INVESTIGATION OF ANTIPROLIFERATIVE ACTIVITY OF GALANTAMINE PEPTIDE DERIVATE GAL – VAL AGAINST 3T3 CELL LINES

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ABSTRACT

In current investigation the aim was the assessment of properties of newly synthesized peptide ester of Galantamine: 6-O-[N-(3,4-dichlorophenyl)-D,L-Alanyl]-L-Valil-Glycine-Galantamine (GAL – VAL) to inhibit cell growth rate of cultured 3T3 mouse embryonic fibroblast cells, triplicate treated separately with different concentrations (1.875 μM ÷ 30 μM). The applied MTT assay determines the ability of viable cells to convert a soluble yellow tetrazolium salt [3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) into purpleblue formazan, which absorbance is measured spectrophotometrically at λ = 570 nm. Cell growth inhibition (%) and the index of cell viability (%) were calculated. Inhibition of 3T3 cell growth obtained after treatment with GAL – VAL at concentration 30 μM is 88.32 %. Inhibitory concentration IC₅₀ is 23 μM. These experimental results proved that against 3T3 cell line ester possesses antiproliferative effect.

Key words. Peptide esters, Galantamine, MTT, 3T3.
INTRODUCTION
Cancer is a disease of tissue growth regulation failure. Cancers are primarily an environmental disease with 90% – 95% of cases attributed to environmental factors and 5% – 10% due to genetics.\(^1\) In order for a normal cell to transform into a cancer cell, the genes which regulate cell growth and differentiation must be altered.\(^2\) In 2008 approximately 12.7 million cancers were diagnosed\(^3\) and in 2010 nearly 7.98 million people died.\(^4\) Many management options for cancer exist with the primary ones including surgery, chemotherapy and radiation therapy.\(^5\)

Galantamine (also called galanthamine) was originally isolated from several plants, including daffodil bulbs, but is now synthesized. Galantamine is a specific, competitive, and reversible acetylcholinesterase inhibitor. It is also an allosteric modulator at nicotinic cholinergic receptor sites potentiating cholinergic nicotinic neurotransmission. Galantamine has received regulatory approval in 29 counties: Argentina, Australia, Canada, Czechia, the European Union (except for The Netherlands), Iceland, Korea, Mexico, Norway, Poland, Singapore, South Africa, Switzerland, Thailand, and the United States.

The acetylcholinesterase inhibitor Galantamine\(^6\) allosterically sensitizes the \(\alpha_7\) – subtype of nicotinic acetylcholine receptors\(^7\), stimulate choline – acetyltransferase activity\(^8\) and possesses antioxidant activity\(^9\) Galantamine is applied for therapy of Alzheimer’s disease\(^10\), Alzheimer's disease with cerebrovascular disease and vascular dementia.\(^11\)

Newly synthesized from prof. Vezenkov Galantamine peptide ester 6-O-N-[N-(3,4-dichlorophenyl)-D,L-Alanyl]-L-Valil-Glycil-Galantamine (GAL-VAL)\(^12\) possess both acetylcholinesterase and \(\gamma\) – secretase inhibitory activity\(^13\) and antioxidant properties in ferric reducing/antioxidant power (FRAP) method.\(^14\)

L-Leucyl-L-Leucine methyl ester induces apoptosis on cell lines.\(^15\) In this connection in current investigation our aim was the assessment of properties of newly synthesized peptide esters to inhibit cell growth rate of 3T3 cell line.\(^16\)

MATERIALS
1. Tested peptide ester – 6-O-N-[N-(3,4-dichlorophenyl)-L-Alanyl]-L-Valil-Glycil-Galanthamine (Fig 1.).
Fig. 1. Structure of 6-O-N-[N-(3,4-dichlorophenyl)-L-Alanyl]-L-Valil-Glycine-Galanthamine.

2.1. In vitro cancer test systems – cell lines.
Dulbecco’s Modified Eagle Medium was used for culturing of 3T3 cells.

2.3. Reagents with analytical grade quality.
Fetal bovine serum, 100 IU/ml of penicillin, 100 µg/ml of Streptomycin, standard MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide), dimethylsulfoxide.

2.4. Preparation of solutions of peptide esters.
For obtaining of solutions of each ester with concentration: 1.875 µM, 3.75 µM, 7.5 µM, 15 µM and 30 µM, an accurately weighted quantities of the examined peptide ester GAL–VAL were dissolved separately in dimethylsulfoxide.

2.5. Preparation of solution of MTT.
An accurately weighted quantity of MTT was dissolved in phosphate buffer solution to obtaining solution with concentration 5 mg/ml MTT. This solution is stable 1 month at storage at 4 °C.

3. Method – MTT test assay line.16
For the assessment of antiproliferative effect of compound the standard MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide) colorimetric assay was applied. 3T3 mouse fibroblast cells were cultured in 96 – well flat – bottomed micro plates in Dulbecco’s Modified Eagle Medium, supplemented with 5% of fetal bovine serum, 100 IU/ml of Penicillin and 100 µg/ml of Streptomycin in 75 cm² flasks and kept in 5% CO₂ incubator at 37°C. 3T3 cells were harvested in exponentially growing phase, counted with
haemocytometer and by dilution with a particular medium the cell density was adjusted to the concentration of $5.10^4$ cells/ml. 100 µl/well of this cell culture were introduced to each well of a 96 – well plates. After overnight incubation at 37°C in 5% CO$_2$ the supernatant was discarded and the cells were exposed to 200 µl of every peptide ester in different concentrations (1.875 µM – 30 µM) of GAL – VAL. To each well after 48 h were added 200 µl 0.5 mg/ml MTT. All samples were incubated further for 4 h. In order to facilitate the solubilization of the formazan product 100 µl of dimethylsulfoxide were added to each well. The absorbance of the obtained from reduction formazan was measured at $\lambda = 570$ nm. The cytotoxicity was recorded as concentration causing 50 % growth inhibition (IC$_{50}$) for 3T3 cells.

RESULTS AND DISCUSSION

The reduction of yellow MTT to purple formazan in the mitochondria of living cells takes place only when mitochondrial reductase enzymes are active. Therefore the conversion can be directly related to the number of viable (living) cells. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals which are insoluble in aqueous solutions. The crystals were dissolved in dimethylsulfoxide and the resulting purple solution was spectrophotometrically measured at $\lambda = 570$ nm. The decrease of cell number results in an decrease in the amount and absorbance of formazan.

3T3 lines are valuable in vitro host systems for oncogenic transformation studies. At the time of their establishment, 3T3 cells were different than most other cell lines in regard to the fact they did not induce tumors to develop when injected into murine species. The unusual behavior of the line enabled researchers to make a clear distinction for the first time between immortal cells and cells that have the ability to form tumors. Due to examination of 3T3 cells it has become widely accepted that immortalization of cells is a process that is not necessarily related to a cell’s ability to undergo oncogenic transformation.$^{17}$

As the positive ($A_{(+)}$) control was used the respective cell line treated with solution of MTT without addition of the examined compounds. As the negative ($A_{(-)}$) control was used the respective cell line dissolved in culture medium without addition of the examined compounds and MTT. On Table 1. are presented the absorbances for positive ($A_{(+)}$) and negative ($A_{(-)}$) controls.
Table 1. Absorbances for positive (A_{(+)} ) and negative (A_{(-)} ) control for different cell lines in MTT – test of Mosmann.

<table>
<thead>
<tr>
<th>N:</th>
<th>( A_{K(+) } )</th>
<th>( A_{K(-)} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1.208</td>
<td>0.098</td>
</tr>
<tr>
<td>2.</td>
<td>1.222</td>
<td>0.096</td>
</tr>
<tr>
<td>3.</td>
<td>1.236</td>
<td>0.090</td>
</tr>
<tr>
<td>4.</td>
<td>1.340</td>
<td>0.098</td>
</tr>
<tr>
<td>5.</td>
<td>1.210</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>1.350</td>
<td></td>
</tr>
<tr>
<td>( \bar{X} )</td>
<td>1.261</td>
<td>0.096</td>
</tr>
<tr>
<td>SD</td>
<td>0.066</td>
<td>0.004</td>
</tr>
</tbody>
</table>

For the estimation of cytotoxic activity the MTT – test of Mosmann is applied triplicate separately for different concentrations (1.875 \( \mu \text{M} \pm 30 \mu \text{M} \)) of GAL – VAL. The absorbances of the obtained formazan are summarized on Table 2.

Table 2. Absorbances of formazan produced from 3T3 cell line treated with GAL – VAL.

<table>
<thead>
<tr>
<th>( C_{\text{GAL - VAL}} ) [( \mu \text{M} )]</th>
<th>Absorbances of formazan [AU]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.</td>
</tr>
<tr>
<td>1.875</td>
<td>1.074</td>
</tr>
<tr>
<td>3.75</td>
<td>1.109</td>
</tr>
<tr>
<td>7.5</td>
<td>1.065</td>
</tr>
<tr>
<td>15</td>
<td>0.814</td>
</tr>
<tr>
<td>30</td>
<td>0.26</td>
</tr>
</tbody>
</table>

On Figure 2. is illustrated the accordance between concentration of peptide ester and absorbance of formazan.

![Absorbance – concentration relation for GAL – VAL.](image-url)
In the applied MTT test of Moosmann the accordance of formazan is proportional to viability cell lines. Index of cell viability $V (%)$ and the inhibition of cell growth (%) were calculated by the following equations

$$V (%) = \frac{A_T - A(-)}{A(+)} \cdot 100$$

$$I (%) = 100 - \frac{A_T - A(-)}{A(+)} \cdot 100$$

$V (%)$ – index of cell viability

$I (%)$ – inhibition of cell growth

$A_T$ – mean absorbance derived from a well added with test solutions of GAL – VAL

$A(+) - A(-)$ – mean absorbance of positive control, derived from a well added with cell culture without test solutions

$A(-)$ – mean absorbance of negative control

Results for effect of GAL – VAL on inhibition of 3T3 of cell growth [%] and index of viability of 3T3 cell line are presented on Table 3.

**Table 3. Effect of GAL – VAL on proliferation 3T3 cell line.**

<table>
<thead>
<tr>
<th>C&lt;sub&gt;GAL-VAL&lt;/sub&gt; [μM]</th>
<th>Inhibition 3T3 of cell growth [%]</th>
<th>Index of viability of 3T3 cell line [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.</td>
<td>2.</td>
</tr>
<tr>
<td>1.875</td>
<td>16.04</td>
<td>11.67</td>
</tr>
<tr>
<td>3.75</td>
<td>13.04</td>
<td>4.72</td>
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<td>7.5</td>
<td>16.82</td>
<td>14.84</td>
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<td>15</td>
<td>38.35</td>
<td>34.92</td>
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<tr>
<td>30</td>
<td>85.89</td>
<td>85.97</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>21.51</td>
<td>22.51</td>
</tr>
</tbody>
</table>

The accordances between concentration of peptide ester and cell growth inhibition (%) and cell viability (%) are illustrated on Fig. 3.
Fig. 3. Index of 3T3 cell viability and cytotoxic effect assessed by an MTT assay following exposure to GAL – VAL.

By using the equations for mean index of cell growth inhibition: $y = 7.890 \cdot e^{0.081 \cdot x}$ is calculated data for the inhibition concentration IC$_{50}$: 23.17 µM for GAL – VAL. IC$_{50}$ of used standard Cicloheximide is 0.26 µM.

In MTT test the decreased concentration and absorbance of formazan indicates growth inhibitory activity of examine compounds. Inhibition of 3T3 cell growth obtained after treatment with GAL – VAL at concentration 1.875 µM is 13.9 % with cell survival 86.1 %. 30 µM GAL – VAL exerts 88.32 % inhibition with index of cell viability 11.68 %.

In comparison with our results the treatments with cytostatic drug Cisplatin causes a cell apoptosis, the incidence of which appeared to be concentration – dependent.$^{18}$ It is described that treatment of 3T3 cells with Zidovudine cause significant concentration and time dependent decreases in the number of viable cells. 1000 µM Zidovudine lead to 83.6 % inhibition of cell growth. The decrease in the number of viable cells could be due to an
increase in necrotic cell death, an induction of apoptosis or an impairment of the cells transiting through the cell cycle. Curcumin has been demonstrated to be an effective inhibitor of tumor promotion in are the oncogene transformed 3T3 cells – immortalized mouse embryo fibroblast NIH 3T3 erb B2, Curcumin possesses inhibitory activity on tumor promotion.

Benzethonium chloride was identified as a novel cancer specific compound. For benzethonium the dose required to reduce cell viability by 50 % is 42.2 μmol/l in NIH 3T3.

CONCLUSION
MTT test of Moosmann was applied for the evaluation of 3T3 cells survival. In concentration 30 μM the examined Galantamine peptide ester GAL – VAL inhibits 88.32 % of 3T3 cell growth. The experimental results proved that against 3T3 cell line GAL – VAL exerts cytotoxic activity with IC₅₀ = 23.17 μM, calculated from the equation y = 7.609.e⁰.⁰⁹⁸ₙ.

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