

Original Article

Novel small-molecule positive allosteric modulator 1 with blood-brain barrier penetration activity exerts anti-cellular senescence effects via the PAC1-R/YY1/SIRT6 pathway

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Abstract

Aging is the primary risk factor for neurodegeneration. SIRT6, a key longevity regulator, is downregulated in aged brain tissue and linked to neurodegeneration. Thus, activating SIRT6 may delay brain aging. Based on this, the present study reveals the molecular mechanism by which the novel small-molecule positive allosteric modulator (SPAM1) antagonizes neuronal senescence through the PAC1-R/YY1/SIRT6 signaling axis, along with its highly efficient brain-targeting properties. In a naturally aging model of long-term cultured retinal ganglion cells (RGC-5), sustained administration of SPAM1 (1 μ M) significantly ameliorates aging phenotypes, including upregulation of SIRT6 and Lamin B1 expressions and suppression of p16 accumulation. Dual luciferase reporter assays confirm that SPAM1 activates the *SIRT6* promoter. Mechanistically, SPAM1 induces nuclear translocation of PAC1-R and releases its 24-kDa C-terminal fragment, which forms a complex with the transcription factor YY1 within the nucleus. ChIP-qPCR confirms that SPAM1 enhances YY1 enrichment on the *SIRT6* promoter, while YY1 knockdown inhibits SPAM1-mediated SIRT6 activation, establishing YY1's pivotal role. Pharmacokinetics and *in vivo* imaging demonstrate that SPAM1 possesses rapid and persistent brain targeting capabilities: the drug is detectable in the brain within 10 min after intraperitoneal injection; following tail vein administration, brain fluorescence signals appear within 10 min, peak at 1 h, and persist for over 12 h. This study elucidates SPAM1's anti-neurogerontogenic mechanism through a complete signaling cascade: inducing PAC1-R nuclear translocation, recruiting YY1, and activating SIRT6 transcription. Its well-defined mechanism and unique brain targeting establish a pharmacological foundation for developing innovative drugs to intervene in age-related central nervous system decline.

Key words PAC1-R, anti-aging, SIRT6, YY1, blood-brain barrier

Introduction

The hallmarks of aging include genomic instability, telomere damage, epigenetic alterations, mitochondrial dysfunction, and cellular senescence [1]. In age-related pathological processes, neurodegenerative diseases and their associated cognitive decline are particularly prominent, with aging identified as the primary risk factor for neurodegeneration [2]. As prototypical age-related brain disorders, the incidence rates of Alzheimer's disease (AD) and Parkinson's disease (PD) exhibit a clear age-dependent upward trend [3].

The sirtuin (SIRT) family comprises seven members, designated SIRT1 to SIRT7, which are key molecules regulating lifespan and cellular aging processes [4,5]. Each member exhibits distinct subcellular localization, with only nucleus-localized SIRT6 demonstrating a significant negative correlation with aging progression. By participating in critical pathways such as DNA damage repair, genomic stability maintenance, and telomere protection, SIRT6 serves as a pivotal node in aging-related neurodegeneration and plays a vital role in longevity promotion mechanisms [5–8]. This conclusion is supported by experimental evidence: *SIRT6* deficiency

accelerates aging phenotypes [9], while *SIRT6* overexpression significantly extends lifespan in male mice [10]. Of particular significance, SIRT6 exhibits marked downregulation in aged brain tissue and neurodegenerative diseases such as AD, with its expression levels negatively correlated with pathological progression [11,12]. At the molecular level, SIRT6 maintains chromatin homeostasis by deacetylating histone sites, including H3K9 and H3K56, while also participating in repairing age-accumulated DNA damage to safeguard neuronal genomic integrity. These functions confer a central role in both brain development and aging processes [13]. Consequently, targeting and activating the SIRT6 pathway represents a promising strategy for delaying brain aging and preventing related neurodegenerative diseases.

To elucidate the transcriptional regulatory mechanisms of SIRT6, we focused on the transcription factor Yin Yang 1 (YY1)—a dual-function factor exhibiting both activating and repressive properties [14,15], critical for neural development [16,17], and significantly downregulated in brain tissue from PD patients [18,19]. Importantly, YY1 has been demonstrated to directly bind the *SIRT6* promoter and drive its expression, with both factors exhibiting synergistic regulatory mechanisms in pathological brain aging pathways [20,21]. Collectively, these findings suggest that YY1 may play a potentially pivotal role in the aging process.

Pituitary adenylate cyclase-activating polypeptide receptor 1 (PAC1-R), a high-affinity specific receptor for pituitary adenylate cyclase-activating polypeptide (PACAP), is widely distributed throughout the central nervous system (CNS), with particular enrichment in brain tissue, the pituitary gland, and the adrenal glands [22,23]. Given the pivotal role of PAC1-R in CNS aging, its mediation of PACAP's neuroprotective effects in AD/PD models [24,25], and the significant age-related downregulation of the PACAP/PAC1-R system in the CNS [26,27] (with PAC1-R exhibiting a pattern of initial compensatory elevation followed by progressive attenuation in AD patients [26]), this receptor emerges as an ideal target for reversing neuro-aging. We previously developed a novel, highly potent small-molecule positive allosteric modulator 1 (SPAM1) targeting PAC1-R (patent No. CN202210388027.7; molecular formula: $C_{20}H_{19}N_3O_4$) [28]. Subsequent studies demonstrated that SPAM1 not only induces nuclear translocation of PAC1-R, thereby upregulating PACAP/PAC1-R expression to counteract age-related decline in this system [29,30] but also effectively elevates SIRT6 levels in the brain tissue of aged mice and participates in neuroprotective and longevity-related pathways [31]. Furthermore, ChIP-seq bioinformatics analysis revealed that the C-terminal motif of nuclear-translocated PAC1-R is significantly enriched in the transcription factor YY1 [30].

However, the underlying molecular mechanism by which the SPAM1-induced PAC1-R nuclear fragment transmits extracellular signals to the nucleus and precisely drives SIRT6 transcription remains incompletely elucidated. This prompted us to focus on two core molecules: PAC1-R and YY1. We hypothesize that PAC1-R, which undergoes nuclear translocation following SPAM1 activation, may mediate this signaling process through functional interactions with YY1, a key transcriptional regulator of SIRT6. Early ChIP-seq data provide a molecular basis for their potential interaction [30]. Based on this, we propose the core hypothesis of this study: SPAM1 activates PAC1-R via allosteric regulation, inducing its nuclear translocation. Subsequently, the C-terminal fragment of PAC1-R specifically recruits the transcription factor YY1

within the nucleus, forming a functional complex that jointly activates *SIRT6* transcription, ultimately exerting an anti-neuronal aging effect.

Therefore, this study aims to systematically validate the aforementioned hypothesis through multi-level investigations, including *in vitro* aging models, molecular interaction validation, and *in vivo* pharmacokinetics. We seek to elucidate for the first time the complete pathway by which SPAM1 antagonizes neuronal aging through PAC1-R/YY1 axis-mediated regulation of *SIRT6* transcription. Additionally, we will systematically evaluate its blood-brain barrier penetration capacity and drug potential for intervening in brain aging. This work will not only reveal a novel mechanism by which membrane receptors mediate nuclear anti-aging programs but also provide crucial pharmacological rationale for developing anti-neuroaging drug candidates with brain-targeting potential.

Materials and Methods

Materials and cell lines

All of the materials for the cell culture and transfection reagents were from Invitrogen (Waltham, USA). The promoter reporter vector pYr-PromDetect was from Yingrun Biological Company (Changsha, China). siRNA was obtained from Jinkairui Biological Company (Wuhan, China). Mouse retinal ganglion (RGC-5) cell lines were provided by Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Peptide SPAM1 was synthesized by GL Biochem Ltd (Shanghai, China) at 95% purity. The purity of the peptides was confirmed using reversed-phase high-performance liquid chromatography (HPLC), and they were characterized using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

Cell culture and natural aging model establishment

RGC-5 cells were cultured in F-12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution. Cells were incubated in a constant-temperature incubator at 37°C with 95% humidity and 5% CO₂.

For the natural aging model, cells were passaged at a 1:3 ratio every 3 days for 40 consecutive days (approximately 13 cumulative passages). In the SPAM1 treatment group, 1 μM SPAM1 (molecular formula: $C_{20}H_{19}N_3O_4$; dissolved in PBS) was added on the initial day of culture and maintained for 40 days. All experiments were independently replicated three times.

SA-β-galactosidase staining

Cells from the control and SPAM1-treated groups cultured for 40 days were collected and seeded into a 12-well plate at 1×10^5 cells/well. After culturing for 24 h until cell confluence reached 80%, the medium was aspirated, and the cells were gently washed once with PBS. Fixation and staining were performed using the Cell Senescence β-Galactosidase Staining Kit (Beyotime, Shanghai, China). Following incubation at 37°C in the dark for 16 h, blue-stained cells were observed and counted under an inverted light microscope (Olympus, Tokyo, Japan). The senescent cell rate was calculated as: senescent cell rate (%) = (number of blue-stained cells/total cell count) × 100%. All experiments were independently replicated three times.

Western blot analysis

RGC-5 cells from the 40-day-cultured control group and SPAM1-

treated group were seeded at a density of 2×10^5 cells/well in a 6-well plate and subjected to protein extraction after 24 h of culture. The medium was removed, and cells were washed twice with pre-chilled PBS. Each well was loaded with 100 μ L RIPA lysis buffer containing protease inhibitors (Beyotime) and incubated on ice for 20 min. Lysates were collected, centrifuged at 4°C and 15,000 g for 20 min, and the supernatant was used for BCA protein quantification. Protein samples (10 μ g) were separated by SDS-PAGE electrophoresis and transferred to a PVDF membrane. The membrane was incubated overnight at 4°C with the following primary antibodies: anti-SIRT6 polyclonal antibody (13572-1-AP; Proteintech, Wuhan, China), anti-YY1 monoclonal antibody (sc-7341; Santa Cruz, Santa Cruz, USA), anti-PACAP receptor/AD-CYAP1R1 400-500 aa antibody (ab28670; Abcam, Cambridge, UK), anti-Lamin B1 polyclonal antibody (12987-1-AP; Proteintech), anti-p16 monoclonal antibody (sc-1661; Santa Cruz), and anti- β -actin antibody (EM21002; HUABIO, Hangzhou, China). Subsequently, HRP-labeled rabbit/mouse secondary antibodies (HA1006; HUABIO/ab150075; Abcam) were incubated at room temperature for 1 h. Protein bands were visualized using the UltraSensitive ECL Chemiluminescence Reagent Kit (Beyotime). All experiments were repeated at least three times.

Construction of the recombinant vector pYr-PromDetect-SIRT6

The *SIRT6* gene promoter region (−2500 bp to +9 bp, total length 2509 bp) was cloned and inserted into the pYr-PromDetect promoter reporter vector (Yingrun Biological Company, Changsha, China) to construct the recombinant plasmid pYr-PromDetect-SIRT6. The specific construction method followed the protocol described in a previous study [32]. In this recombinant vector, the *SIRT6* promoter sequence was inserted upstream of the *Renilla* luciferase reporter gene, while the *Firefly* luciferase gene, driven by the endogenous *HSV-TK* promoter, served as an internal control.

Plasmid transfection

Log-phase RGC-5 cells were digested, seeded into a 12-well plate at 1×10^5 cells/well, and cultured in F12/10% FBS for 24 h until 60%–70% confluence. Transfection was performed using the Lipofectamine 3000 (Invitrogen). For each well, 1 μ g of pYr-PromDetect-SIRT6 or empty vector pYr-PromDetect and 2 μ L P3000 Reagent were diluted in 150 μ L Opti-MEM (Invitrogen). Separately, 1 μ L Lipofectamine™ 3000 was diluted in 150 μ L Opti-MEM. Both were allowed to stand for 5 min at room temperature, then combined and incubated for another 20 min to form the transfection complex. The original medium was replaced by 500 μ L Opti-MEM plus 300 μ L of the complex per well. Cells were incubated at 37°C for 6 h, then the medium was replaced by F12/10% FBS, and culture was continued for 48 h.

Transient siRNA transfection and co-transfection

YY1-specific siRNA sequences were shown as follows: sense strand: 5'-CGACGGUUGUAAUAAGAAGUUTT-3' and antisense strand: 5'-AACUUCUUAUUACAACCGUCGTT-3'. The non-targeted siRNA sequences were shown as follows: sense strand: 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense strand: 5'-ACGUGACACGUUCGAGAATT-3'. Non-targeted siNC served as the control (siNC group), while YY1 siRNA constituted the experimental group (siYY1 group). Prior to transfection, cell culture medium was

replaced by Opti-MEM. For each well, 20 pmol siRNA was mixed with small RNA transfection reagent (Cat# CT0101; Bokei Biotech, Beijing, China) in Opti-MEM according to the manufacturer's instructions. The mixture was incubated at room temperature for 5 min before adding to cells.

siNC + pYr-PromDetect-SIRT6 was set as the control group, and siYY1 + pYr-PromDetect-SIRT6 as the experimental group. For each well, 40 pmol siRNA and 1 μ g pYr-PromDetect-SIRT6 plasmid were diluted in 100 μ L Opti-MEM and mixed thoroughly. Separately, 3 μ L Lipofectamin™ 3000 was diluted in 200 μ L Opti-MEM. After 5 min incubation at room temperature, the diluted Lipofectamine was combined with the siRNA/plasmid mixture and incubated for another 20 min to form the transfection complex, which was then added to cells.

All transfection groups were cultured at 37°C for 6 h post-transfection. The medium was then replaced by F12/10% FBS, and cells were cultured for another 48 h. YY1 knockdown efficiency was assessed by western blot analysis. Co-transfected groups were additionally subjected to dual luciferase reporter assay.

SIRT6 promoter activity assay

Forty-eight hours after plasmid transfection, the medium was replaced by serum-free F12 medium. Both the pYr-PromDetect-SIRT6 group and the pYr-PromDetect group were treated with a gradient concentration of SPAM1 (0, 0.01, 0.1, 1, 10, or 100 μ M; the drug was prepared using DMSO, with the final DMSO concentration being equal across all groups = 0.1%), and incubated at 37°C for 1 h. The medium was removed, and the cells were gently washed twice with PBS. Then, 100 μ L of $1 \times$ cell lysis buffer (Meilunbio, Dalian, China) was added to each well and incubated at room temperature with gentle shaking for 15 min to lyse the cells. The lysate was collected, centrifuged at 4°C and 16,000 g for 5 min, and the supernatant was used for detection.

The activities of firefly luciferase and *Renilla* luciferase were sequentially measured using a Synergy H1 multimode microplate reader (Biotek, Winooski, USA) according to the manufacturer's instructions. The promoter activity was expressed as the ratio of *Renilla* luciferase activity to firefly luciferase activity and normalized to the control group transfected with the empty vector pYr-PromDetect. All experiments were independently repeated three times.

Transcription factor prediction

Based on the human *SIRT6* gene promoter region (−2500 bp to 0 bp), potential transcription factor-binding sites were predicted through multidatabase cross-validation, including UCSC (<https://genome.ucsc.edu/>), hTFtarget (<https://guolab.wchscu.cn/hTFtarget/#/>), and GeneCards (<https://www.genecards.org/>). Transcription factors with common predictions (e.g., YY1, SP1, STAT1, FOS, CTCF, KLF5, and MAZ) were retained as high-confidence binding targets for subsequent mechanism validation.

Co-immunoprecipitation (Co-IP)

Logarithmically growing RGC-5 cells were seeded in a 10-cm culture dish. Upon reaching 80% confluence, the medium was replaced by F12 containing 1 μ M SPAM1 for 1 h. After removing the medium, cells were washed twice with pre-chilled PBS and lysed on ice for 1 h in 1 mL IP lysis buffer (Beyotime). The lysate was centrifuged at 4°C and 13,000 g for 30 min. The supernatant was collected, with 5% reserved as Input control. The remaining lysate was equally

divided. To experimental groups, 2.5 µg of anti-PACAP receptor/ADCYAP1R1 400-500 aa antibody (ab28670; Abcam) or anti-YY1 antibody (sc-7341; Santa Cruz) was added, while equal amounts of isotype IgG were added to negative controls. Samples were incubated overnight at 4°C with gentle rotation. The next day, 20 µL of protein A+G magnetic beads were added and incubated at room temperature for 1 h. Beads were washed five times with lysis buffer. Subsequently, 1× SDS loading buffer was added, and samples were boiled at 95°C for 5 min. After magnetic separation, the supernatant was collected and subjected to SDS-PAGE and western blot analysis to detect protein interactions.

Chromatin immunoprecipitation and quantitative PCR (ChIP-qPCR)

RGC-5 cells in logarithmic growth phase were seeded into a 10-cm culture dish. Once cells reached 80% confluence, the medium was replaced by F12 containing 1 µM SPAM1 for 1 h. Cells were cross-linked with 1% formaldehyde at 37°C for 10 min, then cross-linking was terminated by adding glycine. After cell lysis, chromatin was digested using MNase (P2083S; Beyotime) at 37°C for 20 min. The reaction was terminated by adding 0.5 M EDTA. Samples were then sonicated to thoroughly disrupt nuclear membranes. After centrifugation, the supernatant was collected to obtain chromatin fragments. These were incubated overnight at 4°C with either 5 µg anti-YY1 monoclonal antibody (sc-7341; Santa Cruz) or control mouse IgG. The next day, Protein A/G Magnetic Beads/Salmon Sperm DNA were added, and incubation was continued at 4°C. After incubation, magnetic beads were thoroughly washed for decoupling and DNA purification. The final DNA product was used for subsequent qPCR analysis. qPCR primers were designed targeting the predicted high-scoring YY1 binding site within the *SIRT6* promoter region (located at -1150 to -1139 bp upstream of the transcription start site), with sequences as follows: primer F: 5'-TTTACCCCCACACCTACCAC-3' and primer R: 5'-CCTGTGCTGTGTCACCTCTT-3'.

LC-MS/MS analysis

The animal study protocol was approved by the Ethics Committee of Jinan University (IACUC-20200110-06). Male C57BL/6J mice (6–8 weeks old, purchased from Nanjing Jisihuiyuan Biotechnology Co., Ltd., Nanjing, China) were housed under constant temperature and humidity conditions (22 ± 1°C, 55% ± 10% humidity, 12-h light/dark cycle), with free access to water. Animals were fasted for 12 h prior to dosing. Twenty-four mice were randomly divided into eight groups ($n = 3$) and administered intraperitoneal (IP) injections of SPAM1 solution (100 µmol/kg) dissolved in physiological saline. Blood and brain tissue samples were collected at 10 min, 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h, and 24 h post-administration.

Approximately 200 µL whole blood was collected via the orbital venous plexus into EDTA-K2 anticoagulant tubes. After gentle mixing, plasma was separated by centrifugation (6000 *g* for 5 min at 4°C). Following euthanasia, brain tissue was immediately removed, rinsed with physiological saline, blotted dry with filter paper, rapidly frozen in liquid nitrogen, and stored at -80°C.

Approximately 30 µL of plasma was vortex-mixed with 120 µL of acetonitrile solution containing the internal standard (IS; *m/z* 455.2/165.0) for 3 min, followed by centrifugation (13,000 *g* for 5 min at 4°C). A total of 80 µL of supernatant was transferred and diluted with 40 µL of 50% acetonitrile aqueous solution. The tissue was

homogenized in 5 volumes of 30% acetonitrile in water using a frozen tissue grinder. Subsequently, 30 µL of the homogenate was processed in an identical manner to the plasma samples.

Quantification of SPAM1 was performed using an ExionLC-AD system coupled with an API 4000 mass spectrometer (SCIEX) operating in positive ion multiple reaction monitoring (MRM) mode. Chromatographic separation was achieved on an ACE Excel SC18 column (50 mm × 3.0 mm). The mobile phase consisted of (A) 0.1% formic acid in water and (B) acetonitrile, delivered at a flow rate of 0.3 mL/min. The gradient program was as follows: 0–0.2 min, 20% B; 1–2.2 min, 95% B; 2.21–3 min, 20% B. The mass spectrometric parameters were set as follows: monitor ion transitions for SPAM1 (366.1 → 214.8) and IS (455.2 → 165.0); ion spray voltage: 5500 V; temperature: 500°C.

Using Phoenix WinNonlin 8.1 software, non-compartmental models were employed to calculate pharmacokinetic parameters, including C_{max} , T_{max} , AUC_{last} , $AUC_{infinite}$, $AUC_{%Extrap}$, $T_{1/2}$, CL, and V_{ss} .

In vivo fluorescence imaging analysis

Female C57BL/6J mice (5–6 weeks old, purchased from Nanjing Jisihuiyuan Biotechnology Co., Ltd) were acclimatized and then randomly divided into two groups ($n = 3$ /group): the compound group received a single tail vein injection of 100 µL of CY5-labeled SPAM1 (100 µM, dissolved in 5% DMSO + saline + 1% Tween-80), and the blank control group was administered an equal volume of the vehicle via tail vein injection. Dynamic fluorescence imaging of the brain region was performed using a small animal *in vivo* imaging system at 8 time points post-injection: 10 min, 20 min, 40 min, 1 h, 2 h, 6 h, 12 h, and 24 h (excitation/emission wavelengths = 649/670 nm). The enrichment characteristics of SPAM1 in the brain were evaluated through quantitative analysis of fluorescence signals.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 10 software. All data are expressed as the mean ± standard error of the mean (SEM). Differences between groups were assessed using two-tailed unpaired *t* tests and one-way analysis of variance (ANOVA). Differences were considered statistically significant when $P < 0.05$.

Results

SPAM1 mitigates natural aging in RGC-5 cells

To elucidate the regulatory role of SPAM1 in neuronal cell aging, this study established a natural aging model of RGC-5 cells induced by 40 days of long-term culture and continuously administered 1 µM SPAM1. Based on the molecular mechanism characteristics of the following aging markers, p16 induces irreversible growth arrest by inhibiting CDK4/6, and its accumulation is a core marker of aging [33]; progressive loss of Lamin B1 disrupts nuclear membrane integrity, representing a hallmark molecular event of aging [34]. Western blot analysis revealed: compared to the untreated aging group, SPAM1 significantly upregulated Lamin B1 expression while suppressing p16 level; the key neuroprotective factor SIRT6 expression recovered after SPAM1 intervention (Figure 1A–C). Further validation via SA-β-gal senescence staining showed that the SPAM1-treated group exhibited a significantly reduced positive cell rate (14.32% ± 1.25% vs 31.56% ± 2.28% in the aging group, $P < 0.01$) and diminished staining intensity (Figure 1D). These findings indicate that SPAM1 effectively delays the senescence

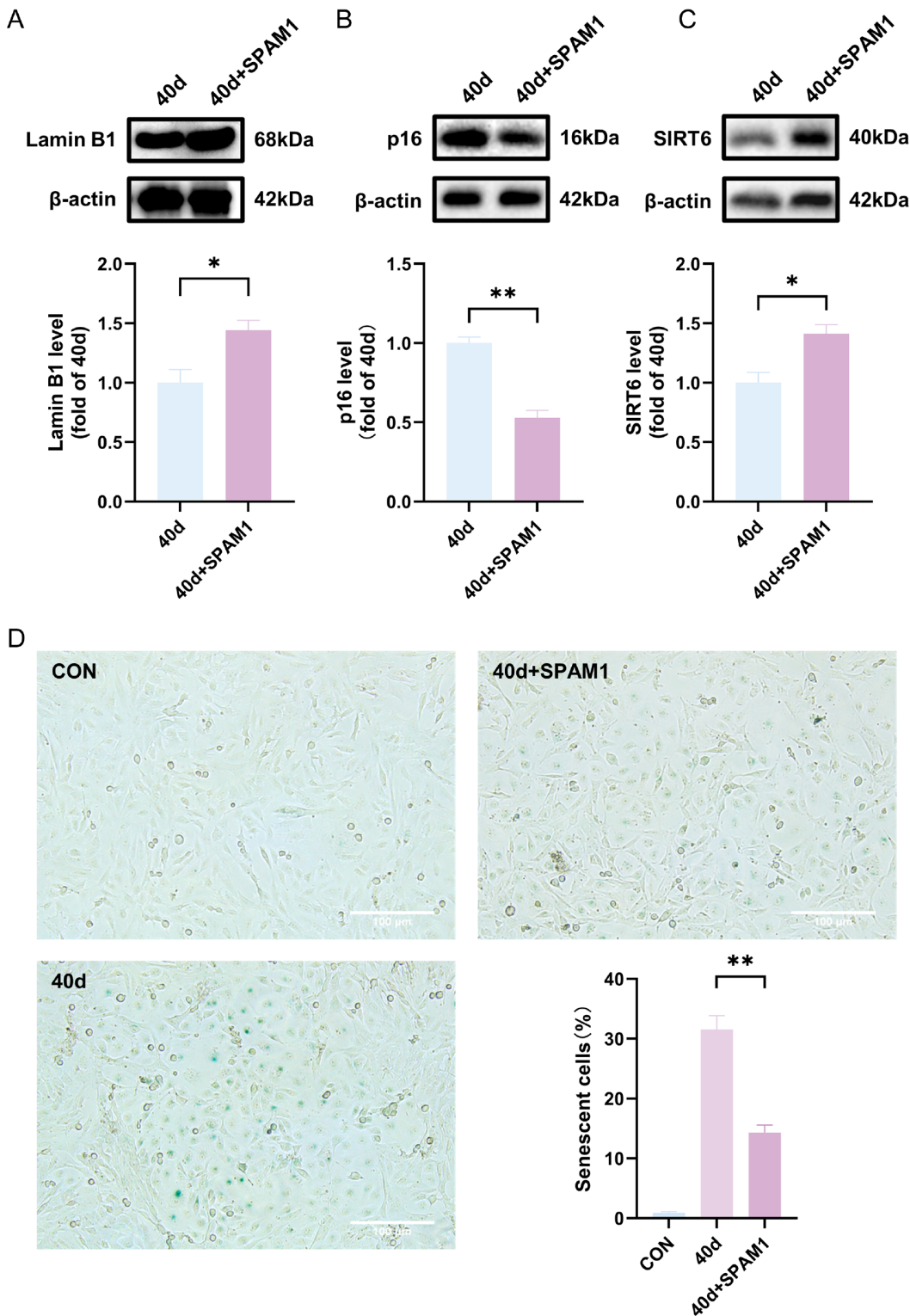


Figure 1. SPAM1 regulates key senescence markers and alleviates the senescence phenotype in RGC-5 cells (A) Western blot analysis and quantitative statistics show that continuous treatment with 1 μ M SPAM1 significantly increases Lamin B1 expression in aged RGC-5 cells cultured for 40 days. * P < 0.05 vs 40 d. (B) Western blot analysis and quantification show that continuous treatment with 1 μ M SPAM1 significantly reduces accumulation of the senescence marker p16. ** P < 0.01 vs 40 d. (C) Western blot analysis and statistics show that SPAM1 (1 μ M) treatment significantly upregulates the expression of the anti-aging factor SIRT6. * P < 0.05 vs 40 d. (D) SA- β -gal senescence staining and statistical analysis of positive cell counts show that SPAM1 (1 μ M) treatment significantly reduces the proportion of β -gal-positive cells. Scale bars: 100 μ m. ** P < 0.01 vs 40 d. Data are presented as the mean $\pm$ SEM of three independent experiments.

phenotype in RGC-5 cells by activating the SIRT6 pathway and coordinating the regulation of p16 and Lamin B1.

SPAM1 enhances *SIRT6* promoter activity

Given that SPAM1 rescues SIRT6 expression in senescent cells, we hypothesize that SPAM1 mediates transcriptional regulation by enhancing *SIRT6* promoter activity. To validate this hypothesis, we constructed a dual luciferase reporter vector, pYr-PromDetect-SIRT6 (Figure 2A), containing the human *SIRT6* promoter region (−2500 to +9 bp) to assess the effect of SPAM1 on *SIRT6* promoter activity. After transfecting RGC-5 cells with this reporter vector, luciferase activity was measured following treatment with SPAM1 (0.01–100 μ M). The results shown in Figure 2B indicate that SPAM1 activated the promoter at certain concentrations: the low-concentration group (0.01–0.1 μ M) showed no significant change in activity ($P > 0.05$), whereas activity was significantly enhanced at $\geq 1 \mu$ M (1.72 ± 0.09 -fold vs 0 μ M, $P < 0.05$). These results confirm that SPAM1 positively regulates *SIRT6* transcriptional activity. Since both 1 μ M and 100 μ M effectively activated the reaction with no statistically significant difference between them ($P > 0.05$), 1 μ M was selected as the optimized concentration for subsequent studies.

Prediction of *SIRT6* transcription factors and YY1 screening

Based on the previously identified SPAM1-induced nuclear translocation of PAC1-R [29] and the upregulation of SIRT6 at both the transcriptional and protein levels, we hypothesize that the nucleus-localized fragment of PAC1-R regulates *SIRT6* transcription by recruiting transcription factors. Cross-analysis of the *SIRT6* promoter region (−2500 to 0 bp) using hTFtarget, GeneCards, and UCSC JASPAR databases identified 20 conserved transcription factor-binding sites (YY1, SP1, STAT1, FOS, CTCF, and KLF5, among others) (Figure 3A). Among these, YY1 has been demonstrated to

directly bind to the *SIRT6* promoter and regulate its expression, and it can co-act with SIRT6 to participate in regulating brain aging, neurodegenerative diseases, and age-related mitochondrial down-regulation [21,35]. This finding corroborates prior ChIP-seq results, which showed that enrichment analysis of the C-terminal motif of PAC1-R identified YY1 as a significant binding factor [30]. Therefore, YY1 is more likely than other transcription factors to be recruited by nuclear PAC1-R and participate in the regulation of the *SIRT6* promoter.

YY1 knockdown inhibits *SIRT6* expression

To elucidate the relationship between SIRT6 and YY1, YY1 protein expression was downregulated in RGC-5 cells via siRNA-mediated gene knockdown (Figure 3B). The results showed that SIRT6 expression was downregulated by $42.3\% \pm 3.6\%$ ($P < 0.01$) in the presence of YY1 knockdown. This confirms that YY1 is a key transcriptional activator of SIRT6.

Interaction between the nuclear-entry C-terminus of PAC1-R and YY1

To confirm whether the PAC1-R C-terminal fragment induced by SPAM1 nuclear translocation can physically interact with the transcription factor YY1, we performed Co-IP analysis on nuclear extracts from RGC-5 cells treated with SPAM1 (1 μ M). Immunoprecipitation using either a monoclonal antibody against YY1 or a specific antibody against the PAC1-R C-terminal fragment revealed that the two proteins bound to each other to form a complex (Figure 3C). Notably, following IP with the YY1 antibody, a specific PAC1-R band was detected only at approximately 24 kDa. This indicates that SPAM1-induced nuclear translocation of PAC1-R is accompanied by its proteolytic cleavage, yielding a 24 kDa C-terminal functional fragment that forms a stable complex with YY1 within the nucleus.

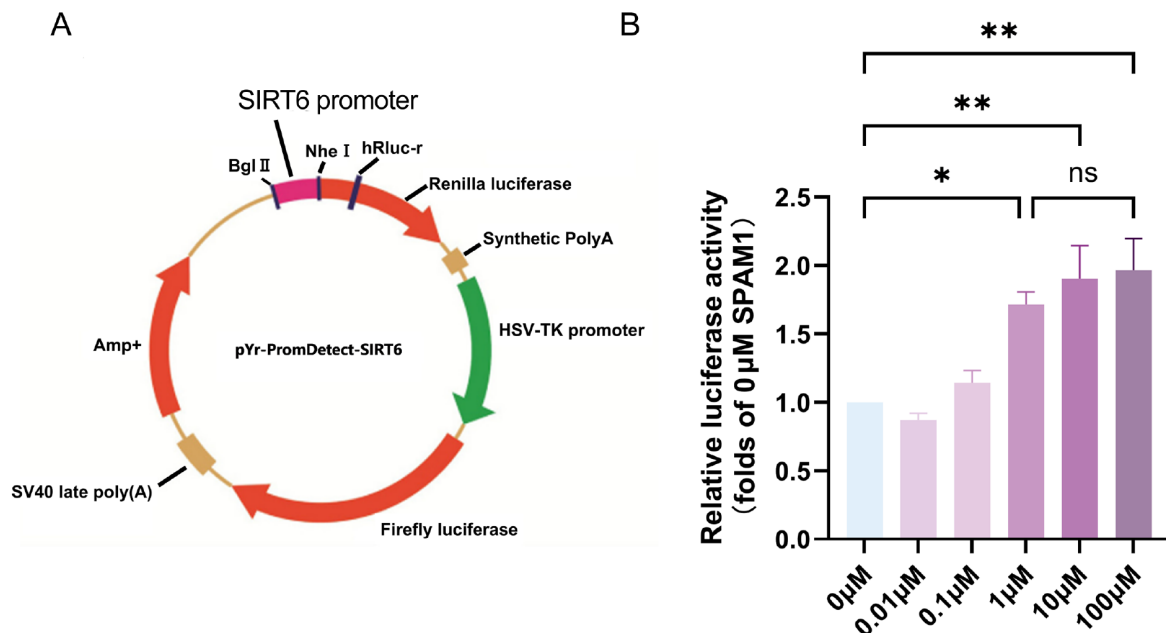


Figure 2. SPAM1 enhances *SIRT6* promoter activity (A) Schematic diagram of the dual luciferase reporter vector pYr-PromDetect-SIRT6 containing the human *SIRT6* promoter region (−2500 to +9 bp). (B) Dual luciferase assay results demonstrate that SPAM1 (1–100 μ M) significantly enhances *SIRT6* promoter activity. * $P < 0.05$ vs 0 μ M; ** $P < 0.01$ vs 0 μ M. Data are presented as the mean $\pm$ SEM of three independent experiments.

SPAM1 enhances YY1 enrichment in the *SIRT6* promoter region

To elucidate whether SPAM1 exerts its regulatory function by promoting YY1 binding to the *SIRT6* promoter, we performed ChIP-qPCR analysis. First, potential YY1 binding motifs within the *SIRT6* promoter region (−2500 to 0 bp) were predicted using the JASPAR database (Figure 3D), and specific primers were designed for the high-scoring binding site (located at −1150 to −1139 bp upstream of the transcription start site). After treating RGC-5 cells with 1 μ M SPAM1 or control solvent, cross-linking was performed, followed by ChIP experiments using the YY1 antibody. As shown in Figure 3E, SPAM1 treatment significantly enhanced YY1 enrichment at the *SIRT6* promoter region compared to the control group. This result directly confirms that SPAM1 promotes the recruitment of the transcription factor YY1 to the *SIRT6* promoter, providing crucial evidence for its molecular mechanism in activating *SIRT6* transcription.

YY1 knockdown inhibits SPAM1-mediated activation of the *SIRT6* promoter

To determine whether YY1 mediates SPAM1's transcriptional regulation of *SIRT6*, the *SIRT6* promoter dual luciferase reporter vector was co-transfected with siYY1 into RGC-5 cells (siYY1 group; due to co-transfection limitations, the knockdown efficiency was $51.5\% \pm 6.6\%$; Figure 3F). The results shown in Figure 3G indicate that promoter activity in the siYY1 group was significantly reduced compared to that in the control group (siNC), consistent with the downregulation trend of *SIRT6* protein observed by western blot analysis (Figure 3B), indicating that YY1 knockdown significantly suppressed basal *SIRT6* transcriptional activity. Subsequently, we compared the siNC and siYY1 groups after SPAM1 (1 μ M) treatment. SPAM1 significantly enhanced activity in the siNC group; however, under YY1 knockdown conditions, the activating effect of SPAM1 was substantially suppressed ($P < 0.01$). This indicates that YY1 knockdown inhibits SPAM1 activation of the *SIRT6* promoter, establishing YY1 as an essential factor for SPAM1-mediated *SIRT6* activation. Interestingly, although YY1 knockdown attenuated the effects of SPAM1, the activity in the SPAM1-treated siYY1 group remained higher than that in the untreated siYY1 group and even exceeded basal siNC levels. This suggests that SPAM1 may partially activate *SIRT6* through alternative pathways (e.g., recruiting other transcription factors), or due to incomplete YY1 knockdown, the nuclear PAC1-R fragment may still recruit residual YY1 to exert its function, thereby partially rescuing the siYY1-induced downregulation of promoter activity.

The above results collectively demonstrate that YY1 serves as the core mediator through which SPAM1 activates *SIRT6* transcription via PAC1-R. Its mechanism of action can be summarized as follows: SPAM1 acts as a positive allosteric modulator of PAC1-R, inducing nuclear translocation and proteolytic cleavage of the receptor to release its 24-kDa C-terminal functional fragment. This fragment directly recruits the transcription factor YY1 within the nucleus, forming a stable YY1-PAC1-R complex. This complex subsequently enhances YY1 occupancy on the *SIRT6* promoter, ultimately driving the transcriptional upregulation of *SIRT6*.

Pharmacokinetic study of SPAM1 crossing the blood-brain barrier

The blood-brain barrier serves as the natural protective barrier of

the CNS, regulating the entry of blood-derived molecules into the brain and maintaining cerebral homeostasis [36]. However, its presence also effectively restricts the transport of the vast majority of small-molecule drugs (>98%) and nearly all macromolecular drugs to the brain, posing significant obstacles to the treatment of CNS-related diseases [37].

To evaluate the blood-brain barrier penetration capacity of SPAM1, we established a rapid and sensitive LC-MS/MS method to quantitatively analyze drug concentrations in plasma and brain tissue following intraperitoneal injection of SPAM1 (100 μ mol/kg) in mice (Figure 4A for the study workflow). Representative LC-MS/MS chromatograms of the drug are presented in Figure 4B, with concentration-time curves shown in Figure 4C. Relevant pharmacokinetic parameters are summarized in Table 1. Following intraperitoneal administration, SPAM1 was rapidly absorbed into the bloodstream, reaching peak plasma concentration ($C_{\max} = 40466.67$ nmol/mL) within 10 min post-administration. The mean terminal half-life ($T_{1/2}$) was approximately 11.78 h. Notably, at the same initial sampling time point (10 min), we also detected a distinct SPAM1 signal in brain tissue ($C_{\max} = 373.50$ nmol/g). This indicates that SPAM1 rapidly penetrates brain parenchyma rather than being completely blocked by the blood-brain barrier. This early brain entry phenomenon provides a crucial foundation for evaluating its potential to cross the blood-brain barrier. Further analysis revealed that the apparent volume of distribution in brain tissue ($V_{ss} = 419.10$ g/kg) significantly exceeded its physical volume, suggesting that SPAM1 may possess specific distribution or binding characteristics within the brain. However, the total drug exposure (AUC) in the brain remained lower than that in plasma, and the elimination half-life in the brain was markedly shorter than in plasma. This suggests that drug retention in brain tissue may be influenced by dynamic equilibrium or active clearance mechanisms. Notably, within the 1- to 6-h time window post-administration, the brain-to-plasma concentration ratio (B/P) exhibited an upward trend compared to the early post-administration period (10 min to 0.5 h) (Figure 4D). This phenomenon may suggest that SPAM1 maintains a sustained, slow process of brain penetration for a period of time following the initial distribution phase. In summary, this study demonstrates that SPAM1 possesses the ability to cross the blood-brain barrier and reach brain tissue. Its rapid initial entry into the brain and subsequent sustained trend of brain penetration support this conclusion. Although the absolute brain exposure levels require improvement, this characteristic indicates that SPAM1 may possess the molecular basis for development as a central nervous system-targeted drug.

In vivo assessment of efficient brain targeting by SPAM1

Based on the brain distribution trend revealed by LC-MS/MS, this study further conducted *in vivo* analysis of SPAM1 using a live fluorescence imaging system to comprehensively evaluate its brain targeting. Time-series imaging analysis revealed that no significant fluorescence signal was detected in control mice administered an equal volume of solvent via tail vein injection, whereas SPAM1-CY5-treated mice exhibited a dynamic fluorescence intensity profile characterized by initial enhancement followed by attenuation (Figure 5). Notably, approximately 10 min after injection, specific fluorescent signals were detected in the brain regions of the treated mice, providing direct evidence that SPAM1 rapidly crosses the blood-brain barrier. This finding is fully consistent with the earlier

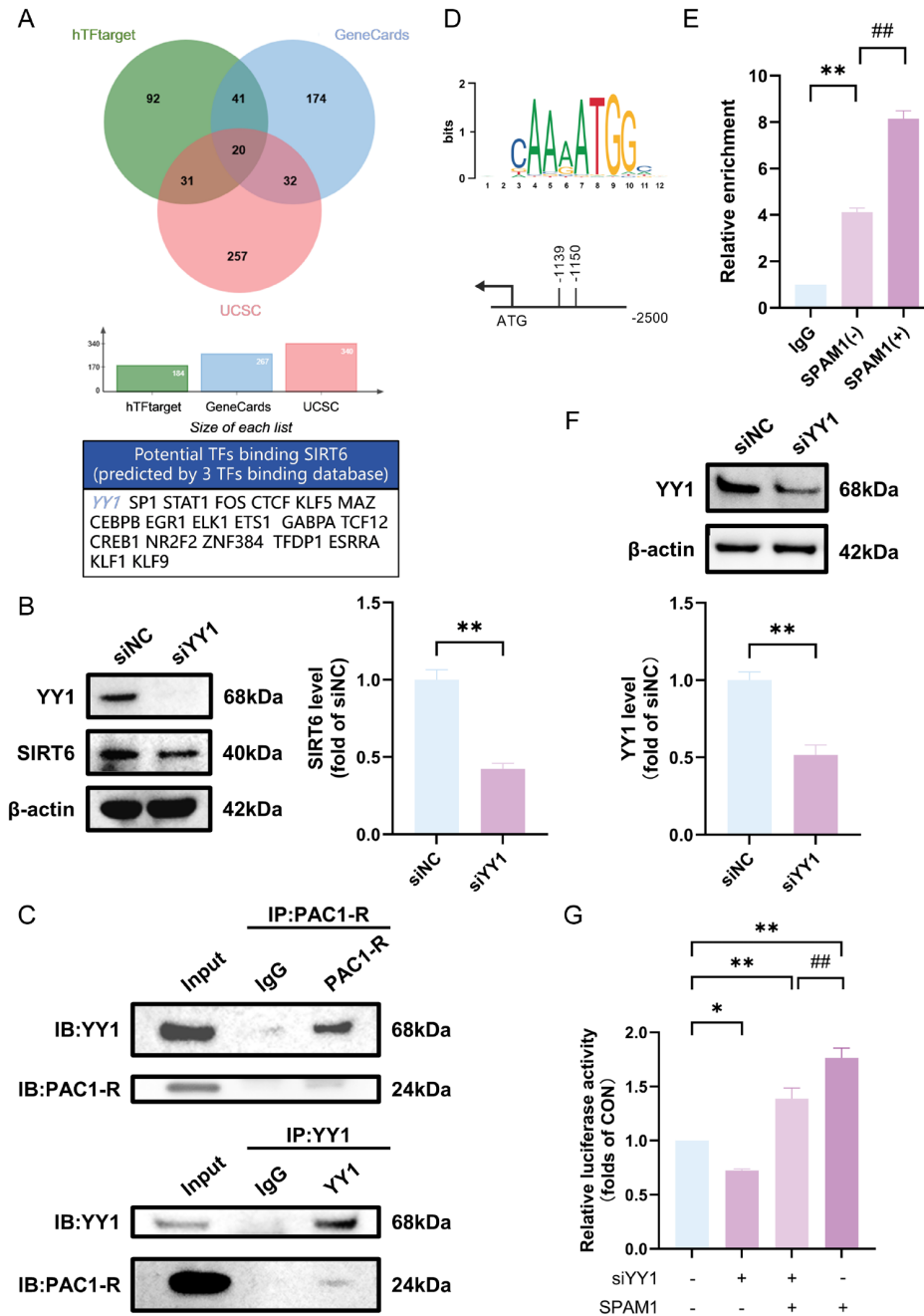


Figure 3. Molecular mechanism by which SPAM1 regulates *SIRT6* transcription via the PAC1-R/YY1 complex (A) By screening the hTFtarget, GeneCards, and UCSC JASPAR databases, 20 transcription factors were identified in the *SIRT6* promoter region. (B) Transfection of YY1 siRNA reduced YY1 protein expression in RGC-5 cells, leading to downregulated SIRT6 expression. **P < 0.01 vs siNC. (C) Co-IP analysis confirmed the formation of the nuclear YY1-PAC1-R complex: positive YY1 signals were detected upon precipitation using an antibody targeting the PAC1-R C-terminal region. Conversely, the PAC1-R C-terminal fragment (24 kDa) was identified in immunoprecipitates obtained using anti-YY1 antibody. (D) YY1 binding motifs predicted based on the JASPAR database (Top) and their potential binding site distribution on the *SIRT6* promoter (~2500 to 0 bp) (Bottom). (E) ChIP-qPCR analysis indicates that SPAM1 (1 μM) treatment enhances YY1 binding to the *SIRT6* promoter. **P < 0.01 vs IgG; ##P < 0.01, SPAM1 (-) vs SPAM1 (+). (F) YY1 knockdown efficiency (51.5% ± 6.6%) in RGC-5 cells co-transfected with siYY1 and the *SIRT6* reporter vector. **P < 0.01 vs siNC. (G) Dual luciferase reporter assays revealed that YY1 knockdown (efficiency: 51.5% ± 6.6%) not only significantly reduced the basal activity of the *SIRT6* promoter (*P < 0.05, siYY1 group vs siNC group) but also markedly inhibited SPAM1-mediated activation of the promoter (**P < 0.01, SPAM1 + siYY1 group vs SPAM1 + siNC group). However, under YY1 knockdown conditions, SPAM1-treated cells still exhibited higher promoter activity than the siNC control group (**P < 0.01, SPAM1 + siYY1 group vs siNC group). Data are presented as the mean ± SEM of three independent experiments.

LC-MS/MS detection of the drug in the brain at the same time point. Subsequently, the fluorescent signal in brain regions peaked

approximately 1 h post-injection and gradually declined thereafter, indicating the initiation of metabolic clearance. Despite the

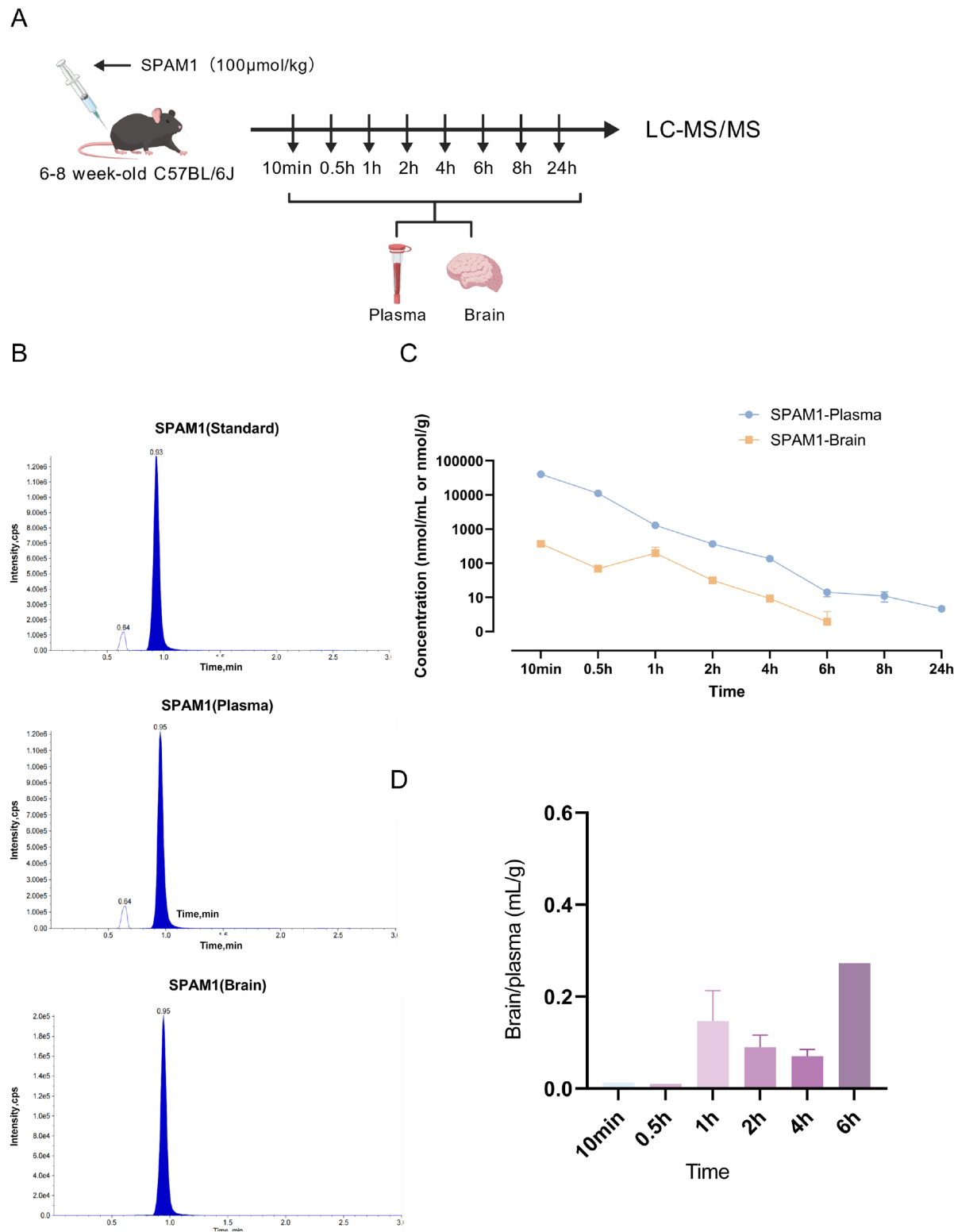


Figure 4. Quantitative analysis of SPAM1 in plasma and brain tissue by LC-MS/MS (A) Schematic diagram of the experimental workflow for detecting SPAM1 concentrations in mouse plasma and brain tissue. Male C57BL/6J mice aged 6–8 weeks received intraperitoneal injections of SPAM1 (100 μmol/kg). LC-MS/MS measurements were performed at 10 min, 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h, and 24 h post-administration. (B) HPLC chromatograms of SPAM1 in standards, plasma, and brain tissue. (C) Drug concentration-time curve in plasma and brain tissue of mice after injection of SPAM1. (D) Quantitative statistical graph of the brain/plasma concentration ratio (B/P) for SPAM1. The ratio showed an upward trend from 1 to 6 h post-administration compared to the 10 min to 0.5 h period. Data are expressed as the mean ± SEM, *n* = 3.

Table 1. Pharmacokinetic parameters following intraperitoneal injection of SPAM1 (100 μ mol/kg) in mice

Item	Plasma	Brain
T_{max} (h)	0.17	0.17
C_{max} (nmol/mL or nmol/g)	40466.67	373.50
$T_{1/2}$ (h)	11.78	0.99
AUC_{last} (h·nmol/mL or h·nmol/g)	16762.38	339.67
$AUC_{infinite}$ (h·nmol/mL or h·nmol/g)	16841.87	342.47
$AUC_{\%Extrap_Obs}$ (%)	0.47	0.82
V_{ss_obs} (mL/kg or g/kg)	100.92	419.10
CL_{obs} (mL/h/kg or g/h/kg)	5.94	292.00

C_{max} , peak plasma/brain concentration; T_{max} , time to C_{max} ; $T_{1/2}$, terminal half-life; AUC_{last} , AUC from time zero to the time of the last measurable concentration; $AUC_{infinite}$, AUC from time zero to infinite time; $AUC_{\%Extrap_Obs}$, percentage of AUC extrapolated from the last sampling time point to infinity relative to the total $AUC_{infinite}$; V_{ss_obs} , apparent volume of distribution; CL_{obs} , clearance.

continuous decline, significant residual levels remained detectable up to 12 h post-administration, ultimately returning to background levels by 24 h. This extended signal retention window of more than 10 h aligns with the tissue-binding properties suggested by the large apparent volume of distribution of SPAM1 in the brain, together confirming its prolonged retention capability within brain tissue.

In summary, *in vivo* imaging provides direct visualization and expands upon pharmacokinetic findings at the spatiotemporal dynamic level: SPAM1 not only rapidly penetrates the blood-brain barrier within the early phase (10 min) but also achieves sustained delivery within the brain for several hours and effective retention exceeding 12 h. This dual characteristic of “rapid penetration” and “long-lasting retention” provides crucial pharmacokinetic rationale and potential therapeutic time window advantages for its development as a treatment for central nervous system disorders.

Discussion

Although intracellular events such as genomic instability and mitochondrial dysfunction are core drivers of neurodegeneration [1], the regulatory mechanisms of membrane receptor-mediated

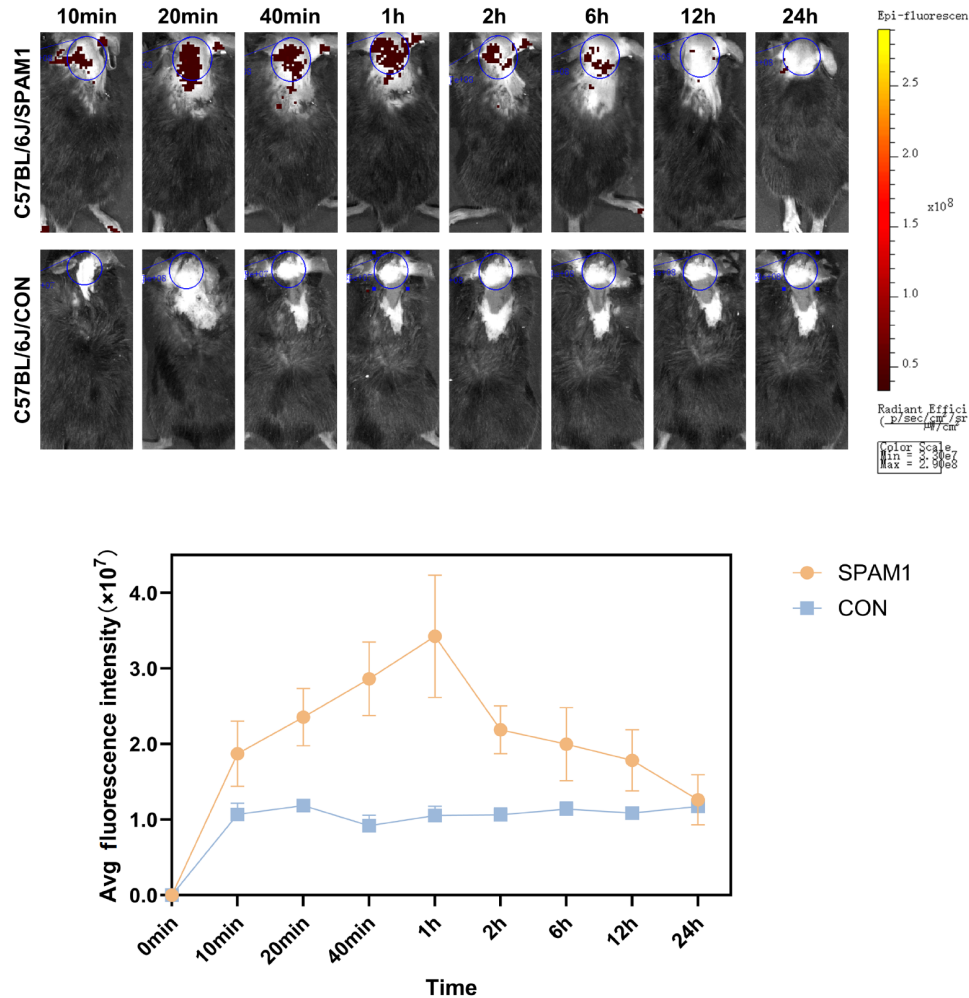


Figure 5. SPAM1 *in vivo* fluorescence imaging tracing brain targeting Time-series *in vivo* imaging of mice following SPAM1-CY5 tail vein injection (Top): from top to bottom, experimental group (SPAM1-CY5) and control group (equivalent volume solvent); from left to right: brain fluorescence distribution at 10 min, 20 min, 40 min, 1 h, 2 h, 6 h, 12 h, and 24 h post-injection. (Bottom) Average fluorescence intensity change curves for both groups at different time points. Data are expressed as the mean $\pm$ SEM, $n = 3$.

nuclear aging programs remain largely unknown. This study first revealed the cascade mechanism by which SPAM1 antagonizes neuronal aging through the PAC1-R/YY1/SIRT6 signaling axis, laying the foundation for the development of receptor-targeted anti-aging drugs. SPAM1 allosterically activates PAC1-R, inducing its nuclear translocation and release of a C-terminal fragment. This fragment recruits YY1 to form a nuclear complex, enhancing YY1 occupancy at the *SIRT6* promoter and driving *SIRT6* transcription. Concurrently, SPAM1 synergistically induces p16 downregulation and Lamin B1 expression recovery, delaying cellular senescence through multiple pathways. Finally, the study confirmed SPAM1's high efficiency in crossing the blood-brain barrier and its persistent retention properties, systematically evaluating its potential as a therapeutic target for intervening in brain aging.

Among Sirtuin family members, the nucleus-localized SIRT6 is crucial for maintaining brain homeostasis and healthy lifespan. SIRT6 deficiency induces accelerated aging phenotypes [9], including RGC loss and optic nerve degeneration [38]; brain-specific defects further lead to DNA damage accumulation, excessive tau phosphorylation, and early cognitive behavioral deficits [12]. Given that neurons, as terminally differentiated cells, possess a critical role in maintaining homeostasis within the CNS—a key focus of anti-aging research—we evaluated the intervention effects of SPAM1 in a long-term cultured (40 days) RGC-5 cell senescence model. The results demonstrated that SPAM1 not only significantly upregulated SIRT6 expression but also synergistically improved multiple aging phenotypes, including reduced SA- β -gal activity, restored Lamin B1 levels, and downregulated p16. Mechanistically, SPAM1 activates the transcriptional activity of the *SIRT6* promoter. This *in vitro* finding aligns with prior *in vivo* experiments demonstrating that SPAM1 restores SIRT6 expression in the hippocampi of aged mice [31], collectively suggesting its potential as a therapeutic strategy for SIRT6-related neurodegenerative diseases. It should be noted that while the naturally aged cell model used in this study can mimic key molecular phenotypes of aging, it remains limited in reflecting the true aging process of neurons *in vivo*. This limitation primarily stems from its inability to fully reproduce the multicellular microenvironment interactions within the brain, as well as the induction method (replicative stress) differing from the multifaceted stresses encountered *in vivo*. Nevertheless, mechanism validation at the cellular level is a necessary step to advance SPAM1 toward translational research. Therefore, subsequent work will focus on further investigating the overall neuroprotective efficacy of SPAM1 in naturally aged or neurodegenerative disease animal models.

To elucidate the transcriptional regulatory mechanism by which SPAM1 activates SIRT6, this study integrated three databases—hTFtarget, GenCards, and UCSC JASPAR—to predict potential transcription factors in the *SIRT6* promoter region. The analysis ultimately focused on YY1, a transcription factor exhibiting dual activating/inhibitory functions. YY1 plays a central role in neural development and homeostasis maintenance: complete knockout causes embryonic lethality, while heterozygous deficiency induces neurogenesis disorders and growth retardation [39]. Furthermore, YY1 is a key factor in cerebellar astrocyte maturation and functional maintenance; its absence results in chronic neuroinflammation and age-related cerebellar degeneration [40,41]. Notably, YY1 and SIRT6 exhibit functional synergy in neurodegenerative contexts: YY1 directly binds to the *SIRT6* promoter to activate its transcription, and both proteins jointly participate in numerous pathways

associated with pathological brain aging [20]. Based on the previously identified SPAM1-induced nuclear translocation of PAC1-R and ChIP-seq data suggesting its C-terminal region may bind to YY1 within the nucleus [30], we hypothesize that SPAM1 may regulate SIRT6 through this pathway. Consequently, our study reveals that SPAM1 induces the release of a ~24 kDa C-terminal fragment following PAC1-R nuclear translocation, which forms a stable complex with YY1 within the nucleus. Further ChIP-qPCR analysis revealed that SPAM1 treatment significantly enhanced YY1 enrichment in the *SIRT6* promoter region, providing chromatin-level evidence for SPAM1's promotion of YY1 transcriptional regulatory activity. Moreover, siRNA-mediated knockdown of YY1 not only reduced SIRT6 expression by 42.3%, but its partial knockdown ($51.5\% \pm 6.6\%$ efficiency) also significantly inhibited SPAM1's activation of the *SIRT6* promoter. Collectively, these findings establish YY1 as the core molecular hub regulating SIRT6 transcription within the SPAM1-PAC1-R axis. Interestingly, under YY1 partial knockdown conditions, SPAM1 still induced *SIRT6* promoter activity significantly higher than the baseline level in the control group, suggesting the potential existence of alternative regulatory mechanisms independent of YY1. We hypothesize that the PAC1-R C-terminal fragment may interact with other transcription factors in addition to recruiting YY1, or partially compensate for function through residual YY1 activity. This hypothesis is supported by several studies: KLF5 can alleviate periodontal inflammatory processes by suppressing the NF- κ B pathway through upregulating SIRT6 expression [42]; furthermore, although there is no direct evidence that the transcription factor SP1 regulates SIRT6, it has known regulatory relationships with other members of the Sirtuin family (such as SIRT1 and SIRT3) [43–45]. Collectively, these clues suggest that the PAC1-R nuclear fragment may constitute a more complex SIRT6 transcriptional regulatory network by recruiting multiple transcription factors.

We further observed dynamic changes in YY1 during the aging process: its expression significantly increased with age, positively correlating with brain pathology, indicating that YY1 overexpression reflects a compensatory response to neural imbalance [46,47]. Notably, *SIRT6* knockout triggers YY1 upregulation, suggesting a potential feedback loop between them. Crucially, while YY1 overexpression effectively elevates SIRT6 levels in young individuals, this regulatory relationship disappears in the brains of aged mice [20]. This indicates that the YY1-SIRT6 regulatory axis exhibits stage-specific imbalances during aging: in early aging, down-regulated SIRT6 expression in brain tissue triggers compensatory YY1 elevation, forming an initial protective response against neurodegeneration. However, as aging progresses, persistently high YY1 expression fails to effectively activate *SIRT6* transcription, leading to a phenomenon of “regulatory dissociation” between the two. A recent study revealed that SIRT6 promotes YY1 ubiquitination and degradation through mono-ADP-ribosylation modification, improving neuromuscular function in aged mice [48]. This feedback mechanism aligns closely with our team's prior findings: SPAM1 treatment not only upregulates SIRT6 expression in the hippocampi of aged mice but also effectively reverses the abnormal elevation of YY1 [31]. This suggests that SPAM1 may activate SIRT6, thereby attenuating the compensatory overexpression of YY1 or indirectly enhancing the YY1 degradation pathway, forming a self-limiting negative feedback loop. Combining the above evidence, this study proposes for the first time a novel mechanism of PAC1-R positive

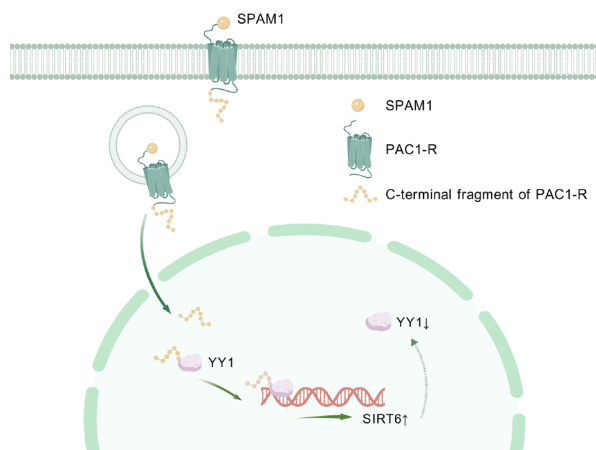


Figure 6. Molecular mechanism of SPAM1-induced nuclear translocation of PAC1-R regulating YY1/SIRT6 The binding of SPAM1 to the N-terminal extracellular domain of PAC1-R significantly triggers the nuclear translocation of PAC1-R. Subsequently, the C-terminal domain of nuclear PAC1-R recruits the transcription factor YY1, promoting YY1 occupancy on the *SIRT6* promoter to enhance promoter activity, which then feedback-regulates YY1 expression.

allosteric regulation mediating resistance to neuronal senescence (Figure 6): SPAM1 induces nuclear translocation of PAC1-R, which recruits YY1 to upregulate SIRT6, followed by feedback-mediated downregulation of YY1 expression. These findings suggest that SPAM1 may intervene in the regulatory dissociation between YY1 and SIRT6 during late-stage aging, thereby enhancing the capacity of the aging brain to maintain neuronal homeostasis. This result provides a novel pharmacological basis for developing SPAM1 as a candidate drug to prevent and treat age-related CNS decline.

Blood-brain barrier, a complex structure composed of brain capillary endothelial cells, pericytes, and astrocytes, forms a multilayered defense mechanism through tight junctions, efflux pumps, and metabolic enzymes. While protecting the CNS from exogenous toxins, it also severely restricts the delivery of the vast majority of therapeutic drugs (>98% of small molecules) to the brain [36,49]. This study, integrating LC-MS/MS quantitative analysis with *in vivo* fluorescence imaging, first demonstrated SPAM1's highly efficient brain targeting that overcomes blood-brain barrier limitations: LC-MS/MS quantification revealed that SPAM1 reached peak concentrations in brain tissue and plasma 10 min after intraperitoneal injection, with sustained and stable brain accumulation between 1 h and 6 h. Fluorescence imaging further demonstrated its spatiotemporal distribution advantage, with brain region signals detectable as early as 10 min post-injection and retention exceeding 12 h. Together, these findings confirm the dynamic characteristics of SPAM1 accumulation and metabolic clearance within brain tissue. Based on SPAM1's molecular properties (small molecular weight of 365.4 Da) and *in vivo* distribution data, it possesses triple potential for developing orally administered brain-targeted drugs: efficient gastrointestinal absorption, active brain penetration, and prolonged brain retention. This discovery provides a molecular foundation for simplifying drug delivery strategies for neurological disorders. To advance translational application, subsequent studies will focus on evaluating the oral bioavailability and brain targeting efficiency of SPAM1. Its preventive and therapeutic effects on age-related decline in the CNS following oral administration will be assessed in both naturally aging and accelerated aging

animal models. This comprehensive evaluation will determine the developmental feasibility of SPAM1 as a novel anti-brain-aging drug candidate.

SPAM1, as a novel positive allosteric modulator targeting PAC1-R, demonstrates multifaceted anti-neuroaging effects. It can simulate endogenous stress response programs without inducing actual tissue damage, exerting central neuroprotective and restorative effects through multiple mechanisms including upregulating SIRT6, downregulating YY1, and enhancing PACAP-PAC1-R system activity [29,31]. Preliminary animal studies in D-galactose-induced brain aging models have confirmed its *in vivo* neuroprotective efficacy. At the omics level, its effects extend beyond the classical PACAP-PAC1-R pathway, demonstrating broad associations with longevity and aging regulatory networks [31]. This study further confirms that SPAM1 enhances SIRT6 transcriptional activation via the PAC1-R/YY1 axis and possesses stable blood-brain barrier crossing capabilities. SPAM1 bridges the gap from well-defined mechanisms to effective intracerebral delivery, demonstrating promising development prospects as a candidate drug for preventing and treating brain aging disorders.

In summary, this study not only reveals the molecular mechanism by which SPAM1 antagonizes neuronal aging through the PAC1-R/YY1/SIRT6 signaling axis by upregulating SIRT6 expression but also elucidates its ability to efficiently cross the blood-brain barrier and exhibit prolonged retention within the brain. This provides a pharmacological and pharmacodynamic foundation for the development of SPAM1 as a therapeutic agent for anti-aging in the central nervous system.

Data Availability

All data supporting the findings of this study, including raw data and original images, are available from the corresponding author upon reasonable request.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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