IBUPROFEN AND LIPOIC ACID DIAMIDE AS CO-DRUG WITH NEUROPROTECTIVE ACTIVITY: PHARMACOLOGICAL PROPERTIES AND EFFECTS IN β-AMYLOID (1-40) INFUSED ALZHEIMER'S DISEASE RAT MODEL

A. DI STEFANO, P. SOZIO, L.S. CERASA, A. IANNITELLI, A. CATALDI[1],
S. ZARA[1], G. GIORGIONI[2] and C. NASUTI[3]

Dipartimento di Scienze del Farmaco, Università "G. d'Annunzio", Chieti; [1]Dipartimento di Biomorfologia, Università "G. d'Annunzio", Chieti; [2]Dipartimento di Scienze Chimiche, Università di Camerino, Camerino; [3]Dipartimento di Medicina Sperimentale e Sanità Pubblica, Università di Camerino, Camerino, Italy

Both oxidative stress and inflammation are elevated in brains of Alzheimer's disease patients, but their pathogenic significance still remains unclear. Current evidence support the hypothesis that non-steroidal anti-inflammatory drugs (NSAIDs) and antioxidant therapy might protect against the development of Alzheimer's disease, and ibuprofen has the strongest epidemiological support. In the present work our attention was focused on (R)-α-lipoic acid considered as a potential neuroprotective agent in Alzheimer's disease therapy. In particular, we investigated a new co-drug (1) obtained by joining (R)-α-lipoic acid and ibuprofen via a diamide bond, for evaluating its potential to antagonize the deleterious structural and cognitive effects of β-amyloid (1-40) in an infused Alzheimer's disease rat model. Our results indicated that infusion of β-amyloid (1-40) impairs memory performance through a progressive cognitive deterioration; however, ibuprofen and co-drug 1 seemed to protect against behavioural detriment induced by simultaneous administration of β-amyloid (1-40) protein. The obtained data were supported by the histochemical findings of the present study: β-amyloid protein was less expressed in 1-treated than in ibuprofen and (R)-α-lipoic acid alone-treated cerebral cortex. Taken together, the present findings suggest that co-drug 1 treatment may protect against the cognitive dysfunction induced by intracerebroventricular infusion of β-amyloid (1-40) in rats. Thus, co-drug 1 could prove useful as a tool for controlling Alzheimer's disease-induced cerebral amyloid deposits and behavioural deterioration.

Alzheimer's disease is an age-dependent neurodegenerative disorder that gradually destroys patient memory and cognition in the geriatric population for which there is no effective treatment at present (1-2). The disease is characterised pathologically by senile cerebral plaques constituted chiefly by extracellular deposition of β-amyloid peptide in hippocampal and cerebral cortical regions accompanied by the presence of thread-like neuronal structures that occupy much of the cytoplasm of pyramidal neurons; these neurofibrillary tangles are aggregates of hyperphosphorilated microtubular Tau

Mailing address:
Prof. Antonio Di Stefano,
Dipartimento di Scienze del Farmaco,
Università "G. d'Annunzio",
Via dei Vestini 31, 66100 Chieti, Italy
Tel: ++39 871 3554708 Fax: ++39 871 3554706
e-mail: adistefano@unich.it

protein. Much evidence has indicated that plaques in the Alzheimer's diseased brain are associated with numerous markers for inflammation, including fibrillary astrocytes, reactive microglia, dystrophic neurites and interleukins (3-5). It is well known that the inflammatory process together the β-amyloid deposition might raise reactive oxygen species levels with simultaneous induction of glutathione depletion, the ultimate factor determining vulnerability to oxidant attack (6-8). Further evidence suggests a reduced prevalence of Alzheimer's disease among users of non-steroidal anti-inflammatory drugs (NSAIDs) substantially delaying its onset, given the importance of the effects of inflammatory processes in the brain of Alzheimer's disease patients (9). It has been proposed that NSAIDs exert their beneficial effects by reducing neurotoxic inflammatory response in the central nervous system (CNS). Weggen et al., investigating the effect of various NSAIDs on the production of β-amyloid (1-42) in cell culture, reported that not all NSAIDs affected its production (10-11); in particular, these investigations found that ibuprofen, indomethacin, and sulindac sulphide reduced β-amyloid (1-42) peptide production by direct modulation of γ-secretase, the enzyme that mediates the final proteolytic cleavage of amyloid precursor protein (APP), which liberates β-amyloid (1-42) peptide (12-14). These NSAIDs exhibit a window modulation where β-amyloid (1-42) production is greatly reduced without inhibition of Notch receptors, the key factor of mechanism-based side effects associated with γ-secretase inhibitors treatment.

As mentioned above, free radicals and oxidative stress have been implicated as prime factor responsible for the neurodegeneration in Alzheimer's disease (15). Antioxidant therapy has shown a slight ameliorating effect on the progression of Alzheimer's disease; in particular, several epidemiological studies revealed beneficial effects of diet supplement with vitamins E and C (16-19). However, the use of these natural antioxidants as therapeutic agents is limited, mainly due to their marginal efficiency in crossing the blood brain barrier (BBB). (R)-α-lipoic acid is a dithiol compound normally bound to mitochondrial α-keto acid dehydrogenases that act as an essential co-factor for mitochondrial enzymes involved in the energy metabolism reactions (20-21).

Recent findings highlighted that the cytosolic and mitochondrial dehydrogenases rapidly reduce lipoic acid to dihydrolipoic acid, the active compound responsible for most of the beneficial effects against Alzheimer's disease, such as the increase of acetylcholine production by activation of choline acetyltransferase, the chelation of redox-active transition metals, the increase of reduced glutathione levels and the down-regulation of redox-sensitive inflammatory processes (22). Starting from these data, and in order to enhance the brain availability of NSAIDs, and to take advantage of the synergistic effect of having a powerful antioxidant and an anti-inflammatory agent combined in an unique chemical entity, we investigated co-drug 1 (Fig. 1) obtained by joining lipoic acid with ibuprofen: this compound might permit targeted delivery of ibuprofen and lipoic acid directly to neurons, where cellular stress and inflammation are associated with Alzheimer's disease. The new co-drug 1 showed a high degree of chemical and enzymatic stability under physiological conditions, for these reasons co-drug 1 can afford more efficacious CNS delivery than lipoic acid and ibuprofen alone (23). In this study compound 1 was investigated for its potential to antagonize the deleterious structural and cognitive effects in the β-amyloid (1-40) infused Alzheimer's disease rat model (24). Although in human Alzheimer's disease patients' different molecular species of the Aβ protein have been identified, Aβ (1-40) has been chosen as it is now clear that β-amyloid (1-42) appears the predominant form deposited during the initial stages of plaque formation in Alzheimer's disease whereas Aβ (1-40) is the predominant species in the more advanced stages of the disease and related more closely to the severe cognitive decline in the later phases (25). Moreover, as Aβ (1-40) has a higher affinity to form amyloid fibrils in rats than Aβ (1-42), the β-amyloid (1-40) infused Alzheimer's disease rat model has proven to be particularly useful in development and evaluation of therapeutics (26).

MATERIALS AND METHODS

Chemicals

Ibuprofen and (R)-α-lipoic acid, acetonitrile, trifluoroacetic acid (TFA), dimethylsulfoxide were

obtained from Sigma-Aldrich (Milan, Italy). Co-drug 1 was synthesized as previously reported (23). β-amyloid (1-40) peptide was obtained from Bachem (Switzerland). Anti-Human Beta-Amyloid 1-40 monoclonal antibody was purchased from Alpha Diagnostic International, TX, USA.

Animals

Pharmacological studies: Male Wistar rats (n = 48) (Harlan, UD, Italy), that weighed 200–225 g at the beginning of the experiments, were used. The animals were individually housed in a room on a 12 h light/dark cycle (lights off at 7:00 a.m.) at constant temperature (20–22°C) and humidity (45–55%). Rats were offered food pellets (4RF; Mucedola, Settimo Milanese, Italy) and tap water ad libitum and were handled once a day for 5 min during the first week after arrival. Each animal was weighed weekly throughout the experimental period. All procedures were conducted in adherence to the European Community Council Directive for Care and Use of Laboratory Animals. Pharmacokinetic and biodistribution studies.

Drug preparation

To fill osmotic pump, the β-amyloid (1-40) solution and vehicle solution (vehicle in pump) were prepared on the same day of intracerebroventricular pump implantation. β-amyloid (1-40) was dissolved in a vehicle solution comprising 35% (v/v) acetonitrile and 0.1% (v/v) TFA to achieve a final concentration of 4,6 nmol/200 µl. Ibuprofen and 1 were both solubilized in sterile saline containing 20% (v/v) dimethylsulfoxide and were daily administered subcutaneously for 28 days at a dose of 5 mg/kg and 10 mg/kg, respectively. A vehicle solution (vehicle for subcutaneous injections) prepared with sterile saline containing 20% (v/v) dimethylsulfoxide or a sterile saline, were also administered subcutaneously for 28 days at a dose volume of 250 µl/kg as ibuprofen and 1.

Surgical procedure

After anaesthesia with 10 mg/kg of a mixture of zolazepam and tiletamine (Zoletil 100, Italmed, Italy) by intraperitoneal injection, a stainless steel cannula was implanted in the animal's lateral cerebroventricle using a stereotaxic instrument at the following coordinates, in millimetres with reference to the bregma: anteroposterior (AP), 1.0; lateral (L), 1.8. Coordinates were taken from Paxinos and Watson and adjusted for the body weight of the animals (27). The cannula, connected by a ø 0.8 mm capillary tube to an osmotic pump (Alzet, model 2004, Charles River, Italy), containing either β-amyloid (1-40) solution or the vehicle alone, was quickly placed subcutaneously under the dorsal skin of the rat. The outlet

of the pump was implanted in the left ventricle 3.5 mm dorsoventral to cranium and attached to the skull with screws and dental cement. The content of the mini-osmotic pump (4.6 β-amyloid nmol/rat) at a final volume of 200 µl was delivered for 28 days at a flow of 0.25 µl/h, according to the protocol reported elsewhere (28). All animals were housed individually and received food and water ad libitum.

Behavioural training

The eight-arm radial maze, made of transparent plexiglass, was located in a dimly lit room 70 cm above the floor, with a rich environment of extra-maze cues. The radial maze had a platform (diameter 30 cm) and 8 enclosed arms (51 cm long x 11 cm x 11 cm high) that were separated from the central platform with guillotine doors. In addition, at the end of each arm a small compartment, separated from the rest of the arms by a perforated plate, always contained fresh food in order to saturate each arm with food odours, whereas a low barrier located 5 cm from the distal end of each arm prevented animals from seeing the possible food reward hidden behind it. One week before training, the quantity of home-cage food was reduced, so that the body weight of each rat progressively decreased to 80% of the free-feeding value. During this period, the animals had ad libitum access to water. Experiments were started at 9:00 a.m. Prior to training, each rat was familiarized with the radial maze, food pellets were scattered over the entire maze surface, and the rat was allowed to explore 15 min and to eat food freely.

The task itself was a simplified version of Stepanichev's radial maze task (29). The radial maze version used in this study (1 baited, 7 non-baited arms) makes a distinction between reference memory errors as initial visits to non-baited arms, and working memory errors as subsequent visits to non-baited arms during the same trial. The reference aspect of maze performance requires the animal to learn that only one of the arms has a food reward, and its spatial location relative to the room remains constant for individual rats over the course of the trials. Investigators have shown that this aspect of the task requires learning the association between constant extra-maze visual cues and a baited arm (30). The working aspect of the task consists of remembering which arms have been explored during a single trial and thus represents a kind of short-term memory.

After adaptation, all rats were trained to enter one baited arm of the maze and to avoid the remaining seven non-baited arms. Each trial began by placing the rat in the center platform with all doors closed. After 10 seconds, all doors were opened. Each trial was completed when the rat found the pellet or 5 min had elapsed. Immediately after a

trial the rat was returned to its home cage. A 5 min inter-trial interval was introduced before the start of the next trial and in that time the maze was rotated 45° and cleaned with a deodorant disinfectant in order to prevent animals from using within-maze cues (tactile and olfactory trails) to construct maps of the environment. This procedure forced them to use extra-maze cues exclusively. The rats were given three daily trials and trained for five days. Arm choices were recorded when the rat had placed all of its paws beyond the threshold of the arm. The number of reference memory errors and the working memory errors were recorded separately. The time spent to perform radial maze was expressed as a percentage of time spent to find the pellet or ratio of time spent to find the pellet in 5 min. The rats were given a total of 15 trials during 5 days, and data for the 15 trials were divided into five training sessions (three trials per session per day). The means of the data from each training session for each group were used for statistics. Group factor was used as a categorical predictor to analyze the influence of type of the treatment.

Experimental protocol

The protocol consisted of the following sequence of events.

Experiment 1. - One week after arrival, all animals were tested pre-operatively on their ability to perform an open field test. One week after a food-deprivation schedule, the rats were trained pre-operatively to perform an eight-arm radial maze task for 5 days.

Experiment 2. - The rats underwent stereotaxic surgery for the preparation of β-amyloid-infused Alzheimer's disease model rats. After intracerebroventricular pump implantation, they were daily administered drugs subcutaneously for 28 days. β-amyloid (1-40) or vehicle (see Surgical procedure) was continuously infused into the lateral ventricle of rats for 28 days and contemporaneously ibuprofen, 1 or vehicle were injected subcutaneously. Rats were randomly divided into 6 groups (8 rats per group) as follow: saline control group, control group, β-amyloid group, β-amyloid + ibuprofen, β-amyloid + (R)-α-lipoic acid and β-amyloid + 1 group.

Given the high lipophilicity of compound 1, it was dissolved in a saline solution containing 20% dimethylsulfoxide. For this reason, two different control groups (one injected with saline and the other with saline containing dimethylsulfoxide) were introduced in order to determine possible effects on memory performance attributable to dimethylsulfoxide.

Experiment 3. - One month after surgery, the rats were submitted to an open field test followed by a radial maze task using the same modalities of experiment 1, to assess the effect of drugs (ibuprofen, (R)-α-lipoic acid and 1) on the impairment of learning abilities in β-amyloid-infused Alzheimer's disease model rats.

Experiment 4. - Two months after surgery, the rats were resubmitted to an open field test followed by a radial maze task.

Pharmacokinetic studies and biodistribution

Ibuprofen and 1 were both solubilized in sterile saline containing 20% (v/v) dimethylsulfoxide (final concentration was 20 mg/ml) and were administered subcutaneously at a dose of 5 mg/kg and 10 mg/kg, respectively. A vehicle solution prepared with sterile saline containing 20% (v/v) dimethylsulfoxide was also administered subcutaneously (2.5 ml/kg).

For the pharmacokinetic studies, after slight anaesthesia with carbon monoxide, the blood of rats was collected for the determination of ibuprofen concentration by cardiac puncture from five rats of each group and then collected in vials containing 20 μl heparin (2500 IU ml^{-1}) as anticoagulant. The blood sampling schedules were 15, 30, 60, 120, 240 and 360 min in tubes containing heparin. 0.3 ml of plasma was diluted with 1 ml of 0.2 M phosphate buffer (pH 2.0) and 5 ml of diethyl ether was added. The mixture was shaken for 3 min, and centrifuged at 10,000 × g for 15 min. The ether layer was collected, and the aqueous layer extracted with 5 ml of diethyl ether. The ether layer was added to that obtained previously. The ether phase was evaporated to dryness, and the residue was dissolved in HPLC eluent and injected into the HPLC (31). Analytical HPLC measurements were run on a Waters 1525 Binary HPLC pump, equipped with a Waters 2996 photodiode array detector, a 20-μL Rheodyne injector and a computer integrating apparatus. The column was a Waters Symmetry RP-C$_{18}$ column (4.6 mm×150 mm, 5 μm), the mobile phase was a mixture of KH$_2$PO$_4$ 0.05 M/acetonitrile (25:75) at a flow rate of 1 ml/min, the UV-detector was set at a length of 264 nm.

For evaluating the biodistribution of co-drug 1, rat brains were removed, washed and weighed. Then they were homogenized and diluted with saline to 1:2 (g/ml). The brain sampling schedules were 30, 60 and 120 min. An aliquot of 0.50 ml tissue homogenates was vortexed with methanol (1.5 ml) for 5 min and then 40 μl of 6 M NaOH was added for performing hydrolysis of prodrug. After 5 min of hydrolysis at room temperature, ibuprofen was released from the prodrug, and 40 μl of 6 M HCl was added to neutralize NaOH. Subsequently, the mixture was vortexed with methanol (1.5 ml) for 5 min and then centrifuged at 14,000 × g for 15 min. The supernatant was injected into the HPLC system (32).

Histological examination

After the completion of the behavioural experiment

rats were sacrificed with anhydride carboxide and their whole brains were removed and dehydrated for immunohistochemical and histological analysis.

Light microscopy

Brain samples were fixed in phosphate-buffered formalin solution, dehydrated through ascending alcohols (ethanol 70°, ethanol 90°, absolute ethanol) and xylene and then paraffin embedded. The samples were then de-waxed (xylene and alcohol at progressively lower concentrations) and processed for haematoxylin-eosin staining, Bielschowsky staining according to the manufacturer's instructions (Bio-Optica, Milan, Italy) and for immunohistochemical analysis (3).

Immunohistochemistry

In order to detect β-amyloid (1-40), immunohistochemistry was performed using an UltraVision LP Detection System HRP Polymer & DAB Plus Chromogen kit (Thermo Fisher Scientific, CA, USA) and processed according to the data sheet. Sections (5 μm) were incubated in the presence of mouse Anti-human Beta-Amyloid 1-40 monoclonal antibody and then in presence of HRP conjugated secondary antibody. Peroxidase was developed using diaminobenzidin chromogen (DAB). Nuclei were hematoxylin counterstained. Negative controls were performed omitting the primary antibody. Samples were then observed with a light microscope (Leica, Heidelberg, Germany) equipped with a Coolsnap video camera for computerized images (RS Photometrics, Tucson, AZ).

Computerized Morphometry measurements and Image Analysis

After digitizing the images deriving from immunohistochemistry stained sections, a Metamorph Software System (Universal Imaging Corporation, Molecular Device Corporation, PA, USA) (Crysel Instruments, Rome, Italy) was used to evaluate Aβ(1-40) expression. Image analysis of protein expression was performed through the quantification of the threshold area for immunohistochemical brown colours per field of light microscope observation. Metamorph assessments were logged to Microsoft Excel and processed for Standard Deviations and Histograms.

RESULTS

Behavioural and memory tests

All rats were pre-operatively trained for the study of their behaviour in an eight-arm radial maze. Rats showed no signs of abnormalities throughout the experimental period; body weight values increased as a function of age and no significant differences were observed before or after surgery among the groups (data not shown).

Two animals were excluded from the study due to their inability to perform radial maze: they committed more than one working memory error on the last day of trials. Performance in radial maze did not differ among groups, and the numbers of reference and working memory errors progressively declined with training in all groups studied, indicating that all groups acquired the task at a similar rate and performed with comparable proficiency. Improvement was also observed in other indices of radial maze learning, such as decreases in time spent to perform the task (data not shown). β-amyloid (1-40) was continuously infused into the lateral ventricle of rats for 28 days, contemporaneously ibuprofen, (R)-α-lipoic acid and 1 or vehicle were injected subcutaneously and their effects assessed one and two months after surgery (24).

Pharmacokinetic profile and biodistribution

Plasma concentrations-time profiles of ibuprofen in rats following subcutaneous administration of co-drug 1 in relation to those of ibuprofen itself are shown in Fig. 2: in both cases, ibuprofen was cleared from the plasma in about 6 h, but with co-drug 1 the plasmatic concentrations at 1, 2 and 4 h were significantly higher compared to ibuprofen itself. In order to evaluate the amount of ibuprofen transported across BBB, we measured its brain concentration after subcutaneous injection of co-drug 1 and parent drug (32). As showed in Fig. 3, administration of co-drug 1 exhibited much higher brain concentration of ibuprofen when compared with equimolar dose of ibuprofen itself (P<0.05 60 min after administration) (11.8 and 7.1 μg/g respectively). The increased distribution in brain after the injection of co-drug 1 suggested that our compound behaves like a bioreversible bioconjugate and could enhance the availability of ibuprofen in the brain.

Histological evaluation

Hematoxylin-eosin, $AgNO_3$ stainings together with immunohistochemical analysis of β-amyloid (1-40) protein were employed to assess whether
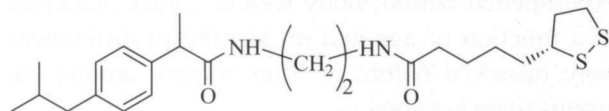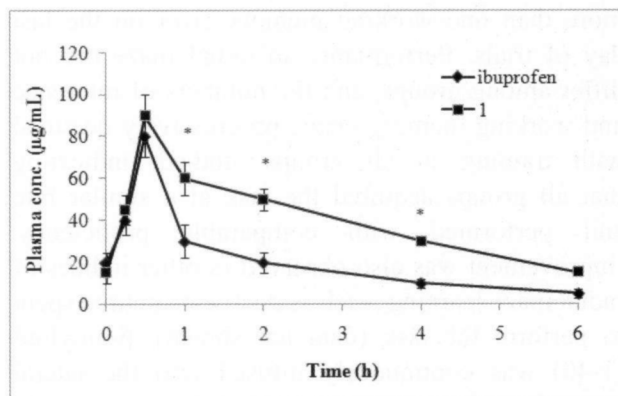
Fig. 1. *Chemical structure of co-drug 1.*

Fig. 2. *Pharmacokinetic profile of ibuprofen and co-drug 1 after subcutaneous administration. Data are expressed as mean ± S.E. Each experiment was performed in triplicate. $^*P < 0.05$ compared to ibuprofen.*
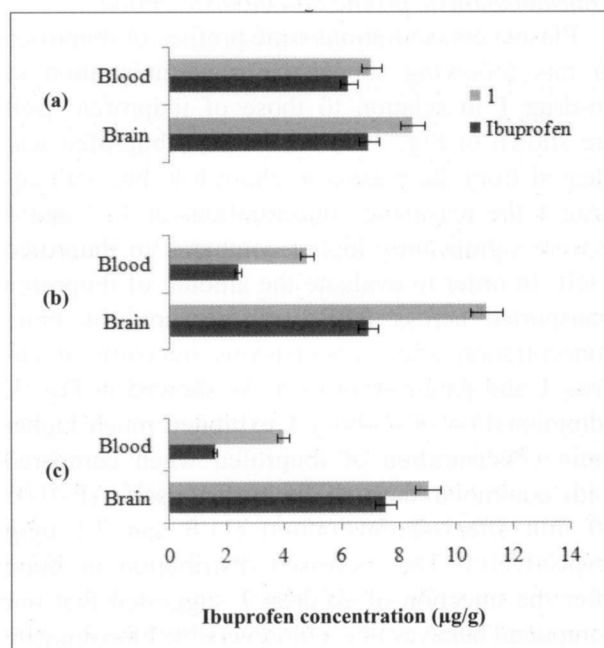

Fig. 3. *Ibuprofen brain and plasma concentration after subcutanueous administration of co-drug 1 and ibuprofen. Data are expressed as mean ± S.E. Each experiment was performed in triplicate. (a): ibuprofen concentration 30 min after administration; (b): ibuprofen concentration 60 min after administration; (c): ibuprofen concentration 120 min after administration.*

inflammatory processes play a role in Alzheimer's disease or in the β-amyloid (1-40) plaques placement (24). Hematoxylin-eosin and $AgNO_3$ stainings in the control sample indicated well-organized cell arrangement within the cerebral cortex, visible nerve connections, and many astrocytes (Fig. 4a and 4g). Cerebral cortices injected with β-amyloid (1-40) and vehicles (Fig. 4c and 4i) showed cell disorganization and dilated capillary vessels. Because the molecular specie β-amyloid (1-40) has a higher affinity to form amyloid fibrils in rats, and its neurodegenerative effect was more pronounced than Aβ (1-42), within the *Cornu Ammonis* (CA1) subfield of the hippocampus, the hippocampal histopathology of β-amyloid (1-40) injected rats was also studied. As showed in Fig. 4l and 4m, hippocampal tissues in rats injected with β-amyloid (1-40) showed cell disorganization and plaque formation (25).

In β-amyloid (1-40) treated rats, the cerebral cortex had disorganized neuronal cells in reduced number, along with few astrocytes and slightly dilated capillary vessels (Fig. 4c and 4i). Ibuprofen and (R)-α-lipoic acid treated cerebral cortex appeared morphologically similar to the previous β-amyloid (1-40) treated ones, even though axon number was also reduced (Fig. 4d, 4f and 4j). In β-amyloid-infused animals treated with co-drug 1, cells appeared well organized and astrocytes and axons seemed well preserved even though capillary vessels were still dilated (Fig. 4e and 4k).

Immunohistochemical analysis

Immunohistochemical analysis of β-amyloid (1-40) protein, which accumulated within capillary vessels, was performed, disclosing many different sized plaques within capillary vessels in the cerebral cortex and hippocampus, depending on rat treatment (Fig. 4l, 4m, 4n-t and Table I). β-amyloid-injected cerebral cortices treated with ibuprofen, (R)-α-lipoic acid or compound 1 showed few plaques within capillary vessels and, in particular, β-amyloid (1-40) protein was less expressed in co-drug 1-treated than in ibuprofen- or (R)-α-lipoic acid-treated cerebral cortex.

Statistical analysis

The results are expressed as means ± S.E. For intergroup differences, the data were analyzed by
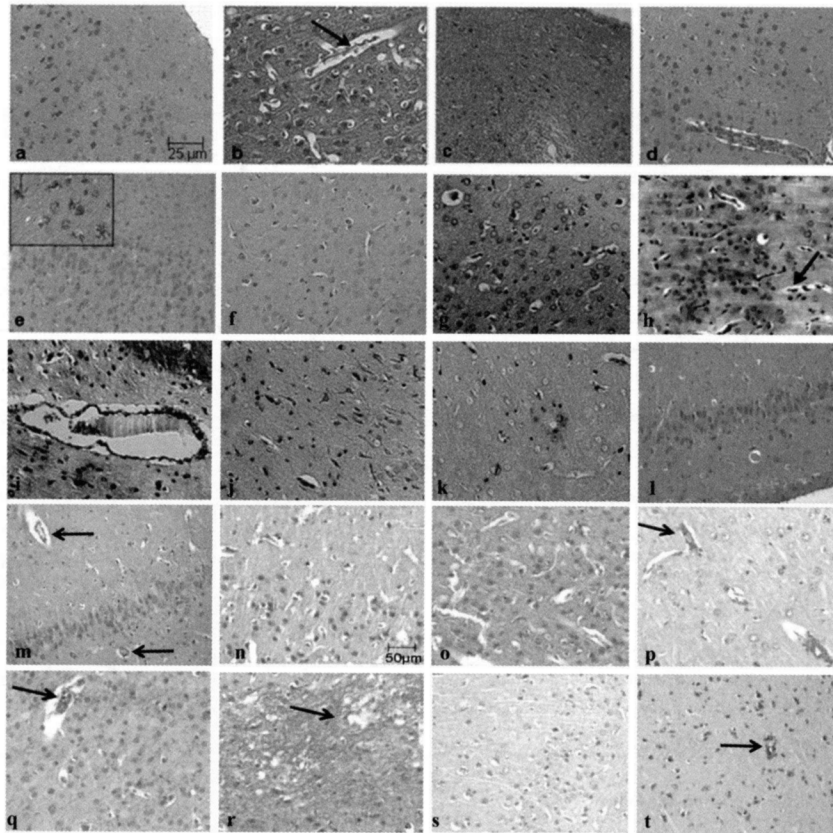
Fig. 4. *Haematoxylin-eosin (a-f), Bielschowsky (g-k) staining and Immunohistochemical detection (n-t) in cerebral cortex of rat brain in different experimental conditions. Magnification 20x. Haematoxylin-eosin staining (l) and himmunohistochemical detection (m) in hippocampal tissue of rat brain injected with β-amyloid. (a, g and n): control; (b, h and o): Dimethylsulfoxide/drug vehicle injected; (c, i, p, l and m): β-amyloid injected; (d, j and q): β-amyloid injected + ibuprofen; [e (the magnification (40x) indicates well-preserved astrocytes), k and r]: β-amyloid injected + 1; (f and t): β-amyloid injected + lipoic acid; (s): negative control. Arrow indicates dilated capillary vessels (b and h) or β-amyloid plaques (m, p, q, r and t).*

one-way ANOVA followed by Newman-Keul test post hoc comparison.

DISCUSSION

In this study, we investigated whether co-drug 1 can improve learning and memory impairment in an infused Alzheimer's disease rat model. One month after surgery we studied the influence of β-amyloid (1-40) and drug treatment on spatial and memory learning. As shown in Fig. 5, data from the same training session did not appear significantly different between the two control groups (saline control = rats infused with β-amyloid (1-40) peptide solvent and then treated subcutaneously with sterile saline,

control group = rats infused with β-amyloid (1-40) peptide solvent and then treated subcutaneously with dimethylsulfoxide, the solvent used to solubilize drugs) ($P>0.05$), thus excluding a possible influence of the different solvents. On the third, fourth and fifth training sessions, the β-amyloid group exhibited a significantly higher number of working memory errors ($P<0.05$) compared with all the other groups (Fig. 5a). In particular, the treatment with 1 or ibuprofen during the same period of β-amyloid (1-40) infusion suggested that the decrease in the working memory errors was attributable to the positive effect of the anti-inflammatory agents. Some differences were observed between the β-amyloid + ibuprofen group and the β-amyloid + 1 group on

a): Working memory errors

b): Reference memory errors

c): % time spent to find pellet
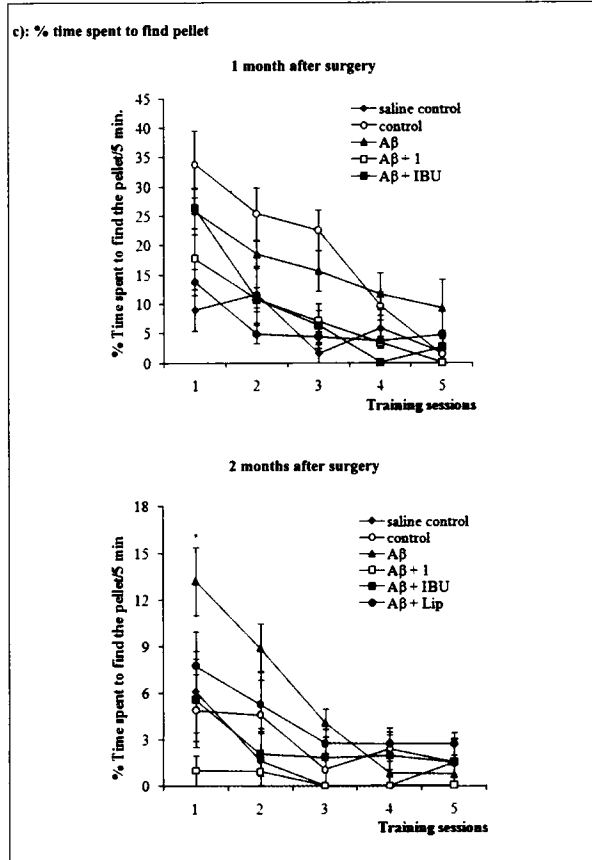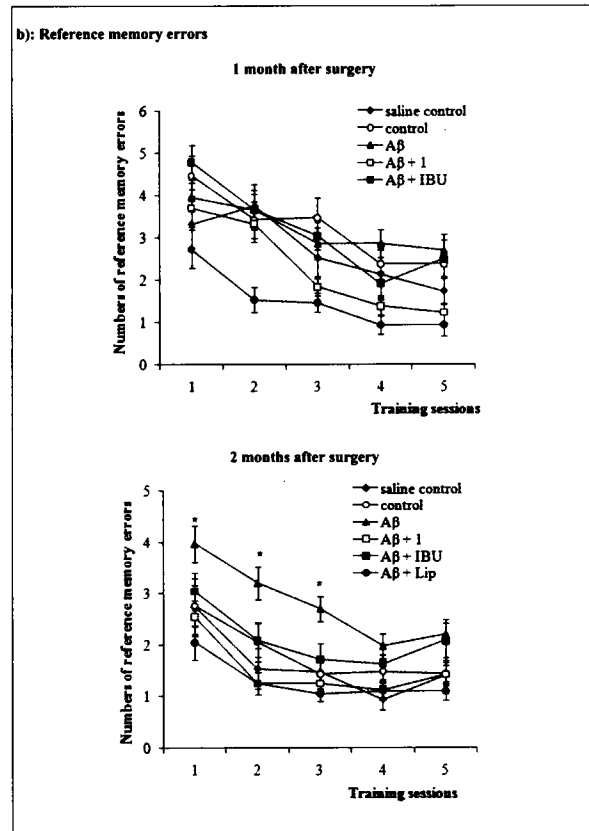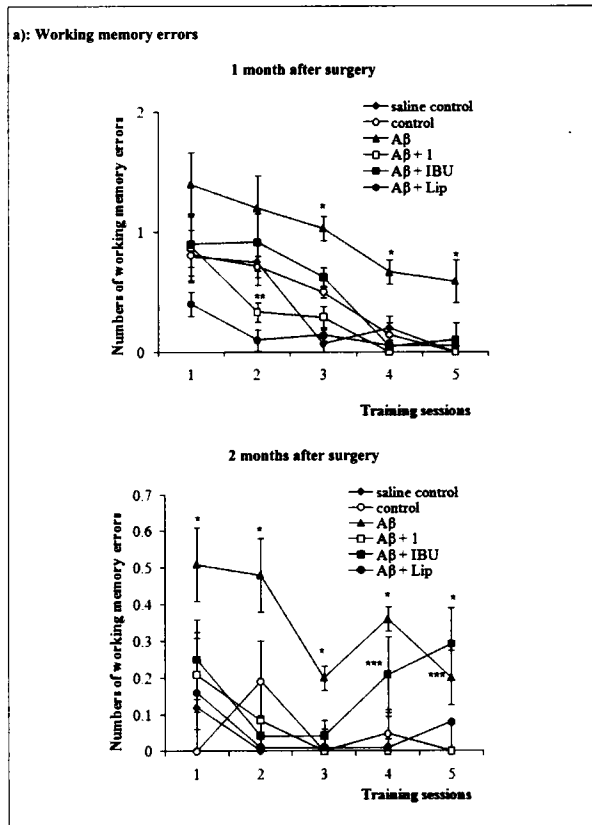
Fig. 5. *Effects of drug administration on the numbers of working memory errors (a), on the numbers of reference memory errors (b) and on the % time spent to find the pellet (c). One and two months after surgery, testing was performed over five training sessions. Each point represents the mean ± S.E. of 3 trials from the daily training session.*

*$P < 0.05$ compared to all group; **$P < 0.05$ compared with β-amyloid and β-amyloid + ibuprofen groups; ***$P < 0.05$ compared with saline control, control and β-amyloid + 1 groups.

the second session day: **1** treatment statistically decreased the number of working memory errors much more than ibuprofen treatment ($P<0.05$), suggesting that **1** affords greater neuroprotective effects (Fig. 5a). As shown in Figs. 5b and 5c, there were no differences in reference memory errors or long-term memory among groups over the training sessions and in the time spent to perform radial maze ($P>0.05$). The ability of the β-amyloid group to re-acquire the previously learned spatial task was

Table I. *Densitometric analysis of β-amyloid positive area/field (76000 μm²), expressed as percentage ± SD, acquired at 20x magnification on fifteen different sections per experimental point using MetaMorph Software System. Differently sized β-amyloid plaques are distributed within the cerebral cortex of ill rats, as shown in Fig. 4n-t.*

Experimental point	Positive area %	Large plaques	Small plaques
Control cerebral corteces	-		
Dimethylsulfoxide/drug vehicle injected cerebral corteces	-		
β-amyloid injected cerebral corteces	1.76 ± 0.09	7.0 ± 0.1	14.0 ± 1.1
β-amyloid injected cerebral corteces + ibuprofen	0.12 ± 0.01	6.0 ± 0.5	4.0 ± 0.3
β-amyloid injected cerebral corteces + 1	0.17 ± 0.01	4.0 ± 0.3	3.0 ± 0.3
β-amyloid injected cerebral corteces + (R)-α-lipoic acid	0.11 ± 0.01	5.0 ± 0.5	4.0 ± 0.3

not impaired following the peptide injection. Thus, intracerebroventricular infused β-amyloid (1-40) induced impairments of working memory without any effect on reference memory, one month after surgery.

Two months after surgery, we reassessed the cognitive abilities of rats using the protocol similar to experiments 1 and 2 in order to monitor progressive behavioural deterioration or recovery in the same animals (Fig. 5a-c). Data of the β-amyloid group for working memory (Fig. 5a) and reference memory errors (Fig. 5b) showed that β-amyloid (1-40) infusion decreased the performance on the first, second and third training sessions compared to all groups (P<0.05). These results indicated the progression of cognitive deterioration induced by β-amyloid infusion, affecting both short-term and long-term memory, two months after surgery. In addition, the β-amyloid treatment markedly increased the time spent to perform radial maze (expressed as % time spent to find the pellet) compared with the data of all groups (P<0.05) on the first training session (Fig. 5c). The treatment with anti-inflammatory drugs was effective in protecting against cognitive deterioration. In particular, the improvement of spatial cognition induced by 1 treatment was statistically more

pronounced than that observed with ibuprofen treatment (P<0.05): the values of working memory errors on the fourth and fifth training sessions of the 1-treated rats were on the same level as those of the control and saline control groups (Fig. 5a).

The open field task is a sensitive tool for detecting possible motor dysfunctions induced by treatment with drugs under study; it also helps ensure that the differences observed in eight-maze performance among groups should not be attributed to locomotor activity alterations or other factors, rather than to differences in spatial memory. In addition, analysis of behavioural activity in the central zone of the field can give general information on the anxiety status of the animal (33).

Locomotor activities of all rats were tested pre-operatively in an open field, and performance among groups was comparable; statistical analysis of pre-operative data did not reveal differences among groups (data not shown). One month and two months after surgery, spontaneous locomotor activity parameters were monitored. No changes of general locomotor activity and behavioural activity in the central zone of the field were observed among the groups (data not shown).

In summary, this report describes the effects of

co-drug 1 on chronic treatment following bilateral intrahippocampal infusion of β-amyloid (1-40) protein. Our results indicated that infusion of β-amyloid (1-40) impairs memory performance through a progressive cognitive deterioration affecting both short- and long-term memory; however, ibuprofen and co-drug 1 seemed to protect against behavioural detriment induced by simultaneous administration of β-amyloid (1-40) protein. In particular, the improvement of spatial cognition induced by 1 administration was more pronounced than that observed with ibuprofen treatment. These data were supported by the histochemical findings of the present study: β-amyloid protein was less expressed in 1-treated than ibuprofen-treated cerebral cortex. Taken together, the present findings suggest that co-drug 1 treatment may protect against the oxidative stress generated by reactive oxygen species and the cognitive dysfunction induced by intracerebroventricular infusion of β-amyloid (1-40) in rats. Thus, co-drug 1 could prove useful as a tool for controlling Alzheimer's disease-induced cerebral amyloid deposits and behavioural deterioration.

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