Laser Speckle Imaging (LSI) (a), and semi-quantitative analysis of regional CBF

in the ischemic core (b) and in the ischemic periphery (c) evaluated by laser speckle imaging (LSI) in mice subjected to 90 min of intraluminal MCAO. Low dose (0.3 mg/kg) or high dose (3 mg/kg) of TAK-063 was orally administered immediately following the onset of reperfusion. Twenty minutes after the onset of reperfusion, 0.3 mg/kg TAK-063 significantly increased regional cerebral microcirculation in the ischemic core territory. Besides this, 3 mg/kg TAK-063 increased cerebral microcirculation in the ischemic core area 70 min after the onset of reperfusion. Although CBF in the ischemic periphery was slightly affected, no statistical difference was observed. Data are represented as mean + S.D. (n = 4-5 mice/group). **p < 0.01/ *p < 0.05 shows significant differences between vehicle and 0.3 mg/kg TAK-063 group, #p < 0.05 shows significant differences between vehicle and 3 mg/kg TAK-063 group.

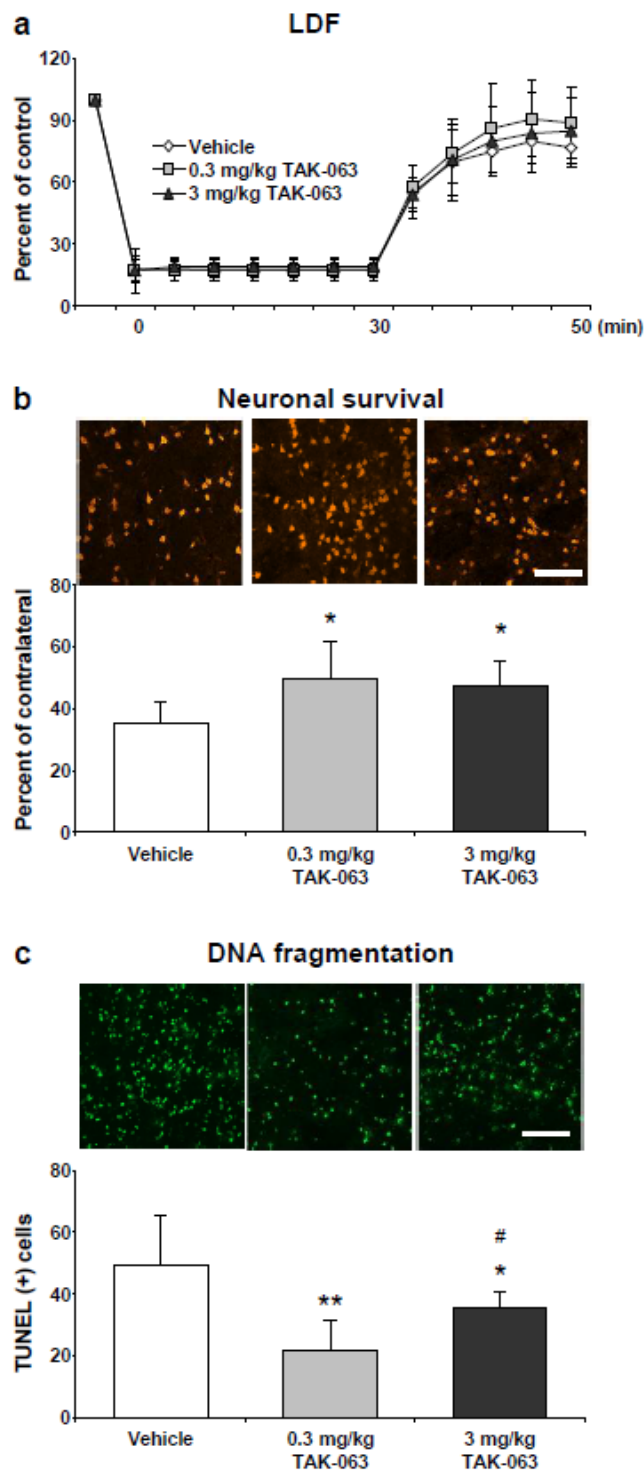


Figure 3

TAK-063 increased neuronal survival and decreased disseminated neuronal injury after cerebral ischemia. Cerebral blood flow (CBF) (a) was controlled via laser Doppler flowmetry (LDF) during ischemia and at the onset of reperfusion. The low dose (0.3 mg/kg) and high dose (3 mg/kg TAK-063) of TAK-063 increased neuronal survival (b) after transient focal cerebral ischemia. In addition, both low and high dose of TAK-063 decreased the number of apoptotic cells (c) in the ischemic striatum. Data are

represented as mean + S.D. (n = 7-8 mice/group). **p < 0.01/ *p < 0.05 compared with vehicle, #p < 0.05 compared with 0.3 mg/kg TAK-063 treated group. Scale bars are 100 μ m (b-c).

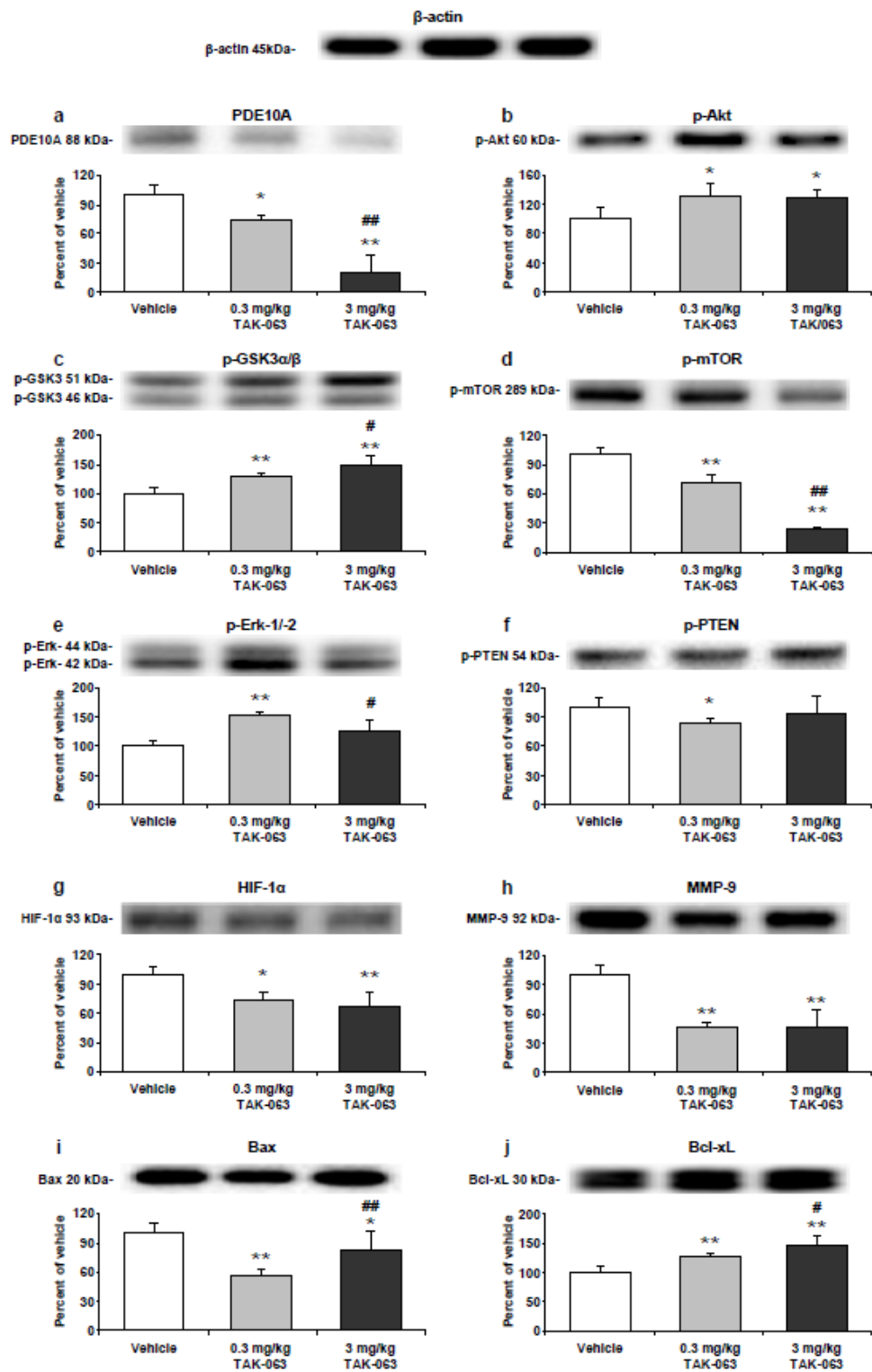


Figure 4

TAK-063 re-regulates neuronal survival-related proteins and pro/anti- apoptotic proteins after focal cerebral ischemia. Western blots for PDE10A (a), p-Akt (b), p-GSK3 α/β (c), p-mTOR (d), p-Erk-1/-2 (e), p-PTEN (f), HIF-1α (g), MMP-9 (h), Bax (i), and Bcl-xL (j) in the ischemic tissue samples obtained from

brains of mice submitted to 30 min of intraluminal MCAO followed by 72 h reperfusion. Note that the level of survival kinase p-Akt, p-GSK3 α/β and anti-apoptotic Bcl-xL protein were significantly increased and PDE10A, p-mTOR, HIF-1 α , MMP-9 and pro-apoptotic Bax protein expression were significantly reduced by TAK-063 treatments. In addition, only low dose of TAK-063 group significantly increased the level of p-Erk-1/-2 and significantly decreased the level of p-PTEN. On top of the figure, a representative β -actin blot is shown. Data are represented as mean \pm S.D. values (n = 3-5 blots/ protein). **p < 0.01/ *p < 0.05 compared with vehicle, ##p < 0.01/ #p < 0.05 compared with 0.3 mg/kg TAK-063 treated group.

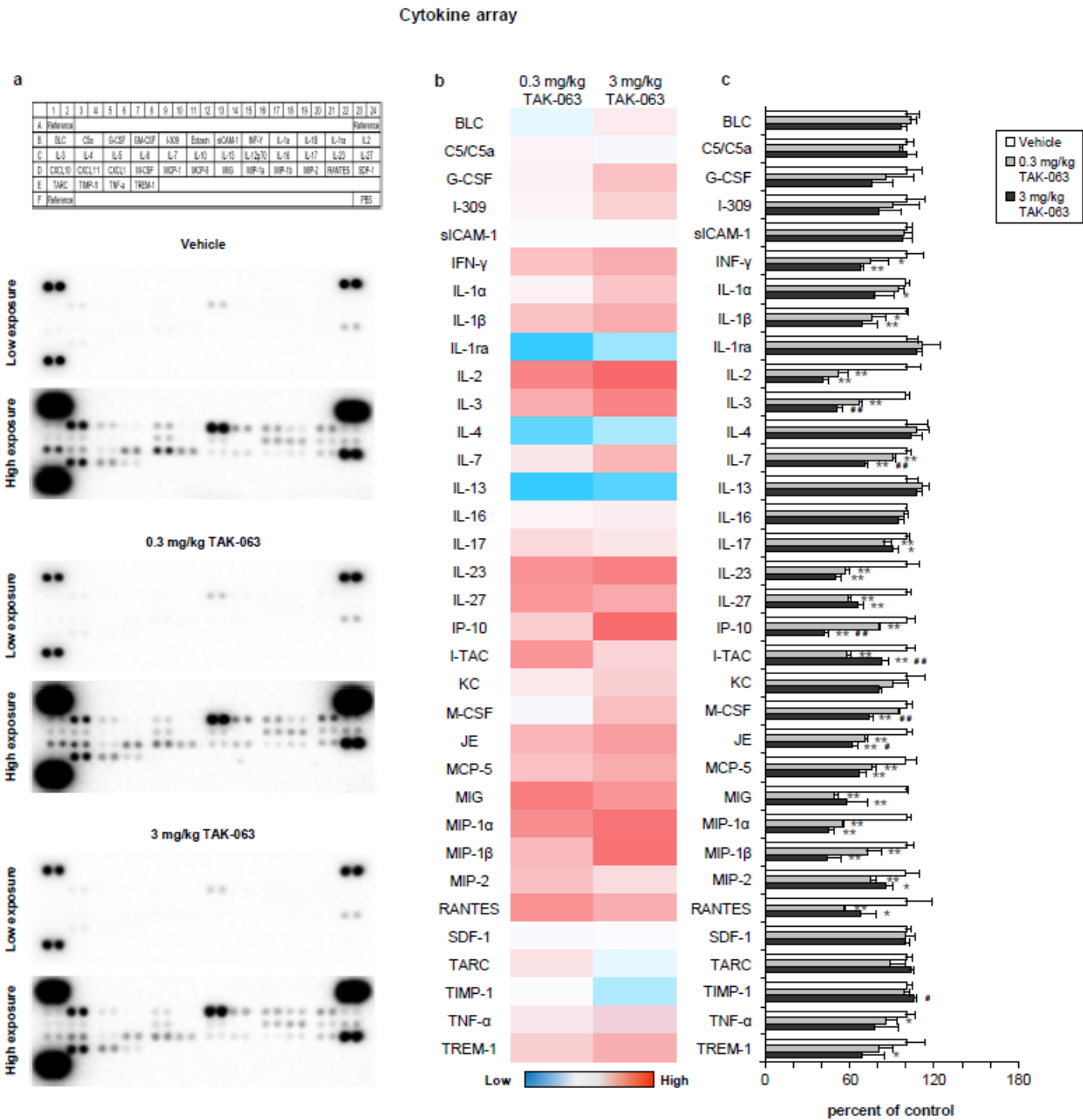


Figure 5

Heat-map and bar graph visualization of the cytokines/chemokine's analysis. Representative images for Cytokine array panels (a). The cytokine and chemokine levels in the ipsilesional striatum from animals submitted to 30 min MCAO followed by 72 h reperfusion, analyzed by a Proteome Profiler Mouse Cytokine Arrays. Results obtained from cytokine/chemokine arrays are visualized in the heat map (b) and in the bar graph (c). Data are represented as mean \pm S.D. values. **p < 0.01/ *p < 0.05 compared with vehicle, ##p < 0.01/ #p < 0.05 compared with 0.3 mg/kg TAK-063 treated group.

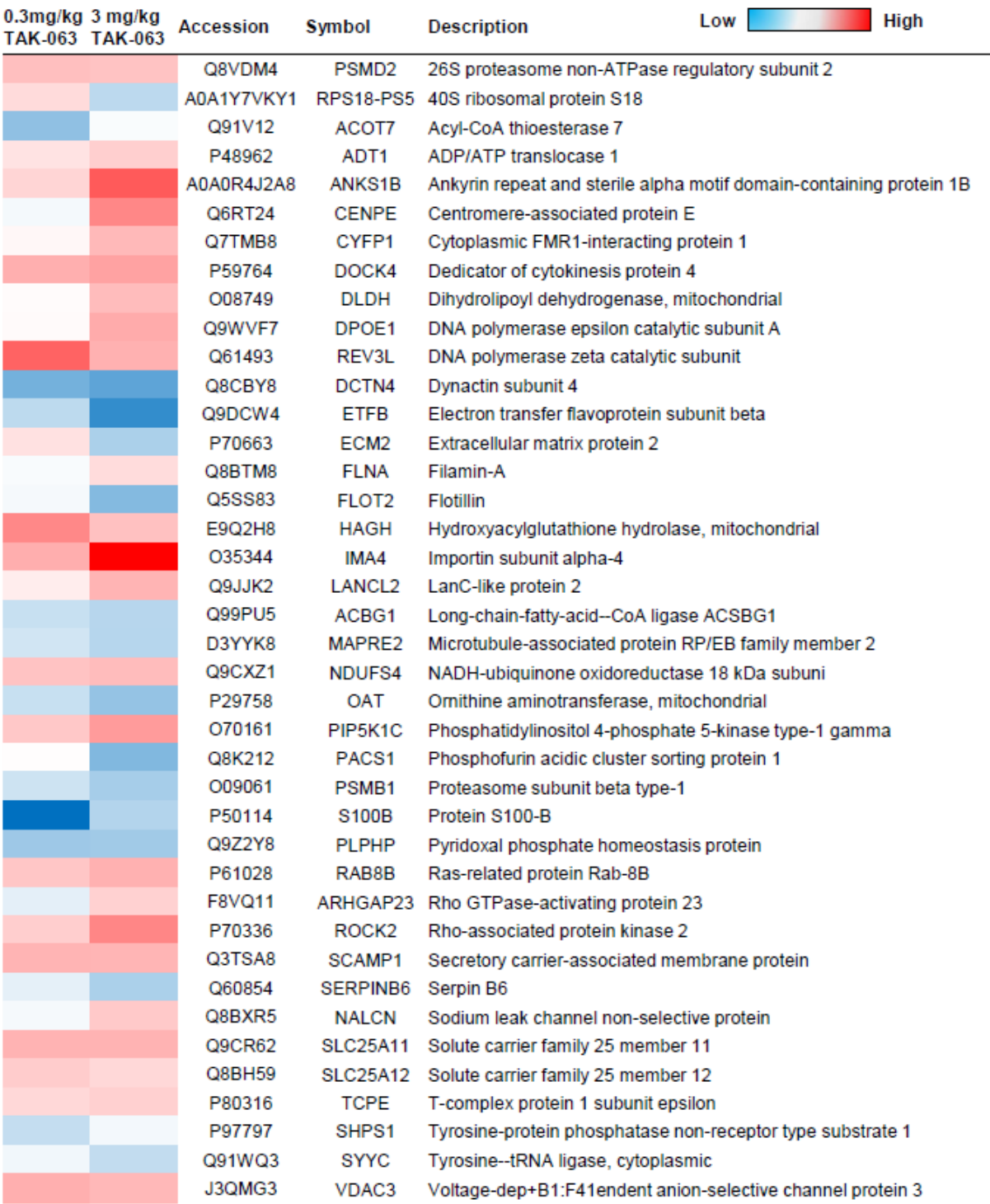


Figure 6

Identification of TAK-063 related proteins and their regulation after 30 min of intraluminal MCAO via liquid chromatography-tandem mass spectrometry (LC-MS/MS). A total of 40 different proteins were identified between vehicle and low-dose/high dose of TAK-063 mediated PDE10A inhibition groups. Comparisons (0.3 mg/kg TAK-063/ Vehicle ratio and 3 mg/kg TAK-063/ Vehicle ratio) of differential protein expression were given by heat map analysis.