Antiangiogenic Potential of Levetiracetam by Blocking N-type Voltage Gated Calcium Channels

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ABSTRACT

Objectives: Angiogenesis is generation of micro vessels. These ion channels on endothelium play vital functions in cell proliferation, migration, cell volume expansion and in related angiogenesis. Our study aims to evaluate the antiangiogenic capability of Levetiracetam, a N-type voltage gated calcium channel blocker. Methods: Anti-angiogenic activity was evaluated by MTT cell viability assay, Morphological screening assay, Zebra fish caudal fin assay and Zebra fish embryo assay using different concentrations of levetiracetam, standard drug Bevacizumab and control. The parameters assessed were percentage viability in MTT, change in the cell shape or volume microscopically in morphological screening assay, percentage regeneration of amputated fin in caudal assay and percentage neovascularization in zebra fish embryo assay. Results: The results suggest that levetiracetam treatment significantly inhibited Hep G2 cells viability and cell volume expansion, which are the further steps in the endothelial cell proliferation. Arrest in neovascularization caused various phonotypical changes in the zebra fish embryo has been captured us by microscope. A significant decrease in fin regeneration was observed. Conclusion: The findings statistically validate the antiangiogenic potential of levetiracetam. The antiangiogenic property might be due to the blocking of N-type voltage gated calcium channels, which are largely located in the endothelial cells.

Key words: Angiogenesis, Levetiracetam, Calcium channels, Cell migration, Neovascularisation.

INTRODUCTION

Angiogenesis, which is the creation of fresh vessels from the already existing vessels, is a normal course which helps in the growth of the body, embryogenesis, development of the female reproductive system and healing of damaged tissue.1,2 It when occurs in abnormal conditions, lead to tumour progression with the help of growth factors. During transition, a tumour undergoes a critical step called angiogenesis. This results in stimulation of capillary growth and development of the ability to synthesize proteins. The most important among such proteins is vascular endothelial growth factor (VEGF). VEGF encourages endothelial cells (EC) to go through a tumour and start the process of neovascularization. EC division further secretes growth factors that result in the development or migration of tumour cells. Consequently, ECs and tumour cells reciprocally fuel each other.3,4 Evidence suggests that angiogenesis occurs when the ratio between angiogenesis activators and angiogenesis inhibitors is less. Angiogenesis inhibitors can be seen as promising therapeutic agents to treat various types of malignancy conditions. Angiogenesis inhibitors, also known as anti-angiogenic agents are the substances that block the process of angiogenesis. They interfere with the various steps in the mechanism of angiogenesis, finally stopping the formation of new blood vessels. As tumour cells need oxygen and nutrients...
produced by the blood vessels, interfering with this essential step leads to decreased tumour progression and hence, used as an effective approach in treating various types of malignancies.5,6

Levetiracetam is a drug used to treat generalized, partial, myoclonic and tonic-clonic seizures. It is also effective in the treatment of wide-spectrum mental disorders related to the nervous system. It acts by irreversibly blocking the pre-synaptic high-voltage activated calcium (Ca$^{2+}$) channels, N-type Ca$^{2+}$ channels, modulating potassium channels and enhancing GABA and glycine neurotransmission. The ion channels on endothelium take parts in a imperative deeds in proliferation and so in the associated angiogenesis.7 As Ca$^{2+}$ channels contribute to cell proliferation,8 Levetiracetam was selected to evaluate its anti-angiogenic effect by various assays.

MATERIALS AND METHODS

Materials

Requirements

Levetiracetam, Bevacizumab (standard drug), 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide solution, Phosphate-buffered saline, pH 7.4, 40% v/v Dimethylformamide, 2% Glacial Acetic Acid, 16% Sodium Dodecyl Sulfate, pH 4.7 and 37°C, 96 well plate, Hep G2 cells, Complete Dulbecco’s Modified Eagle (DME) Medium with 10% fetal bovine serum, phosphate-buffered saline, cytotoxin-Doxorubicin, Human malignant melanoma (A-375) and Human lung adenocarcinoma (A549) (All the chemical and cell lines have been purchased from Sigma-Aldrich). Sensitive analytical balance, camera, Micropipette, Fluorescent microscope, 0.9mm mesh (to catch embryos of zebra fish), Fish system water and tanks.

Experimental animals

Zebra fish were obtained from Scott labs Pvt Ltd, Hyderabad, kept at 28°C on a 10 hr dark and 14 hr light cycle in glass chambers.9 The fish were checked for swimming behaviour daily, body weight gain, twisted vertebral column and throughout look. Fish did not show symptoms of distress for the period of experiment.

Methods

MTT Assay

This cell viability method helps to assess live cells in comparatively high throughput i.e.in 96-well plates, without the want for tricky cellular counting process. So, the maximum use is to evaluate cytotoxicity of numerous chemical at different concentrations. Here, any change in viable cell count can be identified by estimating formazan concentration, which reflects a change in optical density (OD) by means of a plate reader at 540 and 720 nm.

Procedure

MTT solution was stored and covered from light at 4°C and taken measures for no precipitate in the Solubilisation fluid. 25 x 103 Hep G2 cells were seeded in a 96 well plate having 250 µl of DMEM. The test antiangiogenic agent was added. It was incubated for 24 hr. Medium was aspirated and washed with three times PBS. 125 µl of DMEM with 25 µl of MTT solution was added to the well plate. Again it was incubated for 2 hr at 37°C. 100 µl of solubilisation solution was then added. Absorbance was measured at 570 nm by spectrophotometer.10 The inhibitory rate of proliferation was calculated by:

\[
\% \text{ Viable cells} = \frac{\text{Absorbance of sample} - \text{Absorbance of control} - \text{Absorbance of blank}}{\text{Absorbance of blank}} \times 100
\]

Morphological screening

Cell culture

A549 was used, they were sub-cultured. Cell line was maintained in 75 cm$^2$ vented flasks with DMEM and was grown at humid environment of 5% CO$_2$ at 37°C. Cell line was incubated in DME medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% penicillin (1000U/mL), 1% streptomycin (1000µg/mL) and 1% amphotericin (250 U/mL). The cell line was subculture enzymatically in 0.25% trypsin-1mM EDTA and passaged on 75 cm$^2$ plastic flasks at a density of 2.2 × 10$^4$ cells/cm$^2$. Cell count was confirmed by microscopic examination.

Procedure

A-549 cells were treated with 10, 20, 40 µg concentration of Levetiracetam and 25 µg of the standard drug. As a part of antiangiogenic evaluation, we have screened various concentrations of the test drug based on the LD$_{50}$ values against morphological conduct of A549. Cells were observed for 24, 48, 72 hrs, after the testing drug was added. Pictures were taken by Axiovert 200M phase contrast microscope at 10x. The software used was Axiovision Rel.4.2.11

Zebra fish caudal fin assay

Fin regeneration has mainly been studied after a simple removal of the tail fin part using an amputation
procedure. Zebra fish were divided into five groups and grown at 28 ± 0.5°C on a 14 hr light and 10 hr dark cycle in fish tanks. Within few hours of amputating the fin, re-epithelialisation occurs in the wound. Before amputations, zebra fish were anesthetized by 0.04% Tricaine. The caudal fin was amputated to approximately 50% size using a razor blade, at right angles to the cranio-caudal axis. Straight away the amputated fish were placed in the recovery tank and were found recovered within three min. Bevacizumab (25µg/150ml) and Levetiracetam (100, 200 and 400µg/150ml) were added to tanks for seven days post amputation (dpa). The length of fin regeneration was observed and the assay was repeated thrice independently with six fish per group.

**Zebrafish Embryo model assay**

Zebra fish of both the sex were taken in the ratio of 2:1 and kept in separate tanks with unremitting oxygen supply. Fishes were kept without feed for 3 days after which they are fed for 7 days. On the 11th day, both sex are kept in one tank. Fishes are kept in own tanks one day after mating and embryos were harvested use of embryo collector of mesh size 0.9mm. Embryo medium was arranged by dissolving 2.94 g of sodium chloride, 0.13gms of potassium chloride, 0.49gms of calcium chloride, 0.81 g of magnesium sulphate and 10g of methylene blue in 10L of fish system water. Embryos were washes with embryo medium and are taken in a beaker with embryo medium. A microliter plate is taken where each well is filled with 100µL of embryo medium containing blank, test (10, 20 and 40µg/100ml) and standard drug. Using a dropper, embryos were transferred to the microliter plate, one embryo in a well. After adding the drug, embryos were separately placed in culture plates at 28.5°C until 72 hr post fertilization (hpf). 24, 48 and 72 hpf of drug treatment, embryos were examined for neovascularization and also for phonotypical variations such as bending of tail, yolk sac elongation to abdominal region, vessel haemorrhages, delay in hatching, reduced growth and pericardial edema using a trinocular microscope with blue filters and photographs were saved.

**RESULTS**

**Morphological screening (Table 1)**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Code of the Molecule</th>
<th>Morphological screening of Levetiracetam 100 µM against A-549 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td><img src="image1" alt="Image" /> <img src="image2" alt="Image" /> <img src="image3" alt="Image" /></td>
</tr>
<tr>
<td>2</td>
<td>Bevacizumab (25µg)</td>
<td><img src="image4" alt="Image" /> <img src="image5" alt="Image" /> <img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>3</td>
<td>Levetiracetam (10µg)</td>
<td><img src="image7" alt="Image" /> <img src="image8" alt="Image" /> <img src="image9" alt="Image" /></td>
</tr>
<tr>
<td>4</td>
<td>Levetiracetam (20µg)</td>
<td><img src="image10" alt="Image" /> <img src="image11" alt="Image" /> <img src="image12" alt="Image" /></td>
</tr>
<tr>
<td>5</td>
<td>Levetiracetam (40µg)</td>
<td><img src="image13" alt="Image" /> <img src="image14" alt="Image" /> <img src="image15" alt="Image" /></td>
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</tbody>
</table>
Zebrafish fin assay (Table 2) (Figure 1)

Table 2: Effect of treatment groups on zebrafish fin assay.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Treatment Groups</th>
<th>% Regeneration of Fin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control</td>
<td>66.7±0.566</td>
</tr>
<tr>
<td>2.</td>
<td>Bevacizumab (250µg/150ml)</td>
<td>19.6±1.233***</td>
</tr>
<tr>
<td>3.</td>
<td>Levetiracetam (100µg/150ml)</td>
<td>58.3±0.899**</td>
</tr>
<tr>
<td>4.</td>
<td>Levetiracetam (200µg/150ml)</td>
<td>39.6±1.599**</td>
</tr>
<tr>
<td>5.</td>
<td>Levetiracetam (400µg/150ml)</td>
<td>19.7±2.014***</td>
</tr>
</tbody>
</table>

MTT ASSAY (Table 4) (Figure 2)

Table 4: MTT assay of Levetiracetam against A-549 cells.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Conc. in µg</th>
<th>% Viability at 24 hrs</th>
<th>% Viability at 48 hrs</th>
<th>% Viability at 72 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2.</td>
<td>Bevacizumab</td>
<td>20.69</td>
<td>19.84</td>
<td>18.42</td>
</tr>
<tr>
<td>3.</td>
<td>10</td>
<td>40.08</td>
<td>38.68</td>
<td>37.41</td>
</tr>
<tr>
<td>4.</td>
<td>20</td>
<td>48.26</td>
<td>47.29</td>
<td>47.04</td>
</tr>
<tr>
<td>5.</td>
<td>40</td>
<td>52.33</td>
<td>51.61</td>
<td>48.73</td>
</tr>
</tbody>
</table>

Zebrafish embryo assay (Table 3)

Table 3: Effect of treatment groups on Zebrafish embryo assay.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Treatment Groups</th>
<th>% Angiogenic vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control</td>
<td>95.3±1.43</td>
</tr>
<tr>
<td>2.</td>
<td>Bevacizumab (25µg/100ml)</td>
<td>23.5±0.764***</td>
</tr>
<tr>
<td>3.</td>
<td>Levetiracetam (10µg/100ml)</td>
<td>31.5±1.88***</td>
</tr>
<tr>
<td>4.</td>
<td>Levetiracetam (20µg/100ml)</td>
<td>22.2±1.60***</td>
</tr>
<tr>
<td>5.</td>
<td>Levetiracetam (40µg/100ml)</td>
<td>15.3±1.36***</td>
</tr>
</tbody>
</table>

DISCUSSION

Angiogenesis takes place only after proliferation and migration of ECs. This significant step ladder is regulated by extracellular signals. Calcium stimulations control antigenic steps. EC moves from the existing vessels to arrange into a fresh tube. These cells go away from the vessel by shooting into matrix, proliferate and lastly end their mutagenic action. Ca^{2+} ions are secondary messengers mediate many signals in migration and proliferation. Scarcity of extracellular Ca^{2+} causes growth seizure in G1/S signifying Ca^{2+} role in cell cycle progression. Calcium concentration inside the cell has many functions based on its interval, amount and site. Ca^{2+} in endoplasmic reticulum (ER) is essential for the cell functioning. Increased levels of Ca^{2+} in ER stimulates cell division and vice versa. However a continued fall in the Ca^{2+} from ER or sudden decrease in Ca^{2+} from ER, concurrent with discharge of ER-Ca^{2+} into the cytosol, results apoptosis. Voltage gated channels are T-, L-, P-, N- and R-type. Amidst these, T-, L- and N-type are central in pathological environment. Increase in calcium causes the cells shift from G1 and G2/M phases. Opening of N-type stimulate G2/M whereas L-type channel stimulate G0/G1. T-type Ca^{2+} channels are important in cell cycle progression. Opening of Ca^{2+} channels results in down regulation of depolarization at G2/M phase by closing of K^{+} channels. Thus calcium dynamics plays a key role in cell death and proliferation.

Levetiracetam irreversibly blocks high-voltage-activated (HVA) calcium by 18% on an average in newly dissected CA1 hippocampal neurons of rats. Levetiracetam binding to calmodulin protein is significant, resulting in the start of calcium-calmodulin-dependent protein kinases and regulate cAMP a transcriptional protein. Calcium concentration inside the cell has many functions based on its interval, amount and site. Ca^{2+} in endoplasmic reticulum (ER) is essential for the cell functioning. Increased levels of Ca^{2+} in ER stimulates cell division and vice versa. However a continued fall in the Ca^{2+} from ER or sudden decrease in Ca^{2+} from ER, concurrent with discharge of ER-Ca^{2+} into the cytosol, results apoptosis. Voltage gated channels are T-, L-, P-, N- and R-type. Amidst these, T-, L- and N-type are central in pathological environment. Increase in calcium causes the cells shift from G1 and G2/M phases. Opening of N-type stimulate G2/M whereas L-type channel stimulate G0/G1. T-type Ca^{2+} channels are important in cell cycle progression. Opening of Ca^{2+} channels results in down regulation of depolarization at G2/M phase by closing of K^{+} channels. Thus calcium dynamics plays a key role in cell death and proliferation.
specifically blocks N-type Ca\(^{2+}\) channels of CA1 pyramidal hippocampal neurons.

Anti-angiogenic activity was evaluated by MTT cell viability assay. The results suggest that levetiracetam treatment significantly inhibited Hep G2 cells viability. The test drug is 50 percent as potent as the standard drug Bevacizumab. Morphological screening assay, Zebra fish caudal fin assay and Zebra fish embryo assay using different concentrations of Levetiracetam, standard drug Bevacizumab and control. The parameters assessed in morphological screening assay are cell shape and cell volume by microscopic examination. Cell volume expansion, which is the further steps in the endothelial cell proliferation, validates the antiangiogenic potential of the test drug. In this assay, control cells were normal with roughly cuboidal shape, Bevacizumab treated cells with increase in the incubation time, showed decrease in the cell volume hence changing the cell shape to spindle shape. Similar change in the cell shape was also observed with 40µg of levetiracetam. Percentage regeneration of amputated fin in caudal assay was calculated, significant decrease in fin regeneration was observed with the standard and test drug treated fishes. In the zebra fish embryo assay percentage neo micro vascularization was noted from the two major blood vessels, the dorsal aorta and the caudal vein. Decrease in the development of dorsal longitudinal anastomotic vessels and intersegment vessels were observed with the test and standard treated groups.\(^{18-21}\)

**CONCLUSION**

Earlier studies stated the role of Levetiracetam in treating epilepsy. The present study showed a potent antiangiogenic activity of Levetiracetam. Thereresultsof the study will be very useful to develop a cost-effective antiangiogenic compound. However, further research is required for suitable molecular modifications in the lead antiangiogenic drug, Levetiracetam and to deliver a site specific dosage form which can be affective against the pathological conditions resulting due to excessive angiogenesis.

**ACKNOWLEDGEMENT**

We thank the management of CMR College of Pharmacy to allow us to perform the research in the institution.

**CONFLICT OF INTEREST**

Authors declare no conflicts of interest.

**ABBREVIATIONS**

**EC:** Endothelial cells; **VEGF:** Vascular endothelial growth factor; **OD:** Optical density.

**REFERENCES**

Pathologically excessive angiogenesis can be the root cause of many diseased conditions. Ion channels on endothelium have a prime action in multi-stepped activity of angiogenesis. These ion channel functions are responsible for cell proliferation, migration, cell volume expansion, tubulogenesis resulting in micro neovascularization. This study aims to evaluate the antiangiogenic activity of Levetiracetam, a N-type voltage gated calcium channel blocker. Antiangiogenic activity of Levetiracetam was proven statistically significant in all the three methods; significant results were obtained at 20 µg and 30 µg in regeneration of fin growth in the Zebra fish fin assay. Whereas all the three doses i.e. 10 µg, 20 µg and 40 µg have shown potent angiogenic vessel inhibition in the embryo assay. In the MTT assay similar result was observed with 20 µg and 40 µg of the drug. Structural modifications of the channel modulator tested in our study will develop endothelial cell embattled chemical leads with site specificity which reduces the side effects.

**About Authors**

**Dr. Chandana Kamili** has completed her Ph.D from JNTUH, Hyderabad in the year 2018, she has 10 years of experience in teaching and research in various capacities. She is currently working as Assistant Manager, Medical writing for Tata Consultancy Services, Mumbai, India. Her areas of research interest are molecular pathways of angiogenesis, study of modulators of angiogenesis and use of nanoparticles in angiogenesis.

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**Dr. Suresh Merugu** obtained his Ph.D from Indian Institute of Technology Roorkee, He has around 12 years of Teaching experience and 4 years of research experience in the fields of Advance Digital Image Processing, Colorimetry, Computer Vision, Sub-Pixel Classification, Data Mining Techniques and Space Sciences.

**Dr. K. Abbulu** has been severing the field of pharmacy for the past 28 years. He has to his credit 80 research publications and 45 presentations in seminar and conferences. He has guided 14 Ph.D scholars, 55 M.Pharm and 35 B.Pharmacy students.