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ORIGINAL ARTICLE

Anti-Inflammatory Effects of Ladostigil and Its Metabolites in Aged Rat Brain and in Microglial Cells

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Abstract Impaired mitochondrial function accompanied by microglial activation and the release of nitric oxide (NO) and pro-inflammatory cytokines has been reported in Alzheimer's disease, its prodromal phase of Mild Cognitive Impairment (MCI) and in aged rats. The present study showed that 6 months treatment of 16 month old rats with ladostigil (1 mg/kg/day), a novel drug designed for the treatment of MCI, prevented the development of spatial memory deficits at 22 months of age and significantly decreased the gene expression of IL-1β, IL-6, TNF-α and inducible nitric oxide synthase (iNOS) in the parietal cortex. It was also shown that concentrations ranging from 1nM-1 μM of ladostigil and three of its active metabolites inhibited the release of nitric oxide (NO) induced by lipopolysaccharide (LPS) from mouse microglial cells by up to 35–40 %. Ladostigil and its metabolites (10nM) also reduced TNF-α mRNA and protein by 25–35 % and IL-1β and inducible nitric oxide synthase (iNOS) mRNA by 20–35 %. The concentration of 10nM is in the range of that of the parent drug, R-MCPAI and R-HPAI found in plasma after oral administration of ladostigil (1 mg/kg/day) to rats. All the compounds inhibited the degradation of IkB-α and nuclear translocation of the p65 subunit of NF-kB. They also inhibited phosphorylation of p38 and ERK1/2 mitogen-activated protein kinase (MAPK), but had no effect on that of JNK. We propose that the anti-inflammatory activity may contribute towards the neuroprotective action of ladostigil against the development of memory impairments induced by aging or toxin-induced microglial activation.

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Introduction

It is widely held that oxidative-nitrative stress involving the excess production of reactive oxygen and nitrogen species plays a key role in the etiology of neurodegenerative disorders, like Alzheimer's (AD) and Parkinson's diseases (Sayre et al. 2008; Smith et al. 2005; Park et al. 1999). Impaired mitochondrial function in AD is accompanied by microglial activation that increases the release of nitric oxide (NO) and pro-inflammatory cytokines (Maccioni et al. 2009; Mangialasche et al. 2009). Evidence of oxidative stress and neuroinflammation is also seen in Mild Cognitive Impairment (MCI) (Butterfield et al. 2007; Okello et al. 2009) which can develop into AD in a significant proportion of subjects (Palmer et al. 2007; Mitchell and Shiri-Feshki 2009). The drugs most frequently employed for the treatment of AD are acetylcholinesterase (AChE) inhibitors (Wilkinson et al. 2004). One of these, donepezil, has been shown to reduce the cytotoxic effects of oxidative stress (Yuan et al. 2011) and the release of NO and cytokines from activated microglia (Hwang et al. 2010), but both these actions only occur at concentrations 10-100-fold higher than those inhibiting AChE (Darvesh et al. 2003). This lack of protective effect against neurodegenerative processes at relevant therapeutic doses could explain why chronic treatment with donepezil of patients with MCI did not reduce the proportion converting to AD (Raschetti et al. 2007; Persson et al. 2009) and may even have accelerated its onset (Schneider et al. 2011).

Ladostigil (6-(N- ethyl, N- methyl carbamyloxy)-N propargyl-1(R)-aminoindan hemitartrate, is an AChE and brain-selective monoamine oxidase (MAO) inhibitor

(Weinstock et al. 2000) that was shown to protect cells against the toxic effects of oxidative and nitrative stress (Youdim and Weinstock 2001; Maruyama et al. 2003). Treatment for one month of aged female rats with ladostigil (1 mg/kg) also increased the expression of genes that are associated with metabolism and oxidative processes in the hippocampus which had been down-regulated as a result of age (Bar-Am et al. 2009). The same dose of ladostigil administered for 6 months to middle-aged male rats, prevented the increase in OX42 immunoreactivity, an indication of microglial activation in the parietal cortex and hippocampus and the development of memory deficits (Weinstock et al. 2011). This dose was too low to cause any inhibition of AChE or MAO in the rat brain.

Ladostigil is metabolized in rats (Kiss 2007) and humans (Rost 2003) to three major primary active metabolites; R-MCPAI (6-(N-methyl carbamyloxy)-N-propargyl-1(R)-aminoindan) though de-ethylation by CYP 2C19 in the liver, R-CAI (6-(N-ethyl, N-methyl carbamyloxy)-1(R)-aminoindan through depropargylation by CYP 1A2 and R-HPAI (6-Hydroxy–N-propargyl-1(R)-aminoindan) through hydrolysis of the carbamate moiety by AChE, its target enzyme. R-MCPAI and R-CAI are about 30 and 12-fold more potent inhibitors of AChE respectively than ladostigil (Weinstock 2005) while R-HPAI accounts for all the MAO-inhibitory activity of the drug in vivo (Sterling et al. 2002).

It is not known whether the changes induced in the brain of aging rats result from actions of ladostigil itself, its metabolites or a combination of them. While the reduction in glial activation may be an indirect result of the effect of ladostigil on mitochondrial dysfunction and antioxidant enzymes, it is possible the drug or its metabolites could also act directly on age-activated microglial cells and reduce the release of NO and pro-inflammatory cytokines. Toll-like receptors (TLR) in the brain are thought to play a role in neurodegenerative processes and their stimulation by cytokines and pathogens leads to downstream activation of the transcription factor NFκB (Walter et al. 2007). In addition to activation of the TLR4/CD14 complex on the microglial cell surface (Guha and Mackman 2001) by lipopolysaccharide (LPS), cytokine release can also be induced in microglia by interferon gamma (IFN-γ) through activation of the IFN receptor and JAK-STAT pathway (Darnell et al. 1994; Bach et al. 1997).

Therefore the aims of the study were twofold; 1) to determine whether chronic treatment of aging rats with ladostigil can reduce the age-related increase in the expression of inducible nitric oxide synthase (iNOS) and pro-inflammatory cytokines like IL-1β, IL-6 and TNF-α, that have been implicated in memory loss; 2) to determine whether ladostigil and its metabolites can reduce NO, iNOS, IL-1β and TNF-α gene expression and TNF-α protein in cultured microglial cells stimulated by LPS and IFN-γ and to elucidate the molecular mechanism through which they produce their effect.

Methods

Animals and their treatment The study was performed on 16 male Wistar retired breeders (Harlan, Jerusalem) aged 16 months at the start of the study and 8 young adult rats aged 4 months, according to the guidelines of the University Committee for Institutional Animal Care, based on those of the National Institutes of Health, USA. The rats were housed two per cage in the Animal house at an ambient temperature of 22±1 °C and a 12 h diurnal light cycle (lights on at 0700 h), with food and water provided ad libitum. The daily water intake was assessed for each pair and the rats were weighed once weekly. Ladostigil was added to acidified drinking water of 8 of the rats to provide a daily intake of 1 mg/kg/day. This method of dosing was chosen to avoid the considerable stress of daily gavage. The remaining 8, 16-month old rats drank acidified water (pH 3–4). All doses of ladostigil refer to mg/kg of the hemitartrate salt. Treatment was given until the rats were 22 months old since this was previously shown to decrease significantly the number of rats that developed spatial memory deficits and glial activation.

Assessment of spatial memory Spatial memory was assessed in the Morris water maze (MWM) in adult and aged rats after the latter had been treated with ladostigil or water for 6 months. The experiment was performed as described previously (Weinstock et al. 2011). To begin each trial the rat was placed into the water facing the maze wall from one of four start positions evenly spaced around the pool. These were chosen randomly at the beginning of each day for all rats. The time to reach the escape platform was measured. The rats were given 2 trials a day for 4 days between 10:00 and 14:00 h with an inter-trial interval of 30–40 min. If the rats failed to find the escape platform within 120 s they were placed on it for 7 s and then removed from the pool. Both collection and analysis of the data were performed using an automated video-tracking system (HVS, UK Ltd).

Real time reverse transcriptase polymerase chain reaction (RT-PCR) Three days after completion of the MWM test the rats were euthanized by decapitation, the brains rapidly removed and the parietal cortex carefully dissected on ice, frozen in liquid nitrogen and stored at −80 °C until analysis. This area was chosen since in a previous study it showed the largest differences in microglial activation between adult and aged rats and those with ladostigil treatment (Weinstock et al. 2011). Total RNA was isolated from the frozen samples after homogenization in 1 ml of TRIZOL reagent,

extraction with chloroform followed by isopropyl alcohol, centrifugation to obtain RNA pellets which were dissolved in RNAase free water by passing solution a few times through a pipette tip, and processed using RNeasy Mini-kits (Qiagen) following the manufacturer instructions. RNA concentration and quality (A260/A230 ratio) were measured using a spectrophotometer (NanoDrop). Samples were diluted with RNAase free water to a concentration of 500 ng/μl and frozen at −80 until further analysis. cDNA was obtained by using a High Capacity cDNA reverse transcription kit (Applied Biosystems) for random nonamer and oligo-dT primers according to the manufacturer's instructions. Real-time RT-PCR was performed using validated commercially available Taq-man primer-probe sets (Applied Biosystems) and optimized protocols on the Step-One Plus real-time PCR Detection System (Applied Biosystems). For quantification of gene expression Actin-beta amplicons were generated and used as a "house-keeping" internal control gene. Relative gene expression levels were calculated in relation to those of the corresponding Actin-beta gene.

Reagents and cell cultures Ladostigil hemitartrate; R-MCPAI HCl; R-CAI HCl and R-HPAI mesylate were provided by Avraham Pharmaceuticals. All concentrations are expressed in terms of the above salts. Bacterial LPS from Salmonella typhosa was obtained from Sigma (St. Louis, MO, USA). Recombinant mouse interferon gamma (IFN-γ) was purchased from R&D Systems (Minneapolis, MN, USA). Primary microglial cells were kindly provided by Prof. Rotshenker (Medical Neurobiology Department, Hebrew University) or prepared from brains of 1–3 day-old mice as previously reported (Reichert and Rotshenker 2003). Brains were stripped of their meninges and enzymatically dissociated. The dissociated cells were plated on poly-L-lysine-coated flasks for one week and re-plated for two hours on bacteriological plates and non-adherent cells were removed by washing. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Biological Industries, Israel) containing 1 g/L glucose, 10 % fetal calf serum (FCS, Biological Industries), 10 % of Colony Stimulating Factor (CSF), 50 μg/ml, 0.01 % gentamicin and 1 % L-glutamine (Biological Industries).

NO release assay Microglial cells were co-treated with the compounds at concentrations ranging from 1 nM to 1 μM and LPS (10 μg/ml), or IFN-γ (50 ng/ml). NO released into the medium 24 h later was measured spectrophotometrically in the form of nitrites using Griess reagent (0.1 % N-1-naphthyl ethylene diamine dihydrochloride and 1 % sulphanilamide in 5 % phosphoric acid). The supernatant (50 μl) was mixed with an equal volume of Griess reagent in a 96-well plate and incubated at room temperature for 10 min. The concentration of nitrites was measured on a micro plate reader (Labsystems) at 540 nm using serial dilutions of $NaNO_2$ as a standard.

Cell viability assessment Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test. The cells were incubated with concentrations of ladostigil and its metabolites ranging from 1 nM-10 μM and with MTT in final concentration of 0.5 mg/ml in the medium for 30 min. The medium was then discarded and dimethyl sulphoxide (DMSO 100 μl) was added to each well to dissolve the formazan dye. The plate was read at a wavelength of 580 nm with background subtraction at 650 nm.

Total RNA extraction, cDNA production and real-time polymerized chain reaction (RT-PCR) analysis Microglial cells were co-treated with the compounds at concentrations of 10 and 100 nM and LPS (10 μg/ml) in 96-well plates for 2, 8 or 12 h before being scraped from the wells, centrifuged and collected for RNA extraction. Total RNA was isolated from the cells using Total RNA mini kit (Genaid Biotech, Sijhih, Taiwan). Total cDNA was synthesized from the isolated total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Total RNA (1–2 μg) was added to the reagent mix to a final volume of 20 μl for each sample. The RT reaction was run for 10 min at 25 °C, 2 h at 37 °C and 5 min at 85 °C. Real time RT-PCR reaction was performed using TaqMan the probe-based detection approach, in which 6 μl of TaqMan Master Mix (Applied Biosystems) with appropriate TaqMan probes (TNF-α, IL-1β, inducible nitric oxide synthase, iNOS) was added to 4 μl of cDNA solution (final amount of cDNA – 10 ng/well) to a final volume of 10 μl of reaction in each well. The reaction was run for 40 min at StepOnePlus™ RT-PCR System (Applied Biosystems).

Enzyme-linked immunosorbent assay (ELISA) Microglial cells were co-treated with LPS (10 μg/ml) and the compounds (10 nM or 100 nM) for 6 h and TNF-α protein was detected using the sandwich ELISA method. The cells were incubated for 24 h in 48-well plates. Medium (50 μl) was applied to each well which was coated with capture anti-mouse TNF-α antibody (BioLegend, San Diego, CA, USA) in 96-wells plate. Dilutions of recombinant TNF-α protein ranging from 7.8–500 pg/ml were used for a standard curve. The plate was incubated overnight at 4 °C. After 3 washes with PBS/Tween20 solution the cells were blocked with 1 % BSA in PBS solution for 1 h followed by 3 additional washes. Anti-mouse TNF-α antibody was applied for detection for 1 h at room temperature. A chemiluminescence reaction was performed by adding a pNPP One Component Microwell Substrate reagent (Southern Biotech, Birmingham, AL, USA) and color intensity was evaluated on a plate reader at 405 nm.

Western blot analysis In order to evaluate the possible role of the compounds on MAPK signaling we analyzed phosphorylation of three major components of this pathway; extracellular signal-regulated kinases ERK1/2, p38 and c-Jun N-terminal kinase (JNK) 1, 2, 3 proteins which may be activated upstream to NF-κB and are involved in transduction and proliferation of the pro-inflammatory signal induced by LPS at the TLR4 receptor. Microglial cells were seeded 24 h prior to the experiment in 6-well plates at a density of 2×10^6 cells/well. The cells were pre-treated for 90 min with the compounds (10nM), stimulated with LPS (10 μg/ml) for 30 min in presence of the compounds and the degree of phosphorylation of the MAPK proteins was evaluated. The 30 min time point was found in preliminary experiments to give optimal results for phosphorylation by of MAPK by all components and IκBα protein (data not shown). The cells were homogenized with RIPA buffer (Sigma) which protein included a cocktail of proteinase and phosphatase inhibitors and DNAses (Sigma). Total protein concentration was determined using bi-cinchoninic acid (BCA) assay (Sigma) and 20 μg of it was loaded on 12 % SDS-polyacrylamide gel (Sigma) and run for 40 min. The proteins were transferred onto nitrocellulose membranes and blocked for 30 min with 5 % milk solution at Tris-buffered saline Tween-20 (TBST). The membranes were incubated overnight at 4 °C with appropriate primary antibodies on a shaking platform. Mouse anti-mouse ERK2, rabbit anti-mouse phospho ERK1/2, mouse anti-mouse p38, rabbit anti-mouse phospho p38, mouse anti-mouse JNK1/2/3, goat anti-mouse phospho JNK1/2/3 and rabbit anti-mouse IκBα (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used in 2 ml of 5 % milk solution. Mouse anti-mouse tubulin antibody (Sigma) was used as a housekeeping protein at a dilution 1:15000. Goat anti-rabbit Alexa Fluor 680-conjugated IgG and IRDye 800-conjugated goat-anti mouse IgG (dilution 1:10000) were added for 1 h at room temperature before visualization and analysis by the Odyssey IR imaging system (LI-COR Biosciences).

Immunofluorescence staining Microglial cells were seeded on sterile glass coverslips 24 h before they were exposed to the compounds for 90 min at a concentration of 10 nM, followed by activation for 30 min with LPS (10 μg/ml) in presence of the compounds. The coverslips were fixed in 4 % paraformaldehyde for 30 min at room temperature (24 °C) and then in cold methanol for 10 min at −20 °C. Fixed cells were blocked in 1 % bovine serum albumin (BSA). The cells were incubated with mouse anti-p65 antibody (1:100 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4 °C overnight and Alexa Fluor-488 labeled goat anti-mouse IgG (1:2000 dilution; Molecular Probes Inc., Eugene, OR, USA) at room temperature for 1 h. Stained cells were mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and examined on a fluorescent microscope (Nikon Eclipse TE2000-E). DAPI staining was used for nuclei contrasting marking. Images were analyzed by means of Volocity Image Analysis Software (PerkinElmer, USA) and the co-localization of Alexa Fluor-488 signal for p65 (green) and DAPI nuclear staining (blue) was estimated and represents the amount intra-nuclear p65.

Electro-mobility shift assay (EMSA) Microglial cells were seeded 24 h prior to the experiment in 6-well plates, pre-treated for 90 min with 10 nM of the compounds followed by 10 μg/ml LPS plus 10 nM of the compounds for 30 min. Nuclear proteins were isolated from approximately 1×10^7 microglia cells using Cayman Nuclear Extraction Kit (Cayman Chemical Company, Ann Arbor, MI, USA). Total protein concentration was determined using bicinchoninic acid (BCA) assay (Sigma). Double stranded NF-κB IRDye 700 labeled oligonucleotide (LI-COR Biosciences, Nebraska, USA) corresponding to NF-κB specific consensus sequence was used for the binding reaction at concentration of 50 nM per reaction. The double stranded sequence for NF-κB was 5' – AGT TGA GGG GAC TTT CCC AGG C – 3' and 3' – TCA ACT CCC CTG AAA GGG TCC G – 5' while the underlined nucleotides represent the NF-κB binding site. The unlabeled oligonucleotides with the similar sequence (cold probe) or labeled nonspecific sequence were used as controls for specificity of the binding reaction. The binding reaction was performed using Odyssey Infrared EMSA kit (LI-COR Biosciences) according to appropriate protocol and with 50 ng of total nuclear protein per each reaction. For control of binding specificity the non-specific probe was added to vials containing protein extract from LPS treated cells alone, 10 min before adding the specific probe. The reaction took place during 40 min in the dark at room temperature and 2 μl of Orange loading dye X10 (LI-COR Biosciences) was added to each vial before loading to the gel. A 4 % polyacrylamide TBE gel was prepared and pre-run at 70 mV for 40 min before the samples were loaded. Protein-DNA complexes (20 μl) were resolved by electrophoresis for 1 h at 70 mV in the dark. The gels were scanned at 700 μm using LI-COR Odyssey Imaging system. Band densities were quantitated by densitometric analysis using ImageJ software.

Statistical analysis Mean daily escape latencies for two trials in the MWM in each experimental group were analyzed by repeated measures ANOVA for DAY and GROUP followed by Duncan's *post hoc* test on given days where appropriate. Comparisons of means for gene expression data were analyzed by ANOVA for group followed by Duncan's *post hoc* test. All the data represent mean values ± SEM. For microglial analyses all results are expressed as mean ± SEM

from 4–8 independent experiments with 3–4 replicates in each experiment unless otherwise stated. Statistical analysis of data was performed by one-way ANOVA with Duncan's *post hoc* test or Dunnett's test with multiple comparisons of means where appropriate. Differences were considered statistically significant if the P value was <0.05.

Results

Spatial memory Repeated measures ANOVA revealed a significant effect of day ($F_{1,23}$=37.6, P<0.0001) and a day X group interaction ($F_{2,\ 22}$=3.95, P<0.05). The escape latencies of untreated aged rats differed significantly from those of young adults on all four days of testing and from those treated with ladostigil (1 mg/kg/day) on days 3 and 4 (P<0.01) (Fig. 1a).

Gene analysis in rat parietal cortex ANOVA of values for mRNA showed a significant effect of group for IL-1 β ($F_{2,\ 22}$=7.060, P=0.005), IL-6 ($F_{2,\ 22}$=7.207, P<0.005), TNF-α ($F_{2,\ 22}$=24.55, P<0.0001) and iNOS ($F_{2,\ 22}$= 30.97, P<0.0001) in the cortex. Aged controls had a significantly higher expression of all the genes than adult controls. Ladostigil significantly reduced the expression of these genes in aged rats to the level in cognitively intact adult rats (Fig. 1b).

Release of nitric oxide from LPS or IFN-γ activated microglia Activation of microglia by IFN-γ increased NO release 10–15 fold. Neither ladostigil nor its metabolites (1 nM-1 μM) caused any significant reduction in the release of NO after activation of the microglial cells with IFN-γ (Fig. 2a). Therefore, no further experiments were performed with IFN-γ. Exposure of microglial cells to LPS resulted in a 4–8 fold increase in the release of NO which was reduced significantly by 15–20 % by 1 nM all the compounds (Fig. 2b). At concentrations ranging from 10 nM-1 μM NO release was further decreased to a maximum of 35–40 % (P< 0.001) depending on the compound (Fig. 2c). The effect of R-MCPAI was significantly greater than that of ladostigil at a concentration of 1 μM, and that of R-HPAI, at a concentration of 100 nM. Viability of microglia was unaffected by LPS 10 μg/ml alone or when given together with the compounds in concentrations of 1 nM-1 μM in the MTT assay (data not shown).

LPS-induced pro-inflammatory gene expression in microglia cells In preliminary experiments we found that the largest increase (35-fold) in TNF-α gene expression compared to that in control microglia was seen 2 h after stimulation with LPS, (10 μg/ml), while those of IL-1β (1000-fold increase) and iNOS (5-fold increase) were seen at 12 h and 8 h

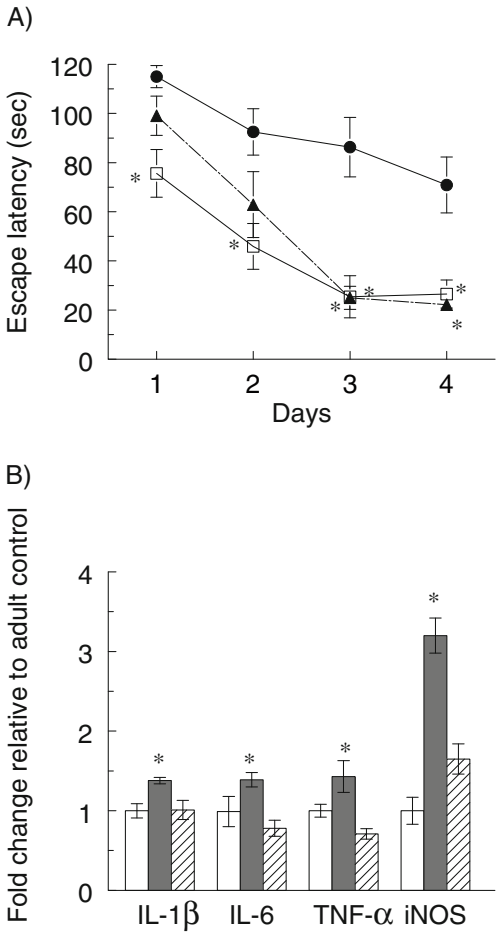

Fig. 1 Effect of chronic treatment of aging rats with ladostigil on spatial learning and proinflammatory gene expression in the parietal cortex. **a**) Open squares: adult control rats; Closed circles: aged control rats; closed triangles: aged rats with ladostigil (1 mg/kg/day). Data represent the mean ± SEM of two daily trials in the Morris water maze. The latency to find the hidden escape platform was significantly longer in untreated aged rats than in adult controls or aged rats treated with ladostigil (1 mg/kg/day). Significantly different from aged control $^{*}P$< 0.01. **b**) Open columns: adult control rats; filled columns: aged control rats; hatched columns: aged rats with ladostigil (1 mg/kg/day). Data show mean ± SEM of mRNA of genes of three cytokines and of iNOS in the parietal cortex taken from the rats three days after completion of the spatial learning test in the MWM. Aging significantly increased the expression of these genes compared to that in adult rats. Chronic treatment with ladostigil (1 mg/kg/day) from the age of 16 months restored gene expression at the age of 22 months to that in 4-month old controls. Significantly different from adult rats and those treated with ladostigil * P<0.01

respectively. Co-treatment of the microglial cells with LPS and 10 nM of each of the compounds for 2 h resulted in a similar significant reduction of 25–30 % in TNF-α gene expression for all metabolites (P< 0.01) (Fig. 3a). When the concentration of each compound was increased to 100 nM the reduction in the expression of TNF-α was significantly lower than with 10 nM. By contrast, the expression of IL-1β was reduced to a similar extent at concentrations of 10 nM

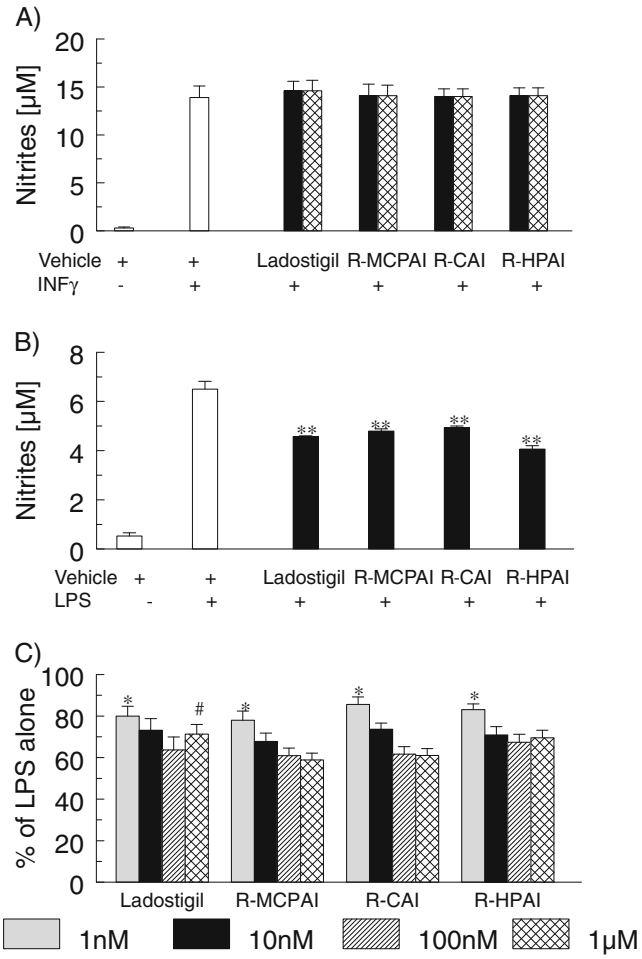

Fig. 2 Effect of ladostigil and its metabolites on the release of NO from IFNγ and LPS activated microglia. **a**) Cells were co-treated with IFNγ (50 ng/ml) and two different concentrations (10 and 1000nM) of ladostigil and its three metabolites. NO release in terms of nitrite was measured with Greiss reagent 24 h later. No significant reduction in NO release was observed at both concentration of the drugs. **b**) Cells were co-treated with LPS (10 μg/ml) and 10nM of all compounds. NO release was measured by Griess reagent 24 h later. Significantly different from LPS alone ** $P<0.01$. **c**) Shows the reduction by different concentrations of ladostigil and its metabolites in NO release induced by LPS (normalized to 100 %). All concentrations of the compounds significantly reduced the release of NO induced by LPS. This reduction reached maximal values of about 35–40 % at concentrations of 100nM and 1 μM. Data represent mean ± SEM of 6–8 independent experiments performed in triplicate. Significantly different from value produced by 10nM-1 μM * $P<0.05$; significantly different from value for R-MCPAI and R-CAI, $^{\#}$ $P<0.05$

and 100 nM by all compounds. Ladostigil reduced the levels of IL-1β mRNA by 35 % at each concentration compared to <25 % for the other compounds (Fig. 3b). All the compounds (10 nM) caused a reduction of similar magnitude in the expression of iNOS after 8 h of co-treatment with LPS (Fig. 3c). This agrees well with their effect on NO release.

LPS-induced TNF-α release from activated microglia cells To determine whether the decrease in gene expression of the cytokines by the compounds is translated into reduced amounts of the protein we measured TNF-α in the medium of the microglial culture. Maximal increase in TNF-α protein was seen 6 h after addition of LPS to microglia but it was not possible to detect sufficient amounts of IL-1β protein from these cells. Ladostigil and R-MCPAI (10 nM) caused a similar reduction in TNF-α of 40–42 %, but R-HPAI and R-CAI were significantly less effective. 100 nM of all the compounds did not further increase their effect (Fig. 3d).

Phosphorylation of MAPK proteins and degradation of IκBα For these experiments we used a concentration of 10 nM of all the compounds since RT-PCR analysis of the TNF-α and IL-1β genes and measurement of NO release did not reveal a significantly greater inhibitory effect at 100 nM. None of the compounds had a detectible effect on phosphorylation of JNK proteins (data not shown). However, all the compounds significantly reduced the phosphorylation of ERK (shown by the ratio of the sum of phosphorylated ERK1 and ERK2 to un-phosphorylated ERK2) (Fig. 4a). The effect of ladostigil was significantly greater than that of R-CAI, but did not differ from that of the other metabolites (Fig. 4a and b). Ladostigil, R-MCPAI and R-HPAI almost completely prevented the phosphorylation of p38 induced by LPS resulting in ratios of phosphorylated p38/p38 protein that were similar to those in untreated cells. By contrast, R-CAI had no effect on the phosphorylation of p38 (Fig. 4c and d).

Since p38 and ERK have been postulated as possible modulators of NF-κB activity (Brown and Jones 2004; Frost et al. 2004; Wang et al. 2010) we examined the effect of the compounds on the degradation induced by LPS of IκBα, the NF-κB inhibitor. This process releases the two functional subunits of NF-κB - p50/p65 - and enables the transcription factor to enter the nucleus culminating in the transcription of wide range of pro-inflammatory elements. Ladostigil, R-MCPAI and R-HPAI almost completely prevented the reduction in IκBα degradation, 30 min after co-treatment with LPS. Although R-CAI had a significant effect on IκBα levels this was considerably weaker than that of the other compounds (Fig. 5a and b).

Nuclear translocation of p65, an NF-κB functional unit The finding in the previous experiment indicated that the compounds promote their anti-inflammatory effect by modulation of the nuclear translocation of p50/p65 subunits. This was further investigated by two methods; immunofluorescence and electro-mobility shift assay (EMSA). As anticipated, microglial cells exposed to LPS increased p65 nuclear translocation by 100 % showing that even a relatively short exposure of the microglia to the pathogen can produce a rapid and substantial response (Fig. 6a and b).

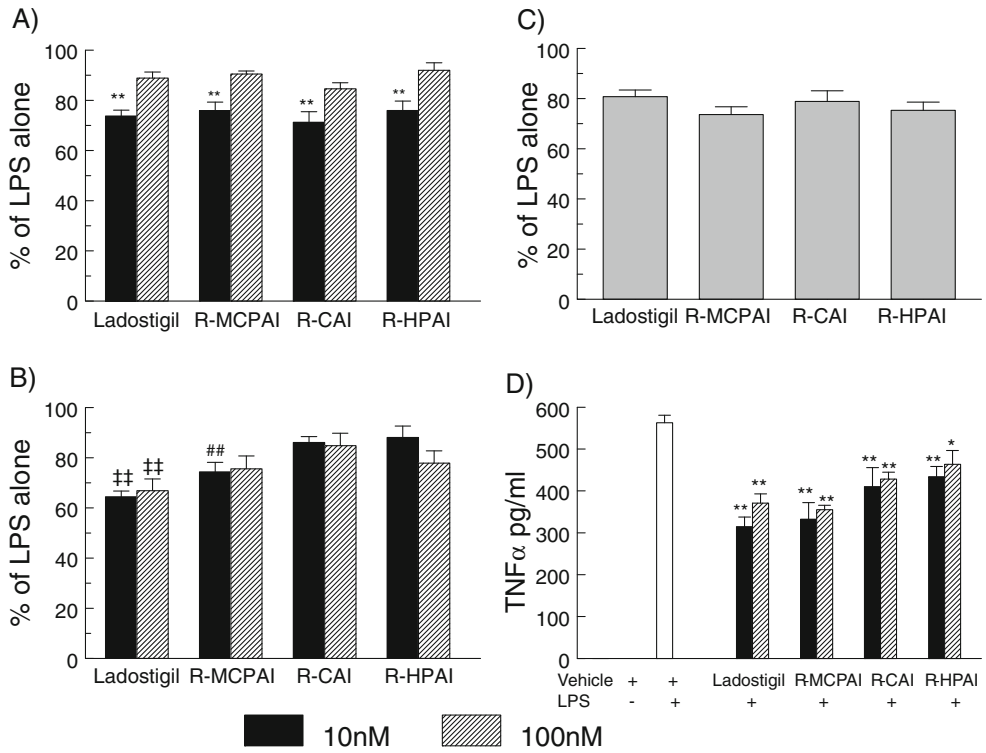

Fig. 3 Effect of ladostigil and its metabolites on expression of TNF-α, IL-1β and iNOS mRNA and TNF-α protein in LPS activated microglia. **a**) TNF-α mRNA was measured 2 h after co-treatment of cells with LPS (10 μg/ml) and two concentrations of the compounds (10nM and 100nM). The mRNA expression of TNF-α was evaluated by RT-PCR and the relative mRNA levels were quantified using TaqMan the probe-based detection approach, with TaqMan Master Mix (Applied Biosystems) and appropriate TaqMan probes. All compounds produced a similar (25–30 %) reduction in TNF-α mRNA at a concentration of 10nM, ** $P<0.01$ but not at 100nM. Data represent the mean ± SEM from 4–6 independent experiments. **b**) IL-1β mRNA measured 12 h after co-treatment of cells with LPS (10 μg/ml) and two concentrations of the compounds (10 and 100nM) were evaluated by RT-PCR (as described in A). All compounds produced a significant reduction of 10–35 % in IL-1β mRNA at a concentration of 10nM and 100nM ($P<0.01$). The effect of ladostigil was significantly higher than that of R-MCPAI, R-CAI and R-HPAI at both concentrations, ‡‡ $P<0.01$. The effect 10nM of R-MCPAI was significantly higher than that of R-CAI and R-HPAI, ## $P<0.01$. Data represent the mean ± SEM from 4–6 independent experiments. **c**) iNOS mRNA measured 8 h after co-treatment of cells with LPS 10 μg/ml and 10nM of the compounds. All compounds produced a similar and significant reduction of 20–25 % in iNOS mRNA, ** $P<0.01$. Data represent the mean ± SEM from 4–6 independent experiments. **d**) TNF-α protein measured 6 h after co-treatment of cells with LPS (10 μg/ml) and two concentrations of the compounds by the ELISA sandwich method. Both concentrations of the compounds significantly reduced TNF-α protein by 35–45 %. All compounds (10 and 100nM) except R-HPAI produced a significant reduction of TNF-α protein expression compared to that in cells treated with LPS alone, * $P<0.05$, ** $P<0.01$. Data represent the mean ± SEM from 5 independent experiments

While all the compounds significantly reduced p65 nuclear entry after LPS, ladostigil and R-MCPAI were the most effective (Fig. 6b). These changes in p65 translocation seen in the immunofluorescence assay were generally confirmed by the EMSA analysis in which a 25–35 % reduction was seen compared to LPS alone (Fig. 6c). Again, the effect of R-MCPAI was significantly greater than that of the other compounds (Fig. 6d).

Discussion

It has been hypothesized that age-related memory impairment may occur as a consequence of oxidative/nitrative stress and sustained glial activation that release pro-inflammatory cytokines (Li et al. 2011; Roy et al. 2008). The current study confirmed previous findings that aged rats show significant spatial memory deficits in the MWM test when compared to young adult rats (Blalock et al. 2003; Gallagher et al. 1993) and that chronic treatment of aging rats with ladostigil for 6 months significantly reduced the memory deficits at 22 months of age (Weinstock et al. 2011). We now show for the first time that aging increases the gene expression of iNOS, IL-1β, IL-6 and TNF-α in the parietal cortex and that chronic treatment with ladostigil can restore expression of these genes to that of cognitively intact adult rats. The parietal cortex is critically involved in cognitive processes utilizing different forms of spatial information (Colby and Goldberg 1999) and lesion of this brain region impairs navigational skills (Nitz 2009). Although others have shown that attenuation of

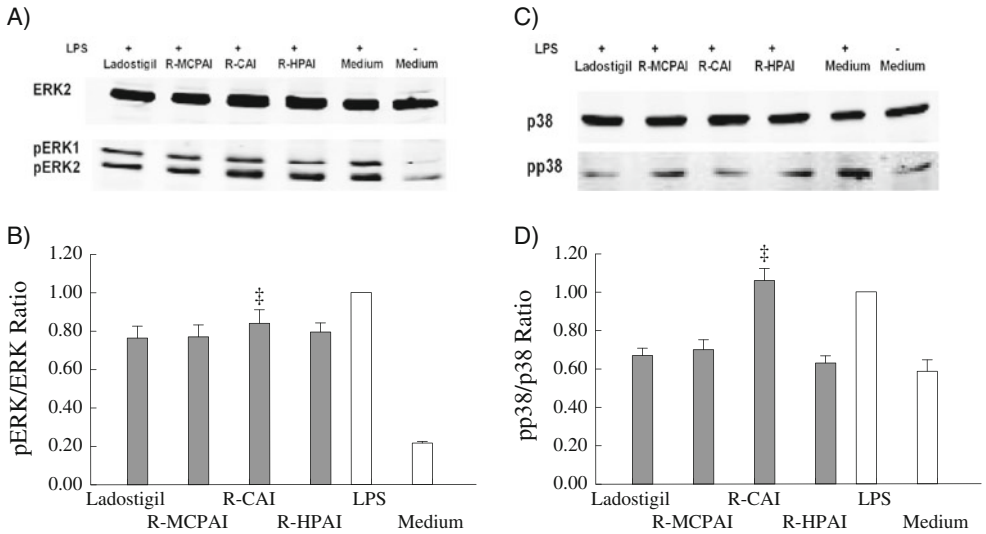

Fig. 4 Effect of ladostigil and its metabolites on phosphorylation of ERK induced by LPS in microglia. **a**) Microglial cells were pre-treated for 90 min with 10nM of compounds then activated with LPS (10 μg/ml) in presence of the compounds for 30 min. Cell lysates were subjected for immunoblot analysis using antibodies against unphosphorylated ERK2 or phosphorylated ERK1/2 (44 and 42 kDa) proteins. **b**) Ratio of phosphorylated (pERK1/2)/ERK2 was significantly reduced by 10nM of all compounds by 20–25 %, $P<0.01$. Data represent the mean ± SD from 4–6 independent experiments. **c**) Microglial cells were pre-treated for 90 min with 10nM of compounds then activated with LPS (10 μg/ml) in presence of the compounds for 30 min. Cell lysates were subjected for immunoblot analysis using antibodies against unphosphorylated or phosphorylated p38 proteins. **d**) The ratio of phosphorylated (pp38)/p38 was significantly reduced by 10nM of all compounds except R-CAI to the level seen in the absence of LPS, $P<0.01$. The effect of R-CAI was significantly less than that of the other compounds ‡ $P<0.01$. Data represent the mean ± SD from 4–6 independent experiments

hippocampal-long term potentiation, an indicator of memory impairment, is accompanied by increased levels of IL-1β (Murray and Lynch 1998) and IL-6 (Li et al. 1997) our data suggest that increased activation of glia (Weinstock et al. 2011) with up-regulation of cytokines and iNOS in the parietal cortex could also be responsible for the impairment of spatial navigation in the aged rat. While the reduction in the expression of pro-inflammatory cytokines by ladostigil could have occurred indirectly following restoration of mitochondrial function and the activity of antioxidant enzymes, it is possible that the drug or its metabolites also had a direct action on microglia to reduce cytokine production.

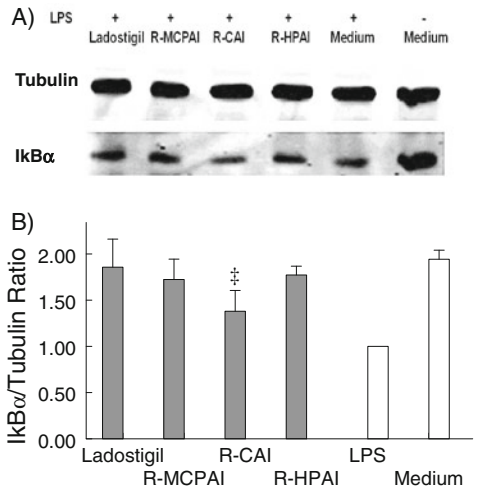

Fig. 5 Increase in the stability of IκBα in LPS stimulated microglia by ladostigil and its metabolites. Microglial cells were pre-treated for 90 min with 10nM of compounds then activated with LPS (10 μg/ml) in presence of the compounds for 30 min. **a**) Cell lysates were subjected for immunoblot analysis using antibodies against tubulin or IκBα proteins. **b**) Quantification of IκBα in the cell cytosol. Ladostigil, R-MCPAI and R-HPAI significantly increased the stability of IκBα in LPS stimulated microglia ($P<0.01$), while R-CAI differed significantly from the other compounds, ‡ $P<0.05$. Data represent the mean ± SD from 5 independent experiments

Cultured primary microglia retain the majority of the known physiological activities of reactive microglia in vivo, and when stimulated by LPS, release NO and pro-inflammatory cytokines (Boje and Arora 1992). We therefore assessed the effect of ladostigil and its metabolites, R-MCPAI, R-CAI and R-HPAI on LPS induced NO and cytokine release in LPS-activated mouse microglial cells. We found that concentrations of 1 nM-1 μM of ladostigil and its metabolites significantly reduced the levels of NO and at 10-100 nM, they reduced TNF-α, IL-1β and iNOS mRNA and TNF-α protein. None of the compounds reduced NO from microglial cells stimulated by IFN-γ. Their immunomodulatory effect does not depend on the presence of the propargyl and carbamate groups since R-CAI and R-HPAI that lack either one of them still reduced NO release and cytokine production. The action of ladostigil, R-MCPAI and R-CAI that contain a carbamate moiety is also independent of AChE inhibition since it occurs at concentrations well below those at which such inhibition is seen in vitro (1–30 μM). The finding of a direct immunomodulatory effect

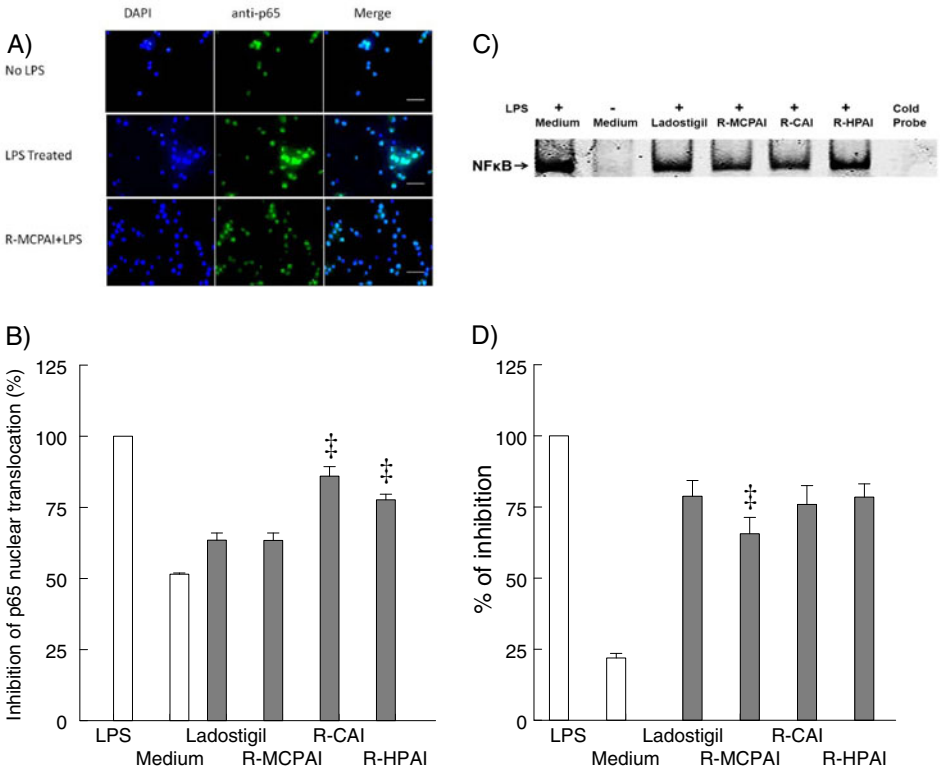

Fig. 6 Inhibition by ladostigil and its metabolites of the fluorescence signal of p65 nuclear translocation. **a)** Representative figure of immunofluorescence staining of intra-nuclear p65 and its reduction by R-MCPAI (10nM). Blue: DAPI nuclear stain. Green: Alexa Fluor-488 anti-p65 stain. Turquoise: co-localization of p65 and DAPI. Scale bar= 100 μm. **b)** Quantification of immunofluorescence staining of intra-nuclear p65. Ladostigil and R-MCPAI reduced the immunofluorescence signal by about 30 %. Both R-CAI and R-HPAI were significantly less effective than ladostigil and R-MCPAI, ‡ $P<0.05$. Data represent the mean ± SD from 3 independent experiments. **c)** Microglial cells were pre-treated for 90 min with 10nM of compounds then activated with LPS (10 μg/ml) in presence of the compounds for 30 min. Cell lysates were subjected for EMSA analysis using specific fluorescently labeled oligonucleotides for NF-κB dimer. **d)** Quantification of nuclear translocation of p65. The compounds reduced intra-nuclear NF-κB by 25–30 %, ($P< 0.01$). The effect of, R-MCPAI was significantly greater than that of ladostigil R-CAI and R-HPAI, ‡ $P<$ 0.05. Data represent the mean ± SD from 5 independent experiments

of ladostigil and its metabolites indicates that the neuroprotective effect of the drug seen in aging rats could occur through a combination of a reduction in oxidative-nitrative stress (Maruyama et al. 2003; Youdim and Weinstock 2001) and of the release of NO and pro-inflammatory cytokines. Furthermore, the low concentrations (1-10 nM) at which the anti-inflammatory effect of ladostigil and its metabolites produce their effects are compatible with those found in the circulation after a dose of 1 mg/kg of the drug is administered. The protective and anti-inflammatory effects of ladostigil in vivo are also unrelated to AChE or MAO inhibition (Weinstock et al. 2011) which is only achieved after administration of higher doses of the drug (Luques et al. 2007).

Cytokine release from microglia activated by IFN-γ is mediated by activation of the JAK-STAT pathway (Bach et al. 1997) which was unaffected by ladostigil or its metabolites. The intracellular cascade that results from the interaction of LPS with TLR4 in microglia involves the activation of the mitogen-activated protein kinases (MAPKs) family, the phosphorylation of IκBα, an inhibitor of the p50/p65 heterodimer and the nuclear translocation of NF-κB (Bhat et al. 1998). MAPKs are classified into at least three components; ERK1/2, JNK, and p38 MAPK (Koistinaho and Koistinaho 2002). We therefore investigated the mechanism of action of ladostigil and its metabolites by examining their effects on members of the MAPK family, phosphorylation of IκBα and the nuclear translocation of NF-κB. In keeping with previous reports, LPS increased the phosphorylation of all three members of this family in mouse microglial cells. However, ladostigil and its metabolites reduced the effect of LPS on the phosphorylation of ERK1/2 and p38 but not of JNK. Furthermore, while at a concentration of 10nM all the compounds were equally effective in reducing the phosphorylation of ERK1/2 they differed in their ability to influence the phosphorylation of p38. Ladostigil, R-MCPAI and R-HPAI almost completely abolished the phosphorylation of p38 by LPS, but R-CAI was virtually inactive. Since the reduction in the release of NO, TNF-α, Il-1β, iNOS mRNA and TNF-α protein by R-CAI did not differ significantly from that of the other compounds, it suggests that

inhibition of ERK1/2 phosphorylation was sufficient for the compounds to produce their pharmacological effects.

In resting microglia NF-κB is present in the cytoplasm as a complex consisting of p50, p65 and IκB subunits. Upon activation of the TLR4 receptor by LPS phosphorylation and degradation of IκB exposes nuclear localization signals on the p50/p65 complex resulting in nuclear translocation. This is followed by gene transcription and cytokine release (Moynagh 2005). Ladostigil, R-MCPAI and R-HPAI restored the levels of IκBα in the cytosol 30 min after LPS activation to those in un-stimulated cells, but in this measure also, R-CAI was less effective. These findings were reflected in the immunofluorescence measure of p65 intranuclear localization which was also inhibited to a greater extent by ladostigil and R-MCPAI than by R-CAI and R-HPAI. The data suggest that while the presence of both a carbamate and a propargyl moiety are not essential for this action they appear to result in more efficient modulation of the effect of TLR4 activation. Since the concentrations of R-CAI that are found in human and rat blood after oral administration of ladostigil are only about 25–30 % of those of the parent drug, R-MCPAI and R-HPAI (Kiss 2007) (Rost 2003), it is unlikely that R-CAI contributes measurably to any anti-inflammatory effect of ladostigil in vivo.

Conclusion

The data from this study show that ladostigil, a drug currently being developed for the treatment of AD and MCI can prevent the development of spatial memory deficits and reduce the overexpression of pro-inflammatory cytokines and iNOS in the aging rat brain. The parent drug and its major metabolites R-MCPAI and R-HPAI are also able to inhibit the production of NO and TNF-α protein and reduce their gene expression together with that of iNOS and Il-1β in LPS stimulated mouse microglial cells. The immunomodulatory activity of these compounds appears to be mediated by a reduction in the phosphorylation MAPKs ERK1/2 and p38 and inhibition of NF-κB intra-nuclear translocation. Since excess production of pro-inflammatory cytokines is believed to contribute to the neurodegenerative changes in MCI (Okello et al. 2009) and AD (Maccioni et al. 2009; Mangialasche et al. 2009) treatment with ladostigil may be able to slow the rate of conversion of MCI to AD.

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