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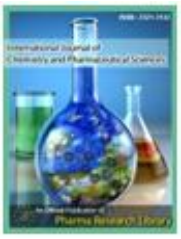

**Research Article** **Open Access**

# Evaluation of Darunavir for Neuroprotective Activity in Alzheimer's Disease

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**A B S T R A C T**

Alzheimer's disease (AD) is associated by deposition of amyloid beta plaques due to improper processing of amyloid precursor protein and tau protein dephosphorylation ending up in formation of neuro fibrillary tangles. Gamma secretase belongs to aspartyl protease family sharing similarity in structure with HIV-Proteases, pepsin. Inhibition of this enzyme plays a key role in targeting this disease. Daurnavir is the drug currently employed in the treatment of HIV infections and its mechanism being the inhibitor of HIV proteases. HIV protease belongs to the Aspartyl protease (ASP) family and gamma secretase also being one of them. Since the test drug has high affinity towards that site, when reaches the brain can target the gamma secretase enzyme. $A\beta_{(1-42)}$ induction led to the development of dementia in experimental animals which was evident from the behavioral tests applied on the induced animals. After treatment with Low dose (60mg/kg, p.o) and high dose (120mg/kg, p.o) animals could recollect their memory and were successfully able to locate the reward and produced consistent results when compared with the negative control, indicating a positive note on drugs effect upon treatment. Histopathological reports showed that, upon treatment with the test gave better results with comparatively more neuronal density and uniformity clearly indicates that the test drug was able to fight the neurotoxicity and protect the brain from neurodegeneration. Results were consistent at higher dose level compared to low dose level group which showed a reasonable degree of degeneration and loss of uniformity in the distribution of neurons. Thus use of HIV proteases is a new targeting for AD.

**Keywords:** Alzheimer's disease, Amyloid beta plaques, Gamma Secretase, HIV Protease, Darunavir

**A R T I C L E I N F O**

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## 1. Introduction

Alzheimer's is one the major cause of death in aged population throughout the world. With recent molecular advances many researchers have provided us with molecular insight about the essential components of brain involved in the cause and progression of disease.[1] Many studies proposed about the activity of enzymes like BACE and γ-secretase and their roles in participation for processing of amyloid precursor protein in the production of amyloid beta plaques. Because of their overexpression in brain it led to the over production of amyloid beta plaques at a higher rate when compared to its clearance rate from the brain leading to their deposition, aggregation and accumulation and causing neuronal toxicity.[2] Targeting these enzymes can prove to be beneficial in the next generation treatment approach to reduce the disease progression and leap to its cure. Use of inhibitors of these protease enzymes was already proved as a novel approach in the treatment of AD.[3]

Darunavir is the current research molecule which available in the market under trade name prezista™ an anti-retroviral agent used as a protease inhibitor for fighting the deadly HIV-AIDS disease. Current study is a structure based approach. Due to many similarities in the structures of these enzymes and owing to the good distribution of this class of drugs in the brain and an approach has been made to target those brain secretases and evaluate the beneficial effects on brain. This marks the possible approach involving the current drug and may open new possibilities to treat AD in near future.[4]

## 2. Materials and Methods

**Animals:**

Adult male wistar rats of narrow weight range weighing between 250-290g were selected for the current study. Studies have suggested that male animals unlike females, which lack estradiol and estrogen makes females less likely to develop AD. Animals were housed in polyacrylic cages of 38×23×10cm dimensions and placing not more than three animals per cage. Rats were housed in an artificially controlled room (23± 2 °C and 55±15% humidity) with food and water available ad libitum, under a 12-h light/dark cycle. Experiments were carried out between 9 a.m. and 5 p.m. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC- JSSCP/ IAEC/M.Pharm/Ph.cology/03/2013-14) of JSS University and performed in accordance with the guidelines of Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India on animal experimentation.[5]

**Chemicals and drugs[6]**

Darunavir (Gift sample from Dr.Reddys), Amyloid beta plaques (catalogue no: A9810, Sigma aldrich)

**Equipment:** Stereotaxic apparatus (Sotelting, USA), IR Actimeter (Prolabs), eight arm radial arm maze.

**Methods**[7,8]

**Preparation of A solution**

Aβ1-42 aliquots were stored at −20 ˚C until use. Aβ1-42 at a concentration of 1mg/ml prepared in Phosphate Buffer Saline was incubated for 5 days at 37 ˚C. Each rat was given 10µl by volume on each site Prepared solution was protected from light and was maintained at ambient temperatures to avoid any damage to the solution.

**Preparation of drug solution**

Darunavir of 60 mg/Kg and 120 mg/Kg dose levels were selected based on human safety dose. Drug was dissolved in 10% poly ethylene glycol (PEG) and administered orally.

**Stereotaxic Surgery**[9]

For the induction of AD, animals were injected with activated amyloid beta plaques solution (10µl of 2.2 nmol) into the brain (Intra Cerebro Ventricular (ICV) injection). Animals were anesthetized with a combination of ketamine (100 mg/kg, i.p.) and xylazine (5 mg/Kg, i.m.) and placed in the stereotaxic apparatus (Stoelting, USA). Stereotaxic apparatus is an instrument with manually adjusted screws and atlas with X, Y and Z coordinates to reach the desired location of brain pointed by precisely placing the syringe head. The syringe used in this instrument was Hamilton syringe-10µL which is similar to HPLC syringe to deliver accurate amounts of Amyloid beta plaques into the brain.

**Experimental Design [**10,11]

7 days after induction of Aβ plaques, animals were tested for their learning and memory behavior in radial arm maze (REM and WME) and IR Actimeter (REM and WME) to confirm the onset of disease. Those animals which showed significant reduction in memory/ showing more number of errors were selected for the study. The animals were divided into four groups of six animals each.

**Behavioral studies**[12]

Post-surgical recovery animals were subjected to a series of behavioral test to estimate their memory status. Their behavioral patterns were evaluated by using IR Actimeter and eight arm radial arm maze.

**IR Actimeter for exploratory behavior analysis in animals [**13,14]: Hole board facilitates to assess the learning and memory and also to evaluate the curiosity and exploratory behavior of rodents. Before the experiment the animals were fed with a minimal amount of food so as to activate their senses and exploratory behavior for food. The hole board has an open field of size 40x40 cm, surrounded by plexiglass wall of 40 cm high. It contained 16 holes in a 4x4 array. Each hole was 2cm deep with a diameter of 3cm. It had two gridded frames of infrared cells. The frame was of 45x45cm. A total of 16x16 infrared beams at a distance of 2.5cm were located on the sides. These frames were held in place by the frame support. The board was elevated from arena so that the animal does not see the bottom when it

pokes its nose into the hole. Each time the animal pokes its nose, the count was recorded by the infrared beams. Each animal was trained for two sessions a day with a time gap of 2 hours for 5 days. The board was cleaned with 70% ethanol to remove the scent clues left by the previous animal. Out of the 16 holes, four holes were baited and the remaining holes were left un-baited. The time duration of each trial was 5 min.

Parameters assessed:

- Hits: Number of pokes into the baited holes
- Errors: Number of pokes into the un-baited holes.

**Eight arm radial arm maze**[16,17]

Animals were placed on the central platform and allowed to explore the maze for 15 min per day for three days. On third day of habituation bait/food was reduced to half of its arms i.e. in four arms and session was ended when all arms were visited. Following habituation animals were trained for eight consecutive days. The trail continued until the animal had entered all eight arms or 10 min elapsed time. When animal found the goal box, it was allowed to remain in there for 1 minute. When the rat inserted only its head into an incorrect opening and remained there for more than a minute, it was replaced at the center of the maze. Animals that proceed through the maze using non-spatial strategies, i.e., repeatedly choosing the arm adjacent or to the arm that was three arms away from the one currently visited, were excluded from the present experiment because they were considered to not have acquired spatial memory.

During the acquisition and retention phases, behavioral performances were measured by latency to find the goal box, the number of working memory errors and reference memory errors. A reference memory error (RME) was registered when the rat visited an arm which was never baited. However, if the rat returned to a baited arm which had been previously visited during the trail, a working memory error was recorded (WME). An arm was considered visited when the rat entered half way down the arm.

The animal was considered to leave an arm when it placed its four paws on the central platform. The following parameters were assessed.

- Working memory errors: Visits to baited arm more than once.
- Reference memory errors: Entry in to the baited arm.

**Histopathology:** Animals were sacrificed; their brains were extracted and hippocampus was isolated from both the hemispheres. Isolated hippocampus was stored in 10% formalin solution until they were processed for fixing and histological evaluation. Finally samples were coded and transferred to the Lifegaurd laboratories TM, Bangalore for the further examination.[18]

## 3. Results and Discussion

**Effect of darunavir on radial arm maze for reference memory error test (RME) in A induced AD rat model**

Darunavir treated animals when tested with RAM for RME is shown in tables (1). The data reveals that pre-trained negative control rats showed a significant ($p<0.001$) significant RME when compared to sham control group as indicated by higher number of errors. Administrations of darunavir (120 mg/kg) have shown a significant ($p<0.001$) decrease in the number of errors when compared to negative control and a dose dependent reduction in errors were observed upon continuation of treatment.[19]

**Effect of darunavir on radial arm maze for working memory error test (WME) in A induced AD rat model**

Darunavir treated animals when tested on RAM for WME were shown in table2. The data reveals that negative control group showed significant errors ($p<0.001$) from day 7 and continued throughout the study when compared to sham control. However, the darunavir treated group (both 60 and 120 mg/kg) have produced significant effect ($p<0.001$) when compared to negative control at the end of 28th day.[20,21]

**Effect of Darunavir on Hole Board (HB) test**

Tables 3 & 4 and show the results for HB after 28 day treatment. The HB test was used to investigate the effects of darunavir on the learning and memory abilities in the Aβ induced Alzheimer's rats. Negative control group exhibits significant ($P < 0.001$) increase in number of visits to the un-baited holes and decrease in number of visits to baited holes when compared to sham control group throughout the treatment. Administration of darunavir causes a decrease in number of visits to the un-baited holes and increase in number of visits to baited holes. Darunavir shows a significant ($P < 0.001$) reversal of learning and memory deficits in the HB test after 28 day treatment at both the test doses. However, mean values indicate that darunavir 60 mg/kg is less effective when compared to 120 mg/kg group.[22,23]

**Effect of darunavir on neuronal density of brain hippocampal slices under neurons selective cresyl violet stain:** The results of histology of brain hippocampus were shown in the figure (1). The sections when observed under cresyl violet stain at 100x magnification which revealed that neuronal density in control group was much higher when compared to negative control group. Density of neurons in the treated group were also higher when compared to negative control group and 120 mg/kg dose treated group showed better neuro protective effect in comparison with 60 mg/kg group.

**Table 1:** Grouping of animals

| Group | Treatment |
|---|---|
| Group-I | Sham operated control |
| Group-II | Negative control (Aβ plaques-1mg/ml) |
| Group-III | Treatment group (Aβ plaques-1mg/ml, Darunavir- 60mg/Kg) |
| Group-IV | Treatment group (Aβ plaques-1mg/ml, Darunavir- 120mg/Kg) |

**Table 2:** Effect of darunavir on radial arm maze for reference memory error test (RME) in Aβ induced AD rat model

| Treatment | Number of Reference Memory Errors | | | | |
|---|---|---|---|---|---|
| | **Day 1** | **Day 7** | **Day 14** | **Day 21** | **Day 28** |
| **Sham control** | 4.167 ±0.4773 | 3.000 ±0.2582 | 2.500 ±0.2236 | 2.167 ± 0.1667 | 1.500 ± 0.2236 |
| **Negative control** | 4.500 ± 0.4282 | 3.667 ±0.2108 | 3.833 ± 0.3073$^{**}$ | 3.500 ± 0.2236$^{**}$ | 3.500 ± 0.2236$^{***}$ |
| **DR LD 60** | 3.833 ± 0.3073 | 3.500 ±0.2236 | 2.833 ± 0.3073 | 2.500 ± 0.2236 | 1.833 ± 0.1667$^{\#\#}$ |
| **DR HD120** | 4.167 ± 0.4773 | 3.167 ±0.1667 | 2.667 ± 0.3333$^{\#}$ | 2.333 ± 0.2108$^{\#}$ | 1.667 ± 0.2108$^{\#\#\#}$ |

Data expressed as mean ± SEM; n=6, Two way ANOVA followed by Bonferonni multiple comparison post-test was applied. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Sham control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. Negative control.

**Table 3:** Effect of darunavir on radial arm maze for working memory error test (WME) in Aβ induced AD rat model

| Treatment | Number of Working Memory Errors | | | | |
|---|---|---|---|---|---|
| | **Day 1** | **Day 7** | **Day 14** | **Day 21** | **Day 28** |
| **Sham control** | 7.333 ±0.3333 | 6.500 ± 0.2236 | 6.167 ± 0.1667 | 5.833 ± 0.1667 | 5.167 ± 0.1667 |
| **Negative control** | 7.500 ±0.4282 | 7.500 ± 0.3416$^{*}$ | 7.833 ± 0.3073$^{***}$ | 7.667 ± 0.2108$^{***}$ | 7.833 ± 0.3073$^{***}$ |
| **DR LD 60** | 7.833 ±0.3073 | 7.000 ± 0.2582 | 6.500 ± 0.2236$^{\#\#}$ | 6.333 ± 0.2108$^{\#\#}$ | 5.500 ± 0.2236$^{\#\#\#}$ |
| **DR HD 120** | 8.000 ±0.3651 | 6.667 ± 0.2108 | 6.333 ± 0.2108$^{\#\#\#}$ | 6.167 ± 0.1667$^{\#\#\#}$ | 5.333 ± 0.2108$^{\#\#\#}$ |

Data expressed as mean ± SEM; n=6, Two way ANOVA followed by Bonferonni multiple comparison post-test was applied.$^{**}P < 0.01$, $^{***}P < 0.001$ vs. Sham control ,$^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. Negative control.

**Table 4:** Evaluation of Errors in induced and treated groups using hole Board apparatus

| Groups | Number of Errors | | | | |
|---|---|---|---|---|---|
| | **Day 1** | **Day 7** | **Day 14** | **Day 21** | **Day 28** |
| **Sham control** | 12.000±0.2582 | 9.000 ±0.3651 | 6.667 ± 0.3333 | 3.833 ± 0.4014 | 2.167 ± 0.3073 |
| **Negative control** | 20.170±0.4014 | 19.670±0.4944$^{***}$ | 19.830±0.3073$^{***}$ | 17.830±0.3073$^{***}$ | 19.670±0.3333$^{***}$ |
| **DR LD 60** | 17.170 ±0.4014 | 15.500 ± 0.9916 | 10.330 ± 0.2108$^{\#\#\#}$ | 8.333 ± 0.2108$^{\#\#\#}$ | 5.667 ± 0.2108$^{\#\#\#}$ |
| **DR HD 120** | 15.170 ± 0.3073 | 14.830 ± 0.4014$^{\#\#\#}$ | 8.000 ± 0.3651$^{\#\#\#}$ | 6.167 ± 0.3073$^{\#\#\#}$ | 3.833 ± 0.3073$^{\#\#\#}$ |

Data expressed as mean ± SEM; n=6, Two way ANOVA followed by Bonferonni multiple comparison post-test was applied. $^{***}P < 0.001$ vs. Sham control ,$^{\#\#\#}P < 0.001$ vs. Negative control

**Table 5:** Evaluation of Hits in induced and treated groups using Hole Board apparatus

| Groups | Number of Hits | | | | |
|---|---|---|---|---|---|
| | **Day 1** | **Day 7** | **Day 14** | **Day 21** | **Day 28** |
| **Sham control** | 5.667 ±0.2108 | 9.000 ± 0.3651 | 13.830 ± 0.3073 | 16.170 ±0.6540 | 18. 830 ± 0.3073 |
| **Negative control** | 6.833 ± 0.8724 | 6.000 ±0.5774$^{***}$ | 6.667 ± 0.3333$^{***}$ | 6.500 ± 0.3416$^{***}$ | 6.667 ± 0.2108$^{***}$ |
| **DR LD 60** | 7.167 ± 0.3073 | 7.500 ± 0.5627 | 9.500 ± 0.4282$^{\#}$ | 11.000 ± 0.3651$^{\#\#}$ | 15.330 ± 0.3333$^{\#\#\#}$ |
| **DR HD 120** | 6.667 ± 0.3333 | 8.333 ± 0.6667$^{\#\#}$ | 12.500 ±0.4282$^{\#\#\#}$ | 14.670 ±0.3333$^{\#\#\#}$ | 7.000 ± 0.2582$^{\#\#\#}$ |

Data expressed as mean ± SEM; n=6, Two way ANOVA followed by Bonferonni multiple comparison post-test was applied. $^{***}P < 0.001$ vs Sham control ,$^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs Negative control

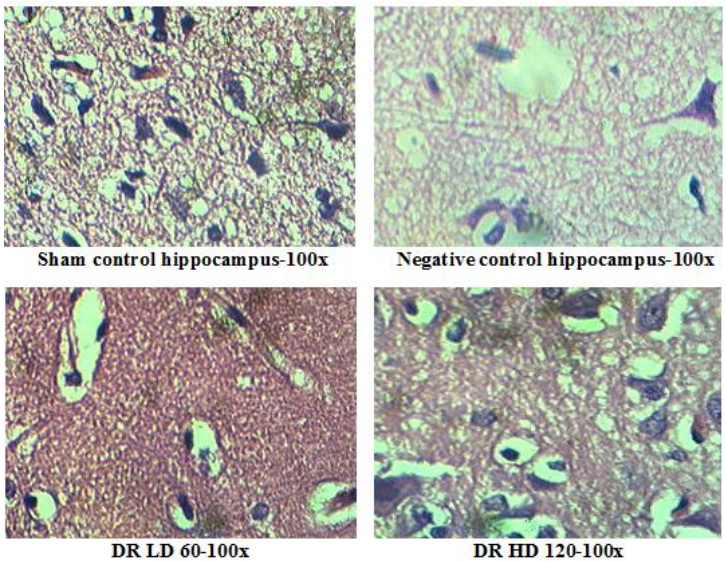


**Fig1:** Effect of darunavir on neuronal density of brain hippocampal slices under neurons selective cresyl violet stain-100x

## 4. Conclusion

In the current study same strategy was employed to deactivate the gamma secretase enzyme which structurally belongs to the same class as of HIV protease i.e., aspartyl protease family. Further insight into the structure of the gamma secretase enzyme reveals that its active sight is made up of subunits like presnillins (PS1 and PS2) where aspartyl sites were located. Studies suggest that HIV proteases have good bioavailability in brain and this factor helped the study in shaping it into a structure based study. However, a line of controversy still exists with the usage of these agents in the treatment for Alzheimer's disease. Owing to their good availability into brain it should be beneficial to evaluate these agents in neurodegenerative diseases but the controversial research facts impedes many researchers to move ahead with these inconsistent results. Our current research motivates us to precisely apply the drug and modern pharmaceutical techniques to land upon a clear insight about these agents for their use in neuro pharmacology.

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