Polycyclitol derivatives restore long-term memory by regulating cdk5/p25 based tau signaling in experimental cerebral malaria

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Abstract

Tau hyperphosphorylation at Ser396/404 and its adverse neurological effects have been evident in animal models of cerebral malaria (CM). As a counter measure, quest for novel pharmacological therapeutics to ameliorate tau hyperphosphorylation in neurodegeneration and restore behavioural and cognitive functions with high efficacy in CM has been at the forefront of neurobiological studies. In this study, using experimental model of cerebral malaria (ECM), we administered four different polycyclitol derivatives, SR4 (01-04) as an adjunctive to ARM therapy resulting in alleviation of cdk5/p25 based tau signaling cascade and restoration of long-term memory. Limitations of scyllo-inositol and rational to synthesize these polycyclitols efficiently has also been captured in the backdrop. Initially, we studied long-term, short term memory and novelty based learning by conducting Barnes maze, T-maze and novel object recognition task in treated animal groups. The cognitive outcomes of SR4-02 (15) and SR4-04 (18) treated groups exhibited better learning and memory compared to SR4-01 (16) and SR4-03 (17) groups. We further evaluated cdk5/p25 and tau phosphorylation protein expression using western blotting, immunohistochemistry and Golgi-cox staining to study neuronal arborization pattern. Immunohistochemical analysis of hippocampal and cortical tissue regions showed reduced phospho tau expression in SR4-03 (17) and SR4-04 (18) groups compared to CM group. Similarly, Golgi-cox images showed increased neuronal density in Cornus Ammonis (CA1) and CA3 regions of hippocampus and cortex of SR4-02 (15), SR4-03 (17) and SR4-04 (18) treated mice. Overall, based on our findings, polycyclitol derivatives have the potential to alleviate tau levels and restore cognition in ECM.

Introduction:

Cerebral malaria (CM) is a fatal form of malaria caused by the infection of protozoan parasite, *Plasmodium falciparum* transferred by a female anopheles mosquito bite. More precisely, CM is a multi-organ disease severely damaging cerebrovasculature and compromises blood-brain barrier (BBB) integrity, resulting in neurological sequelae. In sub-Saharan Africa, children under five years of age are primarily affected with CM, reporting long-term neurological complications (Muppidi et al., 2023). Approximately, 25% of survivors develop neurocognitive and behavioral sequelae (Bruneel, 2019). One of the salient features of CM is leakage of blood brain barrier followed by progressive atrophy of neurons, affecting molecular, cellular and histological functions which in turn hamper the learning and memory functions of the brain. Artemether (ARM) is well known anti-malarial drug used as a therapeutic agent against severe forms of malaria. Despite its effective anti-malarial response, survivors of CM suffer from severe cognitive decline especially with spatial memory deficits (Dai et al., 2010; Reis et al., 2010). Several reports state that administration of ARM can eradicate parasite, improves survivability but cannot alleviate cognitive impairment (Gallego-Delgado et al., 2016; Pena et al., 2012; Serghides et al., 2014). Therefore, previous reports comply with adjunctive therapy (additional therapy that modulates the metabolic pathways) along with ARM for restoration of cognition in

experimental models of CM (Dai et al., 2012a; John et al., 2010; Reis et al., 2010). In the neurodegenerative setting, neuronal loss reduces regional neuronal plasticity in critical cognitive domains such as the cortex and hippocampus, further leading to executive dysfunction of planning, working and spatial memory (McIsaac et al., 2018). Cornus Ammonis (CA) regions are densely packed with pyramidal neurons in hippocampus. CA1 is one of the critical regions which regulates memory consolidation and acts as a bridge for relaying information between hippocampus and sub-cortical areas (Basu and Siegelbaum, 2015; Van Strien et al., 2009). Dentate gyrus (DG), a region in hippocampus which consists of granule cells and long projecting neurons in the hilus region that receives information from entorhinal cortex and sends excitatory information to CA3 region through mossy fibers (Andersen et al., 2006; Jonas and Lisman, 2014). CA3 region is known for retrieval of spatial pattern information during short-term memory tasks including working memory, novelty and one-trial experiments (Kesner, 2007). Spatial memory impairment is dependent on two critical events i.e. Cdk5 overexpression and tau hyperphosphorylation in hippocampus.

Cdk5 is a serine or threonine protein kinase involved in cell cycle progression, ubiquitously expressed in most mammalian tissues, especially in the brain (Kanungo et al., 2009; Tsai et al., 1993). Cdk5 and its activator p35 are vital for maintaining the neuronal activity, migration and growth of neurites thereby regulating neuronal morphology (Dhavan and Tsai, 2001). p25 is the cleaved product of p35 which hyperactivates Cdk5, forming cdk5/p25 complex which phosphorylates tau at Ser396 and Thr231 thereby reducing microtubule assembly in disease states (Patrick et al., 1999; Zheng et al., 2010). Most of the studies have shown a significant accumulation of phospho tau Ser396 in the hippocampus which is critical for long-term depression but not long-term potentiation (Regan et al., 2015; Taylor et al., 2021). Currently, the advanced state of therapy against tau accumulation is immunotherapy using humanized antibodies targeting the amino and carboxy terminus, bound to proline rich or microtubule domains in the neurons. As the strategy of inhibiting Aβ aggregation and hyper phosphorylation of tau has increasingly gained acceptance, greater numbers of inhibitors have been developed and their structure-activity relationship was also explored. These small molecules hold considerable promise as the starting point for the development of new therapies related to Alzheimer's disease (Cummings et al., 2020; Malek et al., 2019). Small organic molecules such as polyphenols like cyanidin (**1**), emodin (**2**) and epigallocatechin gallate (EGCG, **3**), anthraquinone antibiotic daunorubicin (**4**), imidazole derived sulfone (**5**) and carbohydrate related scyllo-inositol (**6**), Fig. 1. have shown substantial potential to disaggregate Alzheimer's disease tissue derived Amyloid-β and tau fibrils in various studies (Bulic et al., 2010; Seidler et al., 2022).

At present about 20% of molecules undergoing clinical trials for AD target Aβ and Tau proteins. Reduction of Aβ aggregation and addressing the tau hyperphosphorylation or the removal of already formed deposits of misfolded proteins has remained the focus of recent research. As a result, several protein aggregation inhibitors, antibodies, and enzyme inhibitors have been investigated (Loera-Valencia et al., 2019). The concept of dual inhibitors, Aβ and tau aggregation inhibitors is also emerging as one of the actively investigated research areas in this domain. For instance, a curcumin derivative, PE859, acts as a dual aggregation inhibitor for restoring the cognitive dysfunction in mouse model (Okuda et al., 2017). Recently, Wieckowska et al. discovered several 1,3-aminoalcohol and indole based compounds as potential dual inhibitors of Aβ and tau aggregation with Aβ disintegrating potential (Szałaj et al., 2020). These studies prompted us to revisit one of the important molecules,*scyllo* -inositol (**6**), discovered several years ago as one of the potential Aβ inhibitors. Evaluation of the*scyllo* -inositol as a potential therapeutic has confirmed the blocking of fibril formation through its direct interaction with the target peptide which eventually results in a pronounced change in the secondary structure and further stabilization of the mono- and oligomeric units of otherwise normal protein. These studies prompted many research groups and industries to devise strategies towards the diversity-oriented synthesis of inositol analogues and probe their molecular interactions with the aggregation cascade of concerned proteins. Keeping in view these observations, there is a scope for more rational efforts in this direction which can address the issues of weak binding interactions and non-specificity of *scyllo* -inositol.

In this article, using the experimental model for CM, we administered SR4-01 to SR4-04 polycyclitol compounds synthesized recently as an adjunctive along with the ARM. After 30 day survivability period, we

studied the expression of cdk5, p35/p25, phospho tau Ser396 in the whole brains of all the experimental groups. Our outcome shows significant improvement in the neuronal morphology with decreased tau hyperphosphorylation levels in the animals that survived after rescue treatment, restoring learning and memory functions especially with SR4-02 and SR4-04, which might have tremendous implications in AD and ECM therapeutics.

Materials and Methods

Ethics Statement: All animal experiments have been conducted after the approval from the Institutional Animal Ethics Committee (UH/IAEC/PPB2014-I/68), University of Hyderabad, India.

2.1 Experimental groups:

Total animals of 30 male and female C57BL/6 mice of 3-4 weeks old (10-15 g) were purchased from the National Institute of Nutrition, Hyderabad. All animals were maintained at appropriate 12 hour light and dark condition and fed with animal feed and sterile water *ad libitum*in the animal house facility, at the University at Hyderabad. Animals were infected with *Plasmodium berghei ANKA* (PbA), intraperitoneally (i.p.) at a concentration of 10^6, a parasite strain that reiterates the human symptoms of CM on day 5-9. Based on the Rapid Murine Coma Behavior scale (RMCBS), we administered the dosage of 3mg/kg of polycyclitol dissolved in sterile water along with ARM (25mg/Kg) dissolved in Arachis oil to animals symptomatic to CM i.p., once per 7 days. Animals infected with PbA, treated and controls were subjected to rapid murine coma behavior score (RMCBS), which is a video based evaluation of symptoms of CM and assessment for rescue drug therapy based on 10 neurobehavioural parameters (Carroll et al., 2010). Out of 30 animals, 26 animals were symptomatic to CM and administered with ARM and SR4-(01-04) (n = 6 animals per group) for 6 days and observed for survivability period of 30 days.

Cognitive tests: We performed cognitive tasks relevant to working and reference memory in all the experimental animals after day 30 in all the treated and control groups. The experimental data was analyzed by a group of blinded researchers using ANYmaze software version 6.0.

Barnes maze

This experiment is performed to evaluate the spatial long-term memory in the rodents. This test consists of a circular platform with 20 equidistantly spaced holes along its perimeter (100 cm in diameter). An escape platform was placed under one of the holes leaving the rest empty. Each animal was guided from the center of the maze to detect escape platform for 4 minutes per session up to 4 days (acquisition phase). The maze and the escape platform were cleaned with 70 % alcohol following each trial. Animals were subjected to probe trial after removing the escape platform on day 5. The time to detect the escape platform (primary latency) and the number of holes entered before primary latency (primary error) were recorded. The mice were video-recorded and tested individually with the ANY-maze behavioural tracking software version 6.0, Stoelting Co, Wood Dale, USA.

T-maze test

Mice were subjected to T-maze consisting of three arms which are left and right sided goal arms measuring 30 cm (diameter) × 15 cm (height) and a start arm of 40 cm (diameter) × 15 cm (height). A forced choice of spontaneous alternation was selected where each mouse was gently placed in the start arm for 3 minutes for include references. The mouse was placed in the start arm of the maze after blocking any one side of the arm. The mouse is forced to explore the L-shaped maze for 5 minutes (acquisition phase). The mouse was placed back in its home cage for 15 minutes time duration. The maze was cleaned thoroughly with 70 % alcohol to remove olfactory cues in the area. During the test phase, the blockage in the arm was removed, and mouse was placed in the start arm and observed for its entry to the arm not visited previously (correct alternation) (test phase). Mouse exploring the arm visited previously during the test phase is considered as wrong alternation. Each mouse was subjected to 6 trials per day for four days to study the "correct alternation" and "wrong alternation." The percentage of correct alternation per animal with side preference rate (actively adapt to one side of the arm) was calculated and compared among the groups.

One-trial novel object recognition test

On day 1, a mouse was placed in an empty square-shaped box made of transparent glass material (dimensions: 30 × 30 × 30 cm) for 20 minutes (habituation phase). The mouse was removed from the arena and placed back in its home cage. The box was cleaned with 70% alcohol. On day 2, two identical objects were placed 5 cm away from the walls. The same mouse was placed in the box for 5 minutes (familiarization phase). Mouse was placed back in its home cage. The walls of the box along with the identical objects were cleaned thoroughly with 70% alcohol. One of the identical objects was replaced with a novel object with a different shape and color in the same position. After 60 minutes, the same mouse was placed in the center of the arena for 5 minutes (test phase). The total time spent by the subject interacting with both the identical objects was recorded in the familiarization and novel object in test phase by sniffing, pawing within a distance of 2 cm.

Golgi cox staining

Golgi cox staining is one of the gold standard methods to study neuronal structure and its arborization. The brains collected from treated and control group and CM were subjected to Golgi cox stain (5% Potassium Dichromate, 5% Mercuric Chloride (sublimate) and 5% Solution of Potassium Chromate were added to distilled water). All the brain samples were stored in Golgi cox solution at room temperature in dark condition for 17 days. All the brains were sectioned at 200 µm and developed according to the protocol designed by Zaquot and Kaindl (Zaqout and Kaindl, 2016). Further, we quantified dendritic length of cortical and hippocampal neurons from Golgi impregnated neurons. The length of eighteen proximal and distal dendrites were measured in hippocampal and cortical Golgi impregnated neurons (n =13 neurons per group) of all the experimental groups. The broken line tool of Image J software was used to measure the dendritic length.

Preparation of brain tissue lysates

Tissues (100 mg) of all the experimental groups were homogenized using Dounce homogenizer with 8 to 10 strokes at 4 °C in Radio-Immunoprecipitation Buffer (RIPA) (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and pH 8). Protease and Phosphatase inhibitors (P8340, P0044 Sigma-Aldrich) (10 µl per 1 ml of lysate) and 1mM phenylmethylsulphonyl fluoride (PMSF) were added to the homogenate. The homogenate was centrifuged at 12,000 rpm for 15 minutes at 4 °C using refrigerated centrifuge. The supernatant was frozen upon collection and stored at – 80 °C in a freezer.

Western blotting

Protein estimation was carried out by Bradford reagent (B6916 Sigma-Aldrich). 50 µg of the protein was resolved by 12% sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE). The gel was transferred onto nitrocellulose membrane (Protran Amersham GE) in Transfer buffer (Tris-HCl- 3 g, glycine-14.4 g, deionized water- 800 ml, methanol- 200 ml, pH 8.3) overnight for 4 °C. The membrane was washed with Tris-buffered saline (TBS) containing 0.05% Tween 20 for 5 minutes and blocked with 5% skimmed milk for 1 hour followed by incubation with the primary antibodies of pCDK5 (sc-377558) from Santa Cruz Biotechnology, CDK5 (sc-6247) from Santa Cruz Biotechnology, p35/25 (C64B10) from Cell signal technology, phospho Tau Ser396 (9632S) from Cell Signal Technology and GAPDH (D16H11) from Cell signal technology overnight at 4 °C. The membrane was washed with TBS and incubated with the anti-mouse and anti-rabbit secondary antibody conjugated to HRP for 2 hours at room temperature. Chemiluminescence signal against HRP conjugated secondary antibodies were analysed by adding Luminol and peroxide. Densitometric analysis of all the protein expression data, normalised with the loading control (GAPDH) was performed using Image J software (version, NIH, USA) and the graphs were plotted using Graph pad prism software.

Immunohistochemistry

Brain samples perfused with 4% paraformaldehyde were subjected to sectioning at 10-15 µm thickness. Brain sections were quenched using 3% hydrogen peroxide in methanol for inhibiting endogenous peroxidase activity. All the sections were blocked by 5% normal goat serum followed by primary antibody Phospho-Tau

(Ser396) at a concentration of 1:1000 overnight at 4 °C. Polydector solution provided in the kit was added to the sections for an hour and covered in DAB (3'-3' diaminobenzidine) buffer for 5 minutes. Hematoxylin was used as a counter stain for observing the nuclear staining and sections were mounted with DPX mountant. Images were captured at 400 X magnification using Leica trinocular DM6B microscope with Leica Application Suite X (LAS X) software.

Statistical Analysis

All the densitometric analysis for Western blots and immunohistochemistry images were quantified using Graph Pad Prism version 5.03 software. The dendritic lengths from Golgi-impregnated images were quantified using Image J software. Statistical differences of all the experimental groups were calculated by the one-way ANOVA with Student Newman-Kuels test using Graph Pad Prism software 5. The ***p values ($p < 0.001$ & $p < 0.005$) were considered as significant. All the results were represented as mean ±standard error mean.

Results

Synthesis of polycyclitols

In order to understand and address the issues of weak and non-specific binding of *scyllo* -inositol with the target proteins, more efforts are required to be channelized in this direction. One possible way-out is to keep the primary backbone of inositol intact and increase the bulk around the molecule by either stitching certain bulky substitutions or more reliably create some related molecules through incisive synthetic interventions like ring annulated conduritol **7** , annulated inositols (**8** -**10**) or even fully functionalized inosito-inositols (**11**-**12**), Fig. 2. Our synthetic endeavour was envisioned from the diepoxide (**13**) which in turn was synthesized from commercially available naphthalene through a well-established reaction sequence (Rashid et al., 2020). Stereo- and regioselective opening of α-epoxide ring in (**13**) under mild acidic conditions led to epoxydiol (**14**). Cis-dihydroxylation of the double bonds with OsO_4/NMMO and base mediated carbonate deprotection furnished the annulated inositol (**15**). Under stronger acidic conditions and extended reaction times, both the epoxide rings of diepoxide (**13**) cleaved regioselectively to furnish tricyclic tetrol (**16**). Concurrent dihydroxylation of both the double bonds resulted in the carbonate protected octol (**17**). Deprotection of the carbonate group in (**17**) delivered the target inositol-inositol (**18**) in excellent yield (Fig 3).

SR4 (1-4) derivatives as adjunct therapy to ARM, restored neurobehavioral parameters in ECM

RMCBS is behaviour scale for evaluating the symptoms of CM (Carroll et al., 2010). Animals post infection with PbA exhibited a decreasing trend in the RMCBS score from day 5. Animals symptomatic to CM showed crouching, piloerection, seizures, and retinal haemorrhage on day 7 with RMCBS score of 6.33±0.88. We observed a significant increase in total RMCBS scores in animals administered with polycyclitols (SR4-01, 02, 03, 04) along with ARM (SR4-01; day 5 (16.33±0.66), day 6 (12.66±1.45), day 7 (11.00±1.15), day 8 (14.33±0.33), day 9 (17.33±0.33) and day 10 (17.00±0.57) SR4-02; day 5 (16.66±0.33), day 6 (12.66±0.88), day 7 (16.0±0.57), day 8 (18±0), day 9 (17±0.57) and day 10 (17.66±0.33), SR4-03; day 5 (17.0±0.57), day 6 (14.0±0.57), day 7 (12.0±0.57), day 8 (16.0±0.57), day 9 (16.3±0.88) and day 10 (17.33±0.33) SR4-04; day 5 (16.33±0.33), day 6 (15.0±0.57), day 7 (12.66±0.33), day 8 (16.66±0.33), day 9 (17.66±0.33) and day 10 (16.33±0.33) ($p < 0.001$) compared to CM group (Fig 4A). All SR4 (01-04) adjunctive treated animals showed better locomotion and rearing behavior with an increasing trend from day 8 to 10. After day 30 of adjunctive therapy, SR4-03 exhibited a significant increase in the survival rate of 66 percent ($p < 0.001$) compared to SR4 (01, 02 and 04) treated animals (50 percent) (Fig 4B). Few rescue treated animals died during the survivability phase. Animals were subjected to cognitive tests after day 30 (Fig 4C). As per the guidelines, a 4-5 day interval period was maintained between each cognitive test to minimize error rate.

Polycyclitol adjunctives restored cognitive function in ECM

Accumulation of hyperphosphorylated tau Ser396 in hippocampal CA1 region is associated with inducing neurotoxicity, promoting spatial learning and memory dysfunction correlating to cognitive decline (Hyman et al., 1990; Regalado-Reyes et al., 2019; Scheff et al., 2006; West et al., 2004). We observed that SR4-02

and SR4-04 treated animals performed primary latency significantly within a shorter time interval (SR4-02; 27±3.42 sec on day 1, 19.0±6.3 sec on day 2, 16.23±7.3 sec on day 3, 9.9±6.89 sec on day 4 and 7.32±7.46 sec on day 5 (probe trail), SR4-04; 35.57±8.6 sec on day 1, 31.74±7.8 sec on day 2, 22.45±6.8 sec on day 3, 16.75±7.2 sec on day 4 and 13.56±7.2 sec on day 5 (probe trail)) ($p<0.001$) compared to SR4-01 and SR4-03 ($p<0.001$) (45.0±5.56 sec on day 1, 40.0±7.96 sec on day 2, 51.6±6.21 sec on day 3, 43.87±7.9 on day 4 and 46.78±7.43 sec on day 5 (probe trail)) and SR4-03 (68.00±8.56 sec on day 1, 54±7.89 sec on day 2, 22.45±6.8 sec on day 3, 16.75±7.2 sec on day 4 and 13.56±7.2 sec on day 5 (probe trail)) in the Barnes Maze experiment (Fig 5A). The error rate on probe trial day was significantly decreased in SR4-02 (1.8±0.01) ($p<0.001$) and 04 treated groups (1.9±0.2) compared to SR4-01 (19.3±3.2) and SR4-03 (3.45±2.45). There was no significance in the error rate between SR4-03 and SR4-04 ($p=0.593$). Frozen behavior was observed especially in SR4-03 treated group (Fig 5 B and D).The heat maps and track plots show that SR4-02 and SR4-04 treated groups spent more time near the escape platform than SR4-01 and SR4-03 (Fig-5 C and E). The CON group was taken as a reference which exhibited primary latency at the earliest time point (18.12±1.2 sec on day 1, 14.12±4.10 sec on day 2, 7.86±3.8 sec on day 3, 4.12±2.89 sec on day 4 and 6.17±0.98 sec on day 5 (probe trail) with a negligible error rate (0.12±0.01). Overall, SR4-02 and SR4-04 experimental groups exhibited a better spatial reference memory with low error rate compared to SR4-01 and SR4-03. In retrieval phase of novel-object recognition test, the track plots revealed that SR4-02 treated group showed a significant increase in the time spent with the novel object (SR4-02: 147±2.5 sec) to the rest of the groups (SR4-04:140±0.5 sec ($p=0.044$), SR4-03: 127.5±7.5 sec ($p<0.001$) and SR4-01: 137.5±7.5 sec ($p=0.022$).We observed that SR4-02 group significantly spent least time with the known object compared to (SR4-01:130.0±10 ($p<0.001$), SR4-03:112.5±2.5 sec ($p=0.001$), SR4-04: 109±4.5 ($p=0.002$) (Fig 5 F and G) in the novel object recognition test. Control animals were used as a reference which showed both novelty and spatial memory at its maximum (155±5 sec). The track plot represents the correct and wrong alternation in spontaneous alternation of T-maze experiment (Fig 5H). Both SR4-02 (43.33±4.4) and SR4-04 treated group (43.33±3.3) exhibited better working memory in T-maze experiment close to 50% correct alternation rate with no significance ($P=0.083$) compared to the rest of the groups SR4-03 (38.33±4.4) and SR4-01 (40.33±2.8) (Fig 5I). Based on the outcomes of the all the cognitive tests conducted, SR4-02 and SR4-04 treated mice have shown a significant improvement in learning and memory functions.

Polycyclitol adjunctives alleviated cdk5/p25 based tau hyperphosphorylation

After performing the cognitive tests, we euthanized experimental animals, collected the whole brain samples and stored at -80^{o}C. Outcomes of IHC staining show a significant decrease in phosphorylated tau at Ser396 inclusions in cortical ***$p<0.001$ (SR4-01; 37.67± 2.728, SR4-02; 55.33±1.764, SR4-03; 31.67± 3.383, SR4-04; 39.67± 2.728) (Fig 6 A and B) and hippocampal regions (SR4-01; 40.33±1.453, SR4-02; 29±2.082, SR4-03; 28.67±1.202, SR4-04; 37±1.528) (Fig 6 A and C) of all the treated groups compared to cortical areas with scattered tau expression (133±3.512) (Fig 6 A and B), superficial, medial entorhinal cortex of CA1 and DG in hippocampal brain regions (89.33± 5.812) in CM group (Fig 6 A and C). Overall, of all the adjunctive therapies, SR4-02 group exhibited a significant downregulation of phospho tau at Ser396 in hippocampal region; whereas SR4-03 exhibited a significant decrease of phospho tau Ser396 expression in cortical regions of CM infected animals. We found excessive deposition of tau inclusions in hilar region of CM. We also identified loss of hippocampal neuronal density especially pyramidal neurons of CA1 and CA3 regions in Golgi impregnated brain sections (Fig 7A). Dendritic arborization fulfils specialized circuit functions during cognitive processes. According to anatomical features, dendrites are categorized as distal and proximal. We observed that length of distal and proximal dendritic lengths of hippocampal neurons was significantly restored in SR4-02; distal:13.5 µm±0.41, proximal:13±0 and SR4-04 group; distal:13±0, proximal: 13±0.27 (***$p<0.001$) compared to rest of the treated groups (SR4-01;distal:9.5±0.139, proximal:8.0µm±0.27, SR4-03;distal:8.5µm±0.13, proximal:10µm±0.27) and CM group (distal:5.5µm±0.13, proximal: 3.5µm ±0.13) (Fig 7 B). Similarly, distal and proximal dendritic lengths of cortical neurons of SR4-02 (distal: 16.0µm ±0.13, proximal: 19.0µm ±0.27) SR4-04 (distal: 17.5µm ±0.13, proximal: 14.5µm ±0.13) (***$p<0.001$) was significantly arborized compared to SR4-01 (distal: 10.5µm ±0.13, proximal: 8.5±0.13) and SR4-03 groups (distal: 7.5±0.13, proximal:11.5µm ±0.13) (Fig 7 C). Western blot data showed a significant reduction in the expression of phospho tau Ser396

in SR4-03 (***p<0.001) (142.18±0.06) and SR4-04 (132.74±0.07) compared to SR4-01 (663.95±0.11) and SR4-02 (333.93±0.08) treated and CM group (2244.84±0.10). Previously our group has shown overexpression of cdk5 and phospho cdk5 at Ser159 (Czapski et al., 2016) during p25 generation in ECM (Kumar and Babu, 2020). Total form of cdk5 expression was significantly reduced in SR4-02 (0.368±0.46), SR4-03 (0.366±0.21) and SR4-04 (0.61±0.0.06) (***p<0.001) compared to CM (1.166±0.10). We observed significant reduction in expression of the activated form of cdk5; phospho Ser159 in SR4-03 (9.15±0.11) and SR4-04 (6.85±0.01) (***p<0,001) compared to SR4-01 (16.0±0.05) and SR4-02 (16.52±0.07). As expected, cdk5 was overexpressed in CM (22.82±0.01). We observed that there was no significance in cdk5 expression between SR4-01(1.037±0.05) and CON group (1.0±0.07). Excessive activation of cdk5 leads to degradation of p35 to p25. Our findings showed that p35 expression was significantly reduced in CM (***p<0.001, 0.32±0.23) and all the adjunctive treated groups show similar restoration of p35 protein (SR4-01: 1.174±0.11; SR4-02: 1.30±0.06; SR4-03: 1.191±0.15 and SR4-04: 1.279±0.10). Further, we found that the cleaved product of p25 was overexpressed in CM (2.54±0.10) and was significantly reduced in SR4-04 (0.068±0.1) (***p<0.001) compared to rest of treated groups (SR4-01: 2.438±0.11; SR4-02: 2.071±0.07; SR4-03: 0.591±0.3). The expression pattern of all the above proteins was quantified after normalization with GAPDH (Fig 8).

Discussion

Tau pathology is reported to be a biomarker for cognitive impairment involving cerebral ischemia and neuroinflammation in the early and late stages of AD (Ballatore et al., 2007; Freude et al., 2005; Iqbal et al., 2005; Kim et al., 2009; Mandelkow et al., 1996). Children less than five years old infected with CM exhibited axonal injury caused by increased plasma tau levels resulting in cognitive impairment and severe mortality (Datta et al., 2021; Medana et al., 2007). A recent study by Oscar B et al. states that dysregulation of tau protein contributes to cerebral vasculopathy and neuronal cell injury impairing cognitive outcomes during CM (Akide Ndunge et al., 2023). According to the Minxian Dai et al., aberrant phosphorylation of tau increased the disease severity by inducing conformational changes in neuronal proteins in experimental models of CM (Dai et al., 2012b). C57BL/6 mice infected with*Plasmodium berghei* ANKA is widely known to reiterate the symptoms of human cerebral malaria. Despite several transgenic models for tau, ECM pathology globally activates tau without transgenic tau approach. Tau hyperphosphorylation is propelled by several kinases including cdk5, glycogen synthase kinase 3 (GSK3β) and mitogen-activated protein kinase (MAPK) (Hatch et al., 2017). Previously, one of our recent studies showed a dysregulation of cdk5 signaling, imbalance between kinases (Ca^{2+}/calmodulin-dependent protein kinase II alpha, CaMKIIα, protein kinase A, cdk5) and phosphatases (protein phosphatase-1 gamma subunit 1, PP-1γ1) in ECM. Based on our previous studies and several reports on vasculopathy based tau hyperphosphorylation, we selected the animal model for CM as an effective model for this study.

Sequestration of infected RBCs cause microvasculature damage inducing chronic hypoxia mediated elevation of VEGF (Vascular Endothelial Growth Factor) which results in expression of calcium-dependent proteases, calpain (Rénia et al., 2012). Chronic hypoxic conditions in the brain are detected in the form of elevated Hypoxia-inducible factor (HIF)-1α levels in terminally ill CM animals and human cerebral malaria (Hempel et al., 2014). Low oxygen levels trigger activation of calpain and several kinases such as mitogen-activated protein kinases (MAPKs) and extracellular signal regulated kinases (ERK1/2) activating brain-derived neurotrophic factor (BDNF) (Gao et al., 2013; Raz et al., 2019; Terraneo and Samaja, 2017). Finally, all the above conditions lead to the imbalance of kinase and phosphatases resulting in tau phosphorylation. We assume that altered vasculature, reduced blood flow and chronic hypoxia upregulate cdk5, one of the key factors in phosphorylating tau at several sites such as Ser202, Ser396/404, Thr181and Thr231 (Noble et al., 2003) in CM. Tau phosphorylation at Ser396 is considered as the earliest event in Alzheimer's disease in which cdk5 has been implicated as potential candidate kinase (Mondragón-Rodríguez et al., 2014; Noble et al., 2003). Similarly, cdk5/p25 signaling is detected early in neurodegenerative diseases like Parkinson's (Ao et al., 2022), amylotrophic lateral sclerosis (ALS) (Bk et al., 2019), brain injuries like subarachnoid haemorrhage (Ding et al., 2022), anxiety, depression (Takahashi et al., 2022), learning disabilities involving cognitive impairment (Kamiki et al., 2018) and cancers like glioblastoma (GBM) (Peyressatre et al., 2020). Several research studies have shown that inhibition of hyperactivated cdk5 based signaling improved hippocampal

neurogenesis and restored cognitive functions in radiation induced cognitive dysfunction (Zhang et al., 2021). Cdk5 inhibition alleviated diabetic neurotoxicity related cognitive deficits in diabetes mellitus animal model (Liu et al., 2019). Drugs such as rolipram (Wachtel, 1983), cilostazol (Schaler and Myeku, 2018), sildenafil (Sanders, 2020), CM-414 (Cuadrado-Tejedor et al., 2017) reduce tau hyperphosphorylation and increased dendritic spine density in hippocampal neurons improving cognition in animal models. Interestingly, recent reports state that D-pinitol, a naturally occurring inositol alleviate hyperactivation of p25/cdk5 activity by regulating cyclin-dependent kinase 5 regulatory subunit 1 (CDK5R1) and GSK-3 beta (tau phosphorylation regulating gene) leading to tau dephosphorylation (Medina-Vera et al., 2022). According to Jeremy Koppel et al., increased extracellular dopamine levels hyperphosphorylate tau and blocking dopamine D_2 receptor results in robust decrease in tau phosphorylation (Koppel et al., 2019). Dopaminergic receptors can modulate dopamine and cyclic adenosine monophosphate regulated phospho protein (DARPP-32), which acts as molecular switch to activate or inhibit kinases (cAMP-dependent protein kinase) by activating protein phosphatase-1. We assume that there is a current necessity to identify the earliest signaling mechanism that trigger tau hyperphosphorylation and drugs that restore the balance between kinases and phosphatases in tauopathies. Our study does not determine phosphatase levels but further research has to be performed whether polycyclitols restore balance between kinase and phosphatase levels in ECM.

Till date, there has been no study conducted on the effect of polycyclitols on cognition and behavior. To our knowledge, our findings represent the first evidence describing the role of polycyclitol derivatives on learning and memory functions in ECM. Christopher D Morrone et al., reported that administration of *scyllo* -inositol reduced the levels of amyloid beta and reversed the cognitive decline in TgF344-AD rats (Morrone et al., 2020). However, failure of*scyllo* -inositol (**6**), in phase-II trials resulted in a setback to this molecule which otherwise exhibited significant Aβ -42 lowering in patient brains. This failure was attributed to its toxicity and non-specific binding with the target protein possibly because of its small size. Considering this failure of *scyllo*inositol (**6**) against Aβ , it was an opportunity to evaluate our recently accessed polycyclitols against the other important concerned protein 'tau' and map their potential in controlling its hyperphosphorylation (Rashid et al., 2020). Due to the bulkier size of polycyclitols (**15** , **16-18**), we expect them to have increased number of interacting sites which might result in a better therapeutic potential for neurodegenerative diseases involving tauopathy. According to a recent reports, tau based therapeutics could play a potential role in reversing the neuronal cell damage and cognitive impairment in ECM (Akide Ndunge et al., 2023; Dai et al., 2012b). ARM alone impairs blood-brain barrier in experimental models of CM despite improving survivability by parasite clearance (Brejt and Golightly, 2019; Dai et al., 2012a; Gul et al., 2021). In agreement to previous research studies, our group showed ARM therapy alone is unable to restore cognition (Kumar and Babu, 2022). ARM monotherapy also holds the current problem of anti-malarial resistance in patients with severe malaria. Therefore, we administered our adjuncts (SR4-01 to 04) along with ARM instead of ARM alone in our study.

RMCBS scores show that rescue therapy successfully alleviated neurobehavioral impairment after few days of administration. We strongly assume that the restoration of RMCBS behavioral parameters is solely due to the ARM as is the first line therapy for CM clearing parasites in the blood. According to Clemmer et al., survivability of ARM treated animals is near to 50 percent (Clemmer et al., 2011), and the same was observed after rescue ARM adjunctive therapy on day 30. Based on outcomes of Barnes maze experiment, we assume that SR4-02 (**15**) and SR4-04 (**18**) treated animals restored the spatial reference memory with minimal error rates. The same experimental groups exhibited a retrieval of working memory in spontaneous alternation method of T-maze experiment. SR4-02 group also showed improved learning and novelty in novel-object recognition test which was similar to the control group. SR4-01 (**16**) and SR4-03 (**17**) groups showed increased side preference while performing T-maze experiment. The increase in error rates in SR4-01 could be due to loss of neuronal density in hippocampal regions and expression of lower levels of phospho tau, p25 inducing neurotoxic stimuli. SR4-03 group exhibited improved neuronal arborization pattern but cognitive impairment still persisted. SR4-03 treated brain sections show improved neuronal arborization with lower restoration of cognition. One of the reasons could be that SR4-01 and SR4-03 contain a carbonate functionality in common compared to SR4-02 and SR4-04, which makes the former compounds highly reactive

and accelerate ROS generation (Juan et al., 2021)(Sparrow et al., 2003). We assume that both SR4-01 and SR4-03 drugs with ARM interacts with hemoglobin, producing toxic oxygen free radicals; the other reason could be the non-compliance of dosage standardization.

Under pathological conditions, hyperphosphorylated tau localized to somatodendritic compartments impair neuronal arborization by dysregulating microtubule regulatory proteins, affecting microtubule stability and resulting in loss of neuronal density (LaPointe et al., 2009)(Kimura et al., 2014). Neuronal loss due to tau hyperphosphorylation is observed in hippocampal, cortical pyramidal neurons and striatal neurons in Alzheimer's, Parkinson's (Kaul et al., 2011) and dementia with Lewy bodies (Hatch et al., 2017). Previously, our group showed extensive neurodegeneration associated with upregulation of NADPH oxidase 2 in CA1 and dentate gyrus regions of hippocampus (Kumar and Babu, 2022) also known as early vulnerable regions of neuronal damage in neurodegenerative diseases (Davolio and Greenamyre, 1995; Planche et al., 2018). Hyperphosphorylation of tau dissociates microtubules affecting the distal ends followed by proximal ends of the dendrites of pyramidal neurons in AD (Regalado-Reyes et al., 2020). As per the findings of Golgi-cox staining, we assume that decreased lengths of distal and proximal neurons of cortical and hippocampus is a key indicator for the loss of neuronal plasticity in CM. p25 and cdk5 hyperactivation followed by tau pathology could deteriorate dendritic arborization pattern of the pyramidal hippocampal and cortical neurons in ECM. We assume that hyperphosphorylated form of tau and accumulation of p25 could be a potential reason for loss of neuronal density in CM. Restoration of neuronal density after administration of adjunctives SR4-02, SR4-03 and SR4-04 could be due to significant reduction of phosphorylated tau, p25 neurotoxicity and total cdk5 levels after ECM.

Conclusion

In conclusion, we report that cdk5-based signaling could lead to tau hyperphosphorylation and polycylitol derivatives (SR4-02 and SR4-04) as ARM adjunctives effectively restore learning and memory functions, reducing active tau levels and improve hippocampal neuronal density in the experimental model of CM. Initially, long-term, short term memory and novelty based learning was studied by conducting Barnes maze, T-maze and novel object recognition task in animal models. Further studies with respect to cdk5/p25 and tau phosphorylation protein expression were carried out using western blotting, immunohistochemistry and Golgi-cox staining. Since polycyclitol derivatives have shown the potential to alleviate tau levels and restore cognition in experimental models of CM and drugs targeting chronic hypoxia and altered vascular remodeling may restore tau levels in neurodegenerative diseases, more in depth studies which focus on molecular signaling pathways associated with tau hyperphosphorylation and cognitive functions in animal models of Alzheimer's and depression must be carried out to further understand the role of SR4-02 and SR4-04.

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Author Contribution

Simhadri Praveen Kumar designed the hypothesis and experimental methodology and performed cognitive tests. Showkat Rashid performed the chemical analysis and synthesis of the adjunctives. Shailaja Karri performed the immunohistochemistry, Western blotting and Golgi-Cox staining studies. Bilal A Bhat helped us with characterization of the adjunctives. Goverdhan Mehta and Phanithi Prakash Babu guided and assisted with editing the manuscript.

Ethical approval

All the animal experiments were conducted as per the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Government of India (Registration No: 48/1999/CPCSEA) after approval from the Institutional Animal Ethics Committee (UH/IAEC/PPB2014-I/68), University of Hyderabad, India.

Conflict of interest

The authors declare that they do not have any conflict of interest.

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Fig.1. Figure representing small molecule inhibitors of tau and Aβ aggregation.

Fig.2. Figure representing bicyclic conduritol and annulated inositols

Fig.3. General synthetic strategy for the synthesis of target Polycyclitols

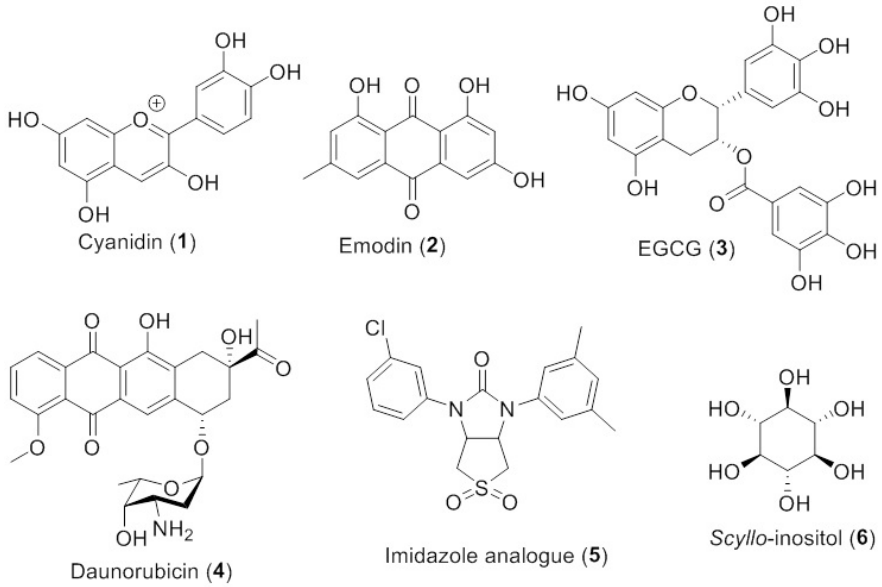
Fig.4. A) Graph representing the RMCBS scores of PbA infected animals from day 5 to 9 and restoration of neurobehavioural parameters after rescue therapy with SR4 (01-04). B) Kaplan-Meier survival curve representing 50-60 percent survival rate after SR4 (01-04) adjunctive therapy until day 30. C) Work plan showing the time points of PbA infection and cognitive tests performed after survival day 30.

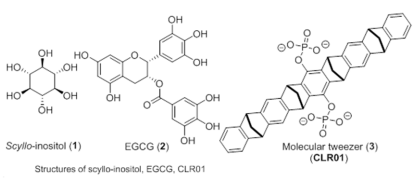
Fig.5. A) Barnes Maze with an escape platform. B) primary latencies exhibited by experimental groups C) Barnes maze trackplots D) number of errors performed till day 5 E) heat maps recorded using ANYmaze tracking software F) representing the trackplots of experimental animals exhibited in novel object recognition test. G) The retrieval phase represents that SR4-02 group exhibit significant increase in exploration time compared to rest of the experimental groups. H) representing correct and wrong alternation in T-maze experiment I) representing the percentage of correct alteration of all the experimental groups exhibited in T-maze along with. Novel object (***p<0.001, **p=0.022, *p=0.044), known object (***p<0.001, **p=0.002, *p=0.001)

Fig.6. A) Representing immunohistochemical staining of phospho tau at Ser396 levels in cortical (B) and hippocampal brain regions (C) of all the experimental groups. ***p<0.001. Scale bar- 15 μm.

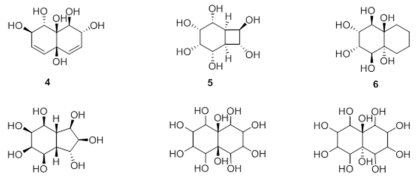
Fig.7. A) Photomicrograph representing the neuronal arborization pattern of cortical and hippocampal regions of Golgi-cox impregnated brain sections of all the experimental groups. B) Graph representing the length of distal and proximal dendrites of hippocampal neurons. C) Graph representing the length of distal and proximal dendrites of cortical neurons ***p<0.001. Scale bar- 20 μm.

Fig.8. Figure representing A) Western blots of phospho tau Ser396, cdk5, phospho-cdk5 Ser159, p35, p25 and GAPDH protein levels in all the experimental groups. B) graphs representing the densitometry of corresponding Western blots. *** p<0.001, **p=0.016, *p=0.038.





Structures of scyllo-inositol, EGCG, CLR01



Bicyclic conduritol and annulated inositols.