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AMYLOID PROTEIN PROCESSING AND POTENTIAL TARGET OF ALZHEIMER DISEASE

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Abstract
Human stem cell models serve as the potential to provide platforms for phenotypic screens to recognize candidate treatments and associated cellular pathways involved in the pathogenesis of Alzheimer disease (AD). Amyloid precursor protein (APP) processing and their derivatives like amyloid-β (Aβ) peptides generation are crucial processes involved in Alzheimer's disease (AD). Identification of modulators of APP processing was processed phenotypically to screen out a small-molecule in TS21 derived neurons. Avermectins identified as modulators in neural cells, can raise the relative production of non-toxic short Aβ peptides in human cortical neurons at the expense of potentially more toxic longer peptides. Further, it was demonstrated that this avermectins effect is not only just because of interaction with the core γ-secretase and it iscrucial for Aβ production. Although this study reflects the avermectinscould be prime targets of GABAA or glycine receptors, and may be possible reasons which affect Aβ peptides processing.
Keywords: avermectin, amyloid, γ-secretase, neural cells, Alzheimer, pathogenesis

1. Introduction

Aging is a complex phenomenon that has been widely accepted as a major risk factor for the development of neurodegenerative disease like Alzheimer. Majority of population around the world are severely affected by Alzheimer disease (AD) (Bos et al. 2018), thereby; impact of this disease on public health system is deliberating growing. Alzheimer's disease cases were almost double every five years after age 65 (Corrada et al. 2008). The basic reason behind this critical situation is due to the lack of approved therapeutic measures as it concerning molecular mechanisms and actual cause are not fully understood (Ballard et al. 2011). Almost 70% cases of AD is actually caused by genetic level affecting various kinds of neuronal cell types in numerous genetic disorders conditions in human cells. For example; dopaminergic midbrain neurons in Parkinson's disease (Sanchez-Danes et al., 2012), and cortical neurons in Alzheimer's disease (AD) (Israel et al., 2012; Shi et al., 2012b; Yagi et al., 2011). Although AD prognosis can be studied with the help of cellular phenotypic character in appropriate human cell types, therefore it is now possible to understand the basic fundamental biology and its relevant disease-modifying pathways using genetic or pharmacological phenotypic screening in a relevant biological context. However, phenotypic screening of smaller compounds in cortical neurons which has potential to modify the production of amyloid-β (Aβ), an aggregation-prone and toxic peptide central cause ofAD pathological conditions.

The aggregation of amyloid-β (Aβ) peptides results from proteolysis cleavage of amyloid precursor protein (APP); on account of rare autosomal dominant missense mutation in encoding

(APP) and presenilin (PSEN-1; PSEN-2) genes; which form catalytic subunits of γ-secretase complex (Ertekin-Taner, 2007). AD pathological condition arises because of *APP* gene over expression or chromosomal trisomy aberrati,on at 21q position are responsible to cause uncontrolled production and accumulation of (Aβ) peptides (Rovelet-Lecrux et al. 2006; Sleegers et al. 2006),) and the early onset of Alzheimer disease pathology. In spite of these facts; it is striking note that no significant explanation was given which establish a concrete link in between the (Aβ) peptides aggregation and neuronal dysfunctions in AD pathology. Thereby; underlying amyloid hypothesis concern to amyloid processing and (Aβ) peptides production became a base of AD therapeutic approach in upcoming future studies. In this context, Findeis, (2007) andKupersteinet al.(2010) showed the dynamic balance in longer and shorter (Aβ) peptides released after proteolytic cleavage by β-secretase and γ-secretase (Takami et al. 2009), as it is a more significant determining factor of AD disease progression and initiation compared to amyloid protein production.In reference to an effective strategy it would be necessary to regulate rather than inhibiting proteolytic processing of other substrates of γ-secretase.The possibility of APP processing augments in support of statement highlighting the association of carboxypeptidaseactivity of γ-secretase with its modulators; which could shift the A-β peptides production away from longer and more toxic species to shorter peptides, without affecting total A-β production or γ-secretase targeting of other substrates. While the results of larger clinical trials are yet to be concluded that modulators may be targeted one in AD patients (Soares et al. 2016; Toyn et al.2016; Yu et al. 2014) and remain a promising possibility for further development.APP first undergoes processing throughβ and γ-secretase, and subsequently follow proteolysisstep in a numerous cellular compartments (Small and Gandy,2006).

This complex events suggested that it may be possibly one of the reasonsto modifyamyloidogenic APP processing in a secretase-independent manner; which couldalter longer A-β toxicpeptide production toward shorter peptides. The present study goal is to detectsecretase-independent, avermectins a smaller modulators of A-β processing that would elevatethe production of A-β peptidefragments in human cortical neurons away from Aβ-42 to lesser toxic shorter peptides.

These scientificdata demonstrate that phenotypic screening in human stem cell models of AD; provides a potentially powerful strategy for identifying error prone disease-modifying pathways and associated compounds, independent of known approaches to modulateAPP processing.

2. Materials and Methods

2.1 Experimental procedure

Preliminarily screening and systematic studies were executed on neuronssegregated from iPSCs isolated from those individuals with TS21 (Park et al. 2008). Additional otherincluded genotypes were undertaken as non-diseased controls (NDCs; Israel et al. 2012), APP duplication (APPdup) (Israel et al. 2012), APP V717I (Moore et al. 2015) and PSEN1 M146I (Moore et al. 2015). iPSCs originator cells were cultured and retained as feeder free in essenetial-8 without supplying any antibiotics. iPSCs cell lines differentiation to cortical neurons was performed by assembling neural stem cells and follows the protocols as per previously described by Shi et al. (2012a), (2012c).

Neural stem cells assembling is essential at this stage as it minimize variability in developing neuronal cells usually seen during experimental procedure. Neural stem or progenitor cells were undertaken for subsequent independent neural differentiations for 15-30 days so as toproduce postmitoticcortical neurons and astrocytes parallel for each trial or drug treatment. Each neuronal differentiation was treated separatelyas a biological replicate. Drug screening was tested on cortical neurons in 96-well plates (Greiner and Ibidi), while other additional experiments were tested in 12-well plates.

2.2 Immunocytochemistry

Cultured cells were fixed in paraformaldehyde 4% (w/v) in PBS and blocked with 5% normal donkey serum in 0.3% (v/v) Tween-20 in Tris-buffered saline before proceeding for immunofluorescent staining. Additionally, primary antibodies used in experimental procedure were anti-MAP2 (ab5392, Abcam), anti-CTIP2 (ab18465, Abcam), and anti-TBR1 (ab31940, Abcam), and one secondary antibody named as Alexa Fluor conjugated is used. Resultant stained cells were observed in an inverted confocal microscope (Olympus FV1000)and obtained results are imported for visualization purpose (PerkinElmer Velocity).

2.3 Drugs

Additional drugs such as (R)-flurbiprofen (Cayman), the γ-secretase modulator E2012 (Chem Express), abamectin (Santa Cruz Biotechnology), ivermectin (Tocris Biosciences), emamectin benzoate (Abcam), selamectin (MicroSource Discovery Systems), moxidectin (Santa Cruz), strychnine HCl (Abcam), picrotoxin(Tocris), muscimol (Tocris), and the γ-secretase inhibitors DAPT (Sigma) and L-685,458 (Merck-Millipore) are also included in this study. The Prestwick Chemical library supplied compounds at a concentration of 10mM in DMSO in a 96-well plates.

2.4 Drug Treatment

In preliminary screening, each drug wasintroduced at a final concentration of 1mMin 0.1% di-methyl sulpho-oxide (DMSO)in neural stem cell culture media. Additionally in other experimental procedure, drugs were mix-up inDMSOorwater, and subsequently applied on neuralcells in such a way, DMSO final concentrations did not surpass 0.4%. Alldrug treatments application started from 50 to 65 days after the beginning of neural cellsinduction, along with this, withdrawn media is further refreshed with the fresh mediaregularly at 48-hr intervals. Compound effects were normalized to taken as vehicle controls within each plate.

2.5 Biochemical Assays

Media supernatant prepared for biochemical analysis, it was centrifuge at 8000g to eliminateunwanted cellular debris as pellet, and supernatant stored at 20°C refrigerated conditions for further use. Other than this, measurement of Ab38, Ab40, and Ab42 was performed with the help of immunoassay (Meso Scale Diagnostics), and determinedLDH activity to check the cell cytotoxicity activity with the help of (Roche) manufactured detection kit.

2.6 Immuno-precipitation

The Aβpeptide profile was determined by immunoprecipitation, using Ab-specific antibodies coupled to magnetic beads. Collected samples were studiedas per previously designed by Portelius et al. (2007). Of the 25 identified A-β species, A-β 1-19 was found to be highlyvariable samples within the same treatment groups therefore, A- β 1-19 were not included for further analysis.

2.7 Immunoblotting detection

After collection in ice-cold PBS buffer solution, cells were furtherlysed in RIPA buffer containing1 mM DTT, protease and phosphatase inhibitors (Pierce), and 25 U/mL DNAse. The cell lysates soluble fraction was further subjected to SDS-PAGE and immunoblotting techniques. N-cadherin (610921, BD Transduction Laboratories), APP (SIG-39152, Covance), and histone H3 (ab1791, Abcam) were used as primary antibodies for detection purposes.

2.8 Cell-free γ-Secretase activity assay

The γ-secretase*in vitro* activity assay was analyzed as perSzaruga et al.(2015) described procedure.

2.9 Statistical Analysis

Statistical analysis was done with help of IBM SPSS and GraphPadPrismsoftwares. Analysis was performed to set to 0.05 level for all significant observation.

3. Results and Discussion

3.1 A Preliminarily Phenotypic Screening todetect modifiers ofA-βproduction

In this study phenotypic screening of neural stem cells originated from cultured TS21, (iPSC) induced pluripotent stem cells in 96-microwell plates to detect the presence of small molecule of modifiers of A-β peptides in cortical neuron of humans, (fig.1). Genetically modified neuronal cells can have ability to over produce A-β type peptides by Shi et al. (2012b), and thus they are sensitive towards drug screening purpose. A single point screening of amyloid peptides (A-β-38, A-β-40, and A-β-42) was done at 1mM concentration of drugs treatment after 4 days immunoassay was performed to check these antibodies presence andin between these events, used media was exchanged regularly with fresh media after 48h time interval. Thereafter activity of lactate dehydrogenase (LDH) in extracellular media was determined after 6 days of drugs treatment as an indicator of cellular toxicity. Each microtitre well plate performance was determined as per coefficient of variation (CoV) of DMSO as control (n=5 culture per plate). In this context, obtained mean (CoV) value for A-β38/A-β42 (9.11%), and A-β40/A-β42 (7.579%) respectively. These mean values reflects the minimal level of variations in A-β ratios in control sample thereby, it is used to quantify the targeted compounds. A control-independent method was used to detect target compounds withthe help of given B-score in order to find out the positional differenceswithin the 96-well plates (Brideau et al. 2003). Adjustment in B-Score can be deduced by using the open-source Bioconductor cell HTS2 package (Boutros et al. 2006).

The present study results shows the LDH activity was elevated in seventy three compounds as it has higher B-score (B score >3) and similar score results were obtained in case of 55 compounds as they reflects the reduction in a Aβ42 production relative to total Aβ production. Similar kind of validation of further compounds identification was repeated again at 1mM in triplicate manner, and significant Fisher's leastsignificant difference test was performed to reproduce their initial effect or at least two of three replicatesreflect increment in >10% in the Aβ38/Aβ42 or Aβ40/Aβ42 ratio compared to DMSO treatment. Additionally among all tested compounds only two compounds were shows the validated hits as these hits (0.1666%) reflects increment in Aβ38 to Aβ42 ratio in a dose-dependent manner.

3.2 Avermectins alter the proportion of Aβ38/Aβ42ratio in humancortical neurons

Avermectins members class compounds with non-steroidal anti-inflammatory drugs (NSAIDs) category GSM (R)-flurbirofen and the GSM E-2012 can have ability to raise the Aβ38 to Aβ42 ratio in TS21 cortical neurons in a dose-dependent manner ($F(7, 16) = 43.766$, $p < 0.0001$; $F(7, 16) = 1633$ at $p < 0.0001$, respectively). Other than this one of the identified compound named as abamectin had minimal level of Aβ38/Aβ42 increment ($F(5, 15) = 2.754$, $p = 0.0586$), whereas, structural analog compounds such as ivermectin significantly raised the Aβ38/Aβ42 level in a dose dependent manner ($F(5, 15) = 4.435$, $p = 0.0221$) respectively. Additionally, avermectin related family compounds namely Emamectin benzoate and selamectin had shown similar effect on Aβ38/Aβ42 level, as per obtained results of these identified compounds; Emamectin benzoate had shown a moderate effect ($F(5, 15) = 6.284$, at $p = 0.0050$) while selamectin shown good potency towards the Aβ38/Aβ42 ratio ($F(5, 15) = 16.18$, at $p = 0.0003$).

All other avermectin related compunds such as moxidectin, a member of macrocyclic lactones do not show any significant effect on Aβ38/Aβ42 ratio as reflected through ($H(5) = 11.77$, $p = 0.0380$; however, for all post hoc comparisons is also made at $p > 0.05$).

All the recorded results of the study had shown that most of the avermectin belonging family of compounds can have higher Aβ38/Aβ42 ratio byincreasing Aβ38 and/or reducing Aβ42, while few of them had shown additional effects on Aβ 40 peptides.

Abamectin, ivermectin, and selamectin had significantly raise Aβ38 level ($F(5, 15) = 6.543$, $p = 0.0040$; $F(5, 15) = 13.05$, $p = 0.0003$; $H(5) = 16.59$, $p = 0.0053$, respectively) comparative toAβ 40 whereas;Emamectin benzoate and selamectin significantlydecreased Aβ 42 level ($F(5, 15) = 5.889$, $p = 0.0033$; $F(5, 15) = 7.22$, $p = 0.0039$, respectively). In addition, abamectin, ivermectin,and selamectinhad shown similar decrement inAβ 40 level ($F(5, 15) = 6.063$, $p = 0.0058$; $H(5) = 12.35$, $p = 0.0303$;$F(5, 15) = 53.38$, $p = 0.0003$, respectively). However, all macrocyclic lactones had a greater tendency of lipophilicity thereby; it was concluded that the avermectins family related compounds had significant effect on Aβ production and it was noteworthy this effect is on account of membrane stress and or due to γ-secretase-APP interaction. Previously encoded study results of (Prichard et al. 2012) reflect that it had no significant correlation between the studied compounds efficacy and lipophilicity on the basis of clogp values. From this results it was concluded that avermection, selamectins shows higher potent nature as

per clogp values (6.0 and 6.3) respectively while other two compounds such as milbemycin and moxidectin had least values <3.0 clogp value

Whileavermectinsabamectin and ivermectinhad a moderately relative values as per recorded cLogP values of 5.3 and 4.8, respectively (Prichardet al. 2012). As per these observed results it was noteworthy that the avermectin effect on Aβ production is not only just because of their own lipophilicity nature, but it requires additional observations to detect possible molecular targets.

3.3 Effects of Avermectinson APP Processing and Aβ Peptide Production

As per the obtained fraction of Aβ peptides produced from human cortical neurons after Aβ38/Aβ42 immunoassay. The studied 3 Aβ peptides fraction originated on account of proteolysis of APP stimulated with the effect of avermectinscompounds and Aβ peptides fraction were further analyzed with the help of immunoprecipitation. Previously encoded studies had consistent increment in Aβ peptides such as Aβ1-14, Aβ1-15,and Aβ1-16, while Aβ1-34 peptide shown decrement when APP processed with a γ-secretase inhibitor (GSI) (Porteliuset al. 2010a, 2012) while GSM modifiers treatment leads toan increment in Aβ1-37 peptidealong with decrement in Aβ1-39, Aβ1-40, and Aβ42 peptides (Portelius et al. 2010b, 2014). As per immunoassay and earlier studied results Aβ1-14, Aβ1-15, Aβ 1-16, Aβ 1-34, Aβ 1-37,Aβ 1-38, Aβ 1-39, and Aβ 1-40 peptides were quantified for further analysis except Aβ 1-42 as it was not reliably detected andquantifiable in all the undertaken samples. Although selamectin (1.5 mM) treatment to TS21 neurons over ten days had significantly raised the Aβ1-37 and a decrease in Aβ 1-40 level, but it has least effects on Aβ 1-14, Aβ 1-15, and Aβ1-16. This increment and decrement in the level of Aβ 1-37 and Aβ 1-40 is occurred consistently as with γ-secretase modulation (Portelius et al., 2010b), while it has shown minimal degree of effect on Aβ 1-14, Aβ 1-15, and Aβ1-16 peptides as it may be due to significant ofγ-secretase activity (Porteliuset al. 2010a, 2012).

Immunoassay experiment reflect avermectin induced a significant effect on Aβ-38 comparative to the Aβ40/42. It is just because of no significant effect of Aβ-38(Porteliuset al. 2010b) on application of GSM E2012 thereby; Aβ38/Aβ42 complex system is more sensitive towards immunoassay detection comparative to graded IP-MALDI technique,particularly for low-abundance and relatively hydrophobicpeptides.

3.4 Effect of Avermectins on Aβ peptidesproduction and its unusual effect on ion channels

This study reflects that avermectinshas significant effect on high affinity glutamate-gated chloride channels. Inflow of Cl^- ions stabilize the open conformations of ions channels in neural cells adversely shown paralyzing effect on nematodes and other parasites (Wolstenholme and Rogers, 2005). In addition, avermectins($F(2, 41) = 176.8$, $p < 0.0001$) also shown agonists effect on ligand-gated chloride channels*i.e.*γ-aminobutyric acid-A (GABAA) and glycine at nanomolar concentration (Dawson et al. 2000) as similarly shown by and picrotoxin ($F(5, 41) = 9.996$, $p < 0.0001$). Other than this, the most potent GABA receptor agonist namely muscimol (100 mM) had no significant effect on the Aβ38/Aβ42 level ($F(6, 17) = 2.007$, $p = 0.1209$)Shan et al.(2001).

In this study GABAA and glycine receptor activity was tested on the basis of the Aβ 38/Aβ42level as this Aβ level is depicted throughivermectin (1 mM) and selamectin (1 mM) absence or presence. Two-way ANOVA analysisshowed the significant main effects of the avermectins($F(2, 41) = 176.8$, $p < 0.0001$) and picrotoxin ($F(5, 41) = 9.996$, $p < 0.0001$) on A-β peptides. Although it has no significant connection in between the avermectins and picrotoxin($F(10, 41) = 0.8645$, $p = 0.5724$). Similarly anothermuscimol; an inducer of GABAA receptor was tested up to 100 mM; and it had no significant effecton the Aβ38/Aβ42 level ($F(6, 17) = 2.007$, $p = 0.1209$). In addition glycine receptor antagonist such as strychnine (0.3–30 mM) in the presence and absence of these avermectins tested at 1Mm had significant effect ($F(2, 41) = 43.55$, $p < 0.0001$) but do not show any interaction between these two compounds $F(10, 41) = 0.6173$, $p = 0.7901$).In addition, no significant inhibition of the concentration-dependent effect of ivermectin or selamectinon the Aβ38/Aβ 42 level was observed when dose response was performed in the presence of strychnine. These study findingsclearly indicate that avermectinscould be prime targetsof GABAA or glycine receptors, and may be affect Aβ peptides processing.

4. Conclusion

This study concluded that the human cortical neurons derived from an individual with induced pluripotent stem cell as TS21 highlighted the probability of identifying phenotypically screening of a small-nontoxic molecule derived from longer toxic form of Aβ peptides responsible to cause AD in humans. In this study, avermectins as modulators were identified a prime precursor of Aβ peptidesproduction, which act independently of γ-secretase to alter APP processing inmanner similar to γ-secretasemodulation. The recent emergence and continued development of human cell models of disease in combination with traditional phenotypic screening approaches open a new avenue to allow the identification of potential drug candidates in addition to uncovering new pathways concerning with Alzheimer disease pathology.

5. References

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