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NEUROBEHAVIORAL AND NEUROCHEMICAL EVALUATION OF EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) INHIBITOR –GEFITINIB IN B-AMYLOID OLIGOMER INDUCED ALZHEIMER'S DISEASE IN MICE MODEL

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Abstract

Background:Alzheimer's disease (AD) is a progressive neurodegenerative disorder that accounts for about 60-80% of dementia cases globally.Currently used AD drugs provide only symptomatic management with lots of adverse effects. A drug with potential curative for AD with negligible adverse effects remains to be investigated. Gefitinib (GE) is anepidermal growth factor receptor(EGFR) inhibitor, commonly used as monotherapy innon-small cell lung cancer and other solid tumours. Very few studies have shown the memory rescuing capacity of GE.***Aim:*** Therefore the present study was designed to investigate the neuroprotective effect of EGFR inhibitor - GEthrough neurobehavioral and neurochemical analysis in A$\beta_{1\text{-}42}$oligomer induced AD in a mice model. ***Methods:***AD induction was done by intracerebroventricular (*i.c.v.*) injection of A$\beta_{1\text{-}42}$ oligomer (4 µg/4 µl) into the lateral ventricles of mice brain. The test compounds *i.e.,*GE (2 and 4 mg/kg of body weight) was administered orally on day 10, 13, 16, 19, 22, 25, and 28; and reference drug *i.e.,* donepezil (DP, 2 mg/kg) were administered orally from the 10th to 28th days. The AD-associated neurobehavioral changes were evaluated by the novel object recognition test (NORT) and the neurochemical biomarker*i.e.,*neuron-specific enolase (NSE) levels were estimated from brain hippocampal, cortex, and cerebellar samples. ***Results:*** The administration of GE was shown to ameliorate the A$\beta_{1\text{-}42}$ induced neurobehavioral and neurochemical changes. These results were similar to the reference drug donepezil-treated group. ***Conclusion:***EGFRinhibitor -Gefitinib ameliorates the A$\beta_{1\text{-}42}$ induced AD pathology via multiple molecular pathways.

Keywords: Donepezil, intracerebroventricular injection, novel object recognition, neurotoxin, neuron-specific enolase.

Introduction

Alzheimer's disease (AD) is one of the major neurodegenerative disorders responsible for more than 60-80 % of dementia cases globally [1]. AD is pathologically characterized by

intracellular neurofibrillary tangles and extracellular amyloid- β (Aβ) deposition in a compact structure between neurons.Amyloid- β is formed from a larger protein unit named amyloid precursor protein (APP) via breakdown by the enzymes, α, β, and γ-secretases, and the breakdown product is deposited as the extracellular plaques known as senile plaques [2,3]. The formation and deposition of amyloid-β is a major cause of neuronal death in vulnerable regions of the brain such as the hippocampus and neocortex, which induces behavioral and functional deficits of AD [4]. The drugs currently available in usage such as cholinesterase inhibitors, orexin receptor antagonists, glutamate regulators, and others provide only symptomatic relief with lots of adverse effects [5-7].Therefore a drug with potential neuroprotectivityand fewer adverse effects is the need for time. Gefitinib (GE)is a selective inhibitor of epidermal growth factor receptor's (EGFR) tyrosine kinase domain. Also referred to as a cancer growth inhibitor. GE is a synthetic compound belonging to the class of Quinazolinamines. Chemically-N-(3 chlorofluorophenyl)-7-methoxy-6-(3-morpholinopropoxy)-quinazolin-4-amine [8]. GE is commonly used as monotherapy in patients withnon-small cell lung cancer(NSCLC), apart from this it is also used in the treatment of certain types of Head and neck, breast, oral, prostate, and colorectal cancers and it selectively targeted the mutant proteins in malignant cells [9-11]. The target protein (EGFR) includes a family of receptors that consist of Her 1 (erb-B1), Her 2 (erb-B2), and Her 3(erb-B3). In certain types of human carcinomas such as lung and breast cancers, the EGFR is overexpressed in the cells which further leads to inappropriate activation of the anti-apoptotic Ras signaling cascade, thereby leading to uncontrolled cell proliferation. GE inhibits EGFR tyrosine kinase by the mechanism of binding to the adenosine triphosphate (ATP)-binding site of the enzyme, through this process it inhibits autophosphorylation of EGFR and blocks the downstream signaling [12]. As well asit inhibits the function of EGFR tyrosine kinase in activation of the anti-apoptotic Ras signal transduction cascade, and thereby inhibits malignant cell proliferation[13,14]. At growth-inhibitory concentrations GE was also shown to inhibit the mitogen-activated protein kinase (MAPK) activity in human A431 and DiFi cancer cell lines, these cancer cell lines overexpress the EGFR [15]. The anti-cancer effect of GE is well explored, however, its other possible therapeutic potential especially related to neurodegeneration is seldom analyzed. In the present study, we used two independent parameters like behavioral screening and neurochemical analysis to explore the ameliorative potential of the EGFR inhibitor GE in A$\beta_{1\text{-}42}$ oligomer-induced neurotoxicity in the AD mice model.

Materials and methods

Animal

Disease-free male Swiss albino mice (12 months old; 20-35 g) were used in this research work. Animals were maintained in the central animal house of AIMST University with a

standard laboratory diet (Soon SoonOilmillsSdnBhd, Penang, Malaysia). The animal was allowed to access the free water *ad libitum*. The 12 hours of natural light and dark cycles were maintained. The macro-environmental temperature and humidity of animal houses were made at 25 °C and 50%. The experimental protocol was approved by AIMST University Animal Ethics Committee (AUAEC/FOM 2020/02 – Amendment No. 1). The caring of animals was done as per the guidelines of AUAEC.

Chemicals

Amyloid ($A\beta_{1-42}$; Biotek Abadi, Cayman Chemicals, USA), gefitinib (SML1657, Sigma, USA), donepezil (Alkem Laboratories Limited, Lower Parel, Mumbai, India), NSE (Biotek Abadi, Elabscience, USA), ketamine hydrochloride injection (Dechra Pharmaceuticals PLC, United Kingdom), xylazine Injection (XYLAMAX®, Bimeda Canada), bovine serum albumin were purchased from Merck & Co., Inc., Japan.

Preparation of $A\beta_{1-42}$ oligomer

Before intracerebroventricular (*i.c.v.*) injection, the $A\beta_{1-42}$ oligomer solution was freshly prepared. Briefly, $A\beta_{1-42}$ protein was dissolved in filtered phosphate-buffered saline (PBS: 1 µg/µl). It consists of 10 mM sodium-dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), and 100 mM of sodium chloride (NaCl) were dissolved in glass-distilled deionized water (pH = 7.5). At a temperature of 37°C,the $A\beta_{1-42}$ solutions were then incubated for over 3 days before use[16].

Induction of AD mice model

AD induction in mice was done by *i.c.v.* injection of $A\beta_{1-42}$oligomer under anesthesia by a mixture of ketamine (75 mg/kg) and xylazine (5 mg/kg). According to the established procedure by Paxinos and Franklin (2004)[17], a total of (4 µg/4 µl) of $A\beta_{1-42}$ oligomer solution was injected into the lateral ventricles of mice brain (on each side 2 µl) at stereotaxic coordinates (anteroposterior- 0.2 mm; mediolateral - 1.0 mm; dorsoventral - 2.5 mm) taken from the atlas of the mouse brain [18]. Animals in the normal group received a 0.9% NaCl injection and animals in the other groups were injected withan $A\beta_{1-42}$ solution.

Experimental protocol

Five groups of adult male Swiss albino mice (n = 8) were used in this study. Group-I served as normal control. Group IIwas the AD group, where AD induction was done by *i.c.v.* injection of $A\beta_{1-42}$ oligomer (4 µg/4 µl)into the lateral ventricles of mice brain (2 µl on each side). Group III and IV served as test compound treatment groups *i.e.,*GE atdoses 2 and 4 mg/kg respectively,with oral administration on 10, 13, 16, 19, 22, 25, and 28th days. Group V served as a reference drug treatment group *i.e.,* donepezil (DP, 2 mg/kg) was orally administered fromthe

10^{th} to 28^{th} days. Thereafter, from the 19^{th} to the 21^{st} day, behavioral training for the novel object recognition test (NORT) was given and on the 22^{nd}-day NORT test was carried out. On the 28^{th} day, the animal was sacrificed and brain tissue samples were collected for neurochemical estimation of neuron-specific enolase (NSE) level in the hippocampus, cortex, and cerebellum tissue of mice brain.

Neurobehavioral assessment bynovel object recognition test (NORT)

The NORT was modified from a previously described method by Yuede*et al.* (2009)[19]. A sound-proof evenly illuminated solid plastic box (45 cm × 45 cm × 45 cm) apparatus was used in this experiment. The NORT behavior assessment pattern was illustrated in Figure 1.The NORT assessment procedure consisted of 4 phases: pre-habituation phase, habituation phase, training phase, and testing phase. On the first day of the experiment, 30 min before the commencement of experiment, animals were brought to the testing room to familiarize themselves with the experimental environment. Mice were allowed freely to explore the box for 5 min in the absence of any objects.On the 2^{nd} and 3^{rd} days,mice werehabituated to the empty box for 20 min each day. The training phase and the testing were conducted on the 4^{th} day; each mouse was given a training trial followed by a testing trial. During the training trial, two identical objects (A and A1) were placed at two opposite positions within the box at equal distances from the nearest corner. The mice were allowed to explore the identical objects for 10 min afterthatmice were returned to their home cages. One hour later, the testing phase was conducted, the animals were placed back in the same box, out of the two familiar objects, one was replaced by a novel object (N), and testing was conducted for 10 min. It was made sure that all the objects used in the study were different in colors and shapes but almost identical in size. To avoid displacement of the object, the objects were fixed to the floor of the box. After each trial, all the objects and the entire box were thoroughly cleaned with 70% vol/vol ethanol to exclude the olfactory cues. Object exploration time was defined as the time duration when an animal points its nose withina 2-3 cm distance to the object, or pawing or sniffing the object. Mere sitting or standing near the object without active sweeping of vibrissae or sniffing does not count as exploration time. Using 2 stopwatches, the exploration time was analyzed manually. In the training session, the object exploration time for the two similar objects (A and A1)was recorded individually, from this the location preference (LP) for objects (A and A1) was calculated. In the testing phase, the object exploration time for one of the familiar objects and the exploration time for the novel object (N) were recorded. In the present study, the familiar object A was kept as such,and object A1 was replaced with a novel object. Therecognition index (RI) was calculated from the object exploration time, using the following formula:

$$Location\ preference\ (LP)\ = \frac{Time\ exploring\ one\ of\ the\ identical\ objects}{Time\ exploring\ the\ identical\ object\ pairs} X\ 100\%$$

$$Recognition\ index\ (RI) = \frac{Time\ exploring\ the\ novel\ object}{(Time\ exploring\ novel\ object\ +\ Time\ exploring\ familiar\ object)} X\ 100\%$$

The Location preference was used as an environmental control; it should be 50%, to rule out the influence of the location of the object. Animals that showed a total exploration time of less than 20 seconds during the testing phase were excluded from the study analysis.

Figure 1: NORT behavior assessment pattern.

Neurochemical estimation

On the 28th day, the animals were anesthetized with diethyl ether. Thereafter, all the animals were sacrificed and brain tissues – cortex, hippocampus, and cerebellum were collected for the estimation of brain neurochemical NSE level.

Estimation of NSE as an indication of neuronal damage

Using the commercial enzyme-linked immunosorbent assay (ELISA) kit method, the brain tissue NSE levels were estimated. Briefly, 50 µl of standard samples were placed in appropriate wells of a microtiter plate. Thereafter, 50 µl of antibody cocktail were added to all wells and incubated at room temperature for 1 hour. Then, the microtiter plate fluid was aspirated and washed all the wells three times with 350 µl of wash buffer (1 X). Finally, 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) solution was added. The reaction of TMB and horse reddish peroxidase (HRP) enzyme was then stopped by the addition of 100 µl of stop solution containing the hydrochloric acid. TMB acted as a chromogenic substrate for the HRP enzyme. The colourless TMB was turned to blue colour (TMB^{+}). Further, this blue colour turned to yellow

colour (TMB^{2+}) upon the addition of the stop solution. Using a spectrophotometer (DU 640B Spectrophotometer, Beckman Coulter Inc., CA, USA) at 450 nm, the changes in absorbance were recorded. The absorbance of blank (zero standards) with the substrate was recorded. The measurement of absorbance with variable standard NSE concentrations *i.e.,* 0, 312.5, 625, 1250, 2500, 5000, 10000, and 20000 picograms per milliliter (pg/ml) was prepared as per the commercial ELISA kit instructions. With absorbance value (y-axis) versus each standard concentration (x-axis) the standard curve was prepared. The NSE activity level was quantified by using the following formula.

$$\mathbf{NSE} = \delta\,\mathrm{O.D.\ blank\ control} - \delta\,\mathrm{O.D.\ value\ against\ the\ standard\ curve\ X\ DF}$$

In the above formula, 'δ O.D.' represented the changes in absorbance/minutes; and DF represents the dilution factor. The NSE activity level was recorded as ng/ml.

Statistical analysis

All the results were expressed as mean ± standard deviation (SD). The behavioral data were statistically analyzed using two-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test and data of tissue biomarkers *i.e.,* NSE levels were analyzed using one-way ANOVA followed by Tukey's Multiple Range test using a Graph pad Prism version-5.0 software. The value of $p < 0.05$ was considered to be statistically significant.

Results

Effect of GE in AD-induced neurobehavioural changes

The present study revealed that*i.c.v.* injection of oligomeric $A\beta_{1-42}$ into the lateral ventricles of mice brain as shown significant ($p < 0.05$) loss of memory and cognitive function in NORT in comparison with the normal animal group. The administration of GE (2 & 4 mg/kg) significantly ameliorated the above behavioral changes in a dose-dependent manner when compared with the AD group. GE (4 mg/kg) showed a similar effect to the comparison reference drug *i.e.,* DP (2 mg/kg) treated group. The details are described in the following section.

Effect of GE in NORT

The $A\beta_{1-42}$oligomer (4 µg/4 µl; *i.c.v.*) induced AD mice showed significant ($p < 0.05$) impairment of memory and cognitive dysfunction in the NORT in comparison with the normal control group animals. During the training phase the object exploration for the two similar objects (A and A1), showed no significant difference in all the groups. However, during the testing phase with two different objects, one novel object (N) and one familiar object showed significant recognition ability forthe novel object than the familiar object in the normal group. But $A\beta_{1-42}$ treated AD group did not show any differences in the recognition ability when compared with the normal group. The administration of GE (2 and 4 mg/kg; *p.o.*) showed a high novel object recognition response. Among these, GE (4 mg/kg; *p.o.*) has shown a more significant novel object recognition ability thereby indicating a good cognitive ability and rescue

of memory when compared with the AD group. The ameliorative effects were showna similar effect to the effect of the reference drug *i.e.,* DP (2 mg/kg; *p.o.*) treated group. The results were tabulated in Table 1.

Table 1. Effect of GE in LP and RI responses in NORT assessment.

	Training Session		**Testing Session**	
Groups	**LP -A**	**LP-A1**	**LP -A**	**RI**
Normal	50.5 ± 2.4	49.4 ± 1.4	31.8 ± 1.3	68.1 ± 1.8
AD	51.2 ± 2.1	48.9 ± 1.9	51.4 ± 1.6 [a]	48.9 ± 1.9 [a]
AD + GE (2)	49.4 ± 1.5	50.5 ± 1.3	42.8 ± 1.5 [b]	57.1 ± 1.5 [b]
AD + GE (4)	49.3 ± 2.8	50.6 ± 1.8	32.7 ± 1.1 [b]	67.2 ± 1.4 [b]
AD + DP (2)	51.8 ± 2.3	48.7 ± 1.6	33.6 ± 1.2 [b]	66.3 ± 1.7 [b]

Digits in parenthesis indicate a dose of mg/kg. Data were expressed as mean ± SD, n = 8 mice per group. [a]$p < 0.05$ Vs normal group. [b]$p < 0.05$ AD group. *Abbreviation:* AD, Alzheimer's disease; DP, donepezil; GE, gefitinib; LP-A, location preference for familiar object A; LP-A1, location preference for familiar object A1; and RI, recognition index.

Effect of GE in Aβ oligomer induced AD neurochemical changes

The administration of $A\beta_{1-42}$-oligomer (4 µg/4 µl; *i.c.v.*) showed significant ($p < 0.05$) alteration of brain tissue biomarkers *i.e.,* an increase in NSE levels in all regions of the brainsuch as the hippocampus, cerebral cortex, and cerebellum in comparison with the normal control group. The administration of GE (2 and 4 mg/kg; *p.o.*) significantly attenuated the $A\beta_{1-42}$-oligomer-induced brain neurochemical changes when compared with the AD group in a dose-dependent manner. These ameliorative effects of GE were shown similar to the effect ofthe reference drug *i.e.,* DP (2 mg/kg; *p.o.*) treated group. The results were indicated in Table 2.

Table 2. Effect of GE on the NSE level of brain tissues.

Groups	**Hippocampus**	**Cortex**	**Cerebellum**
Normal	24.7 ± 1.7	19.2 ± 1.5	17.1 ± 1.2
AD	44.2 ± 2.9 [a]	36.8 ± 2.4 [a]	32.3 ± 1.9 [a]
AD + GE (2)	33.7 ± 2.1 [b]	27.9 ± 1.6 [b]	25.1 ± 1.3 [b]
AD + GE (4)	25.2 ± 1.7 [b]	18.5 ± 1.2 [b]	18.1 ± 1.9 [b]
AD + DP (1)	30.1 ± 1.6 [b]	23.9 ± 2.1 [b]	21.2 ± 1.4 [b]

Digits in parenthesis indicate dose mg/kg, and the value of NSE level was expressed as ng/mg of protein. Data were expressed as mean ± SD, n = 8 mice per group. $^{a}p< 0.05$ Vs normal group. $^{b}p< 0.05$ Vs AD group. *Abbreviation:*AD, Alzheimer's disease; DP, donepezil; and GE, gefitinib.

Discussion

The administration of $A\beta_{1-42}$-oligomer has shown significant ($p< 0.05$) induction of AD which was reflected in the neurobehavioral and neurochemical changes.Data analysis of the present study showed that GE exhibited a potential ameliorative effect against the $A\beta_{1-42}$ oligomer-associated ADchanges and restored the behavioral and neurochemical changes close to the normal level. These indicates a potential ameliorative effect of GE against the $A\beta_{1-42}$ oligomer-associated AD. Previous research reports statedthe neurotoxic effect of $A\beta_{1-42}$ oligomer in gradual enhancing of the β-amyloid deposition and tau protein accumulation. Moreover, it also enhances the formation of senile plaque in the brain regions of the hippocampus and cortex with cognitive dysfunction and neuronal death, which leads to the progression of mild to severe AD [20,21]. Further studies state that $A\beta_{1-42}$ accumulation also producesoxidative stress and promotes microglial activation [22,23]. Inflammatory mediators and the accumulation of free radicals lead to the neurodegenerative process [24]. Behavioral assessment from NORT showed that GE group animals exhibited a significant recognition ability of novel objects in NORT in comparison with the AD group and the effects were similar to the effect of reference drug *i.e.,* donepezil treatment, indicating thatGE rescued the $A\beta_{1-42}$ oligomer-induced memory loss and cognitive dysfunctions.Our study result lies in parallel with a few other research reports that state the memory rescuing potential of GE [25,26]. Though a large volume of data is available regarding the anti-tumor mechanism of EGFR inhibitor –GE, very seldom its effect on memory and neurodegeneration has been studied. Wang et al conducted a study to determine the molecular mechanisms underlying the pharmacological and genetic effects of EGFR in Aβ-induced memory loss, they assayed the EGFR activation level in the hippocampus region of double transgenic mice through Western blotting.Observations showed that the activated form of phosphorylated EGFR(p-EGFR) level was significantly increased in the mice hippocampus. After 18 days of treatment with GE, the increased p-EGFR level was brought back to a similar level to that of the control group mice, this showed that elevated EGFR activity is well correlated with the Aβ-induced memory loss. Immunoprecipitation studies showed that both Aβ42 monomers and oligomers were pulled down with wild-type EGFR (EGFRwt). The results obtained from this mechanism-guided study support the hypothesis that EGFR functions as a cell membrane receptor of Aβ peptides, also the Aβ oligomers-induced activation of EGFR plays a crucial role in leading to memory loss [26]. Moreover, the administration of $A\beta_{1-42}$ oligomers induced potential alteration of the neurochemical *i.e.,* raised NSE levels in the hippocampus, cerebral cortex, and cerebellum of mice brain samples. NSE is expressed in central and

peripheral neurons and also in neuroendocrine cells, which can exist as either γγ or αγ dimeric isozymes.The γγ form of NSE is predominant in neurons, whereas the supporting glial cells such as microglia, oligodendrocytes, and astrocytes express both the αγ form of NSE and non-neuronal enolase (NNE, α-enolase) [27,28]. Early studies suggested that NSE could be a more potent biomarker for assessing and evaluating neuronal damage and the prognosis of brain injury and brain lesions [29-31]. Previous studies conducted on the investigations of NSE with relevance to AD, as revealed inconsistent findings withfew studies stating elevated NSE levels as a biomarker for AD [32,33] and some studies stating severity-dependent levels [34], also few other studies stating unaltered levels of NSE [35,36], or even decreased levels of NSE [37]. In the present study, we estimated the NSE level from the brain homogenate of animals in all the groups. AD group mice's brains exhibited an increase in the level of NSE when compared with the normal control group mice; these results were similar to the previous study results [32,33]. However, the administration of GE (2 and 4 mg/kg; *p.o.*) significantly ameliorated this $A\beta_{1-42}$-oligomer-induced neurotoxicity by restoring to the normal level of NSE and the effects were similar to the reference drug DP (2 mg/kg) treated group. Few studies conducted on the antioxidant potential of GE have shown properties like DPPH(2,2-dipheny l-1-picrylhydrazyl) radical scavenging and hydroxyl radical scavenging[38]. This antioxidant property can be a factor in bringing back the neurochemical NSE close to the normal level.

Conclusion

The administration of EGFR inhibitor *i.e.,*GE has shown to ameliorate the $A\beta_{1-42}$ oligomer-induced neurotoxicity due to its potent inhibitionof activated EGFR, anti-oxidant, and anti-lipid peroxidative effect. Therefore GE can be a novel synthetic medicine for the management of $A\beta_{1-42}$-induced neurodegeneration like AD and other kinds of dementia.

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