EFFECT OF 6-O-N-[N-(3,4-DICHLOROPHENYL)-D,L-ALANYL]-L-LEUCYL-GLYCINE-GALANTAMINE ON 3T3 CELLS VIABILITY

D. Tsvetkova[1], R. Klisurov[2], D. Obreshkova[1], P. Atanasov[3]

[1]Department of Pharmaceutical Chemistry, Faculty of Medicine, Medical University –Sofia
[2]Department of Pharmacology and Toxicology, Faculty of Medicine, Medical University – Sofia
[3]Clinic of THK, UMHATEM „N.I.Pirogov"– Sofia

Summary. The aim of current study was the estimation of the effect of newly synthesized Galantamine peptide ester: 6-O-N-[N-(3,4-dichlorophenyl)-D,L-Alanyl]-L-Leucyl-Glycine-Galantamine (GAL – LEU) on cell growth rate of cultured 3T3 mouse embryonic fibroblast cells. 3T3 cells were in triplicate treated separately with different concentrations: 1.875 μM ÷ 30 μM of GAL – LEU. The applied MTT assay is based on the capacity of mitochondrial succinyl dehydrogenase of living cells to convert the yellow tetrazolium salt (3-(4',5'-dimethylthiazol-2'-yl)-2,5-diphenyl-tetrazolium bromide) into an purpleblue formazan, which absorbance is measured spectrophotometrically at λ = 570 nm.
Cell growth inhibition (%) and the index of cell viability (%) were calculated. GAL – LEU in concentration 30 μM inhibits 99.9 % of cell growth. From equation: $y = 7.603.e^{0.098.x}$ was calculated the inhibition concentration IC_{50}: 19 μM. IC_{50} of used standard Cicloheximide is 0.26 μM. These experimental results proved that against 3T3 cell line the examined ester exerts cytotoxic activity.

Key Words: Peptide ester, Galantamine, MTT, 3T3

Introduction

The most significant risk factor for developing of cancer include tobacco (25 % – 30 %) [1], infections (15 % – 20 %) [2], radiation, environmental pollutants, obesity (30 % – 35 %), lack of physical activity, stress [3], old age [4], but many cancers are developed in childhood too [5]. Acetylcholinesterase inhibitor Galantamine [6] potentiates the release of neurotransmitter acethylcholine [7] and possesses antioxidant activity [8, 9] and positive allosteric modulatory effect on α7 – subtype of nicotinic acetylcholine receptors [10]. Galantamine is applied for therapy of Alzheimer's disease [11, 12, 13]. Newly synthesized from prof. Vezenkov Galantamine peptide ester: 6-O-N-[N-(3.4-dichlorophenyl)-D,L-Alanyl]-L-Leucyl-Glycil-Galantamine (GAL – LEU) [14], possess both acethylcholinesterase and γ – secretase inhibitory activity [15] and antioxidant properties in ferric reducing/antioxidant power (FRAP) method [16].

L-Leucyl-L-Leucine methyl ester induces apoptosis on cell lines [17]. In this connection in current investigation our aim was the assessment of properties of newly synthesized peptide ester to inhibit cell growth rate of 3T3 cell line [18].

Materials and methods

Materials

I. Tested peptide ester: 6-O-N[N(3,4-dichlorophenyl)-D,L-Alanil-L-Leucil-L-Glycil]-Galantamine (GAL – LEU), syntetized from prof. Vesenkov from Department of Organic Chemistry, University of Chemical Technology and Metallurgy (Fig. 1.) [14].
II. In vitro test system – 3T3 mouse embryonic fibroblast cells.
III. Reagents with analytical grade quality.

Dulbecco's Modified Eagle Medium, fetal bovine serum, 100 IU/ml Penicillin, 100 μg/ml Streptomycin, standard MTT (3-[4.5-dimethylthiazole-2-yl]-2.5-diphenyl-tetrazolium bromide), dimethylsufoxide, phosphate buffer.

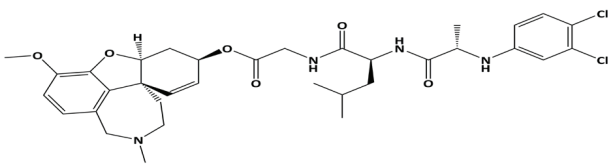

Fig. 1. *Structure of 6-O-N[N(3,4-dichlorophenyl)-D,L-Alanil-L-Leucil-L-Glycil]- Galantamine*

IV. Preparation of solutions of peptide ester.

An accurately weighted quantities of GAL – LEU were dissolved separately in dimethylsufoxide for obtaining solutions with concentration: 1.875 μM, 3.75 μM, 7.5 μM, 15 μM and 30 μM.

V. Preparation of solution of MTT.

5 mg/ml solution of MTT in phosphate buffer was prepared. At storage at 4°C solution is stable 1 month.

Method – MTT test assay [18].

3T3 mouse fibroblast cells were cultured in 96 – well flat – bottomed micro plates in Dulbecco's Modified Eagle Medium. 5 % of fetal bovine serum, 100 IU/ml Penicillin and 100 μg/ml Streptomycin were added. Samples were incubated in 5% CO_2 at 37°C. In exponentially growing phase 3T3 cells were harvested and counted with haemocytometer. The cell density was adjusted to the concentration of 5.10^4 cells/ml by dilution with Dulbecco's Modified Eagle Medium. 100 μl of this cell culture were introduced to each well. Samples were incubated at 37°C in 5% CO_2 for 24 h. After incubation the supernatant was discarded. The cells were exposed to 200 μl of peptide ester in different concentrations (1.875 μM – 30 μM).

After 48 h to each well 200 μl 0.5 mg/ml MTT were added and samples were incubated for 4 h. In order to facilitate the solubilization of the formazan product 100 μl of dimethylsufoxide were added to each well. The absorbance of the obtained from reduction of MTT formazan was measured at λ = 570 nm. The cytotoxicity was recorded as concentration causing 50 % growth inhibition (IC50) of 3T3 cells.

Results

The positive (A(+)) conrol was 3T3 cell line treated with solution of MTT without addition of the examined compounds. As the negative (A(-)) conrol was used 3T3 cell cell line dissolved in Dulbecco's Modified Eagle Medium without addition of GAL – LEU and MTT. On Table 1. are presented the absorbances for positive (A(+)) and negative (A(-)) controls.

For the estimation of cytotoxic activity the MTT – test of Mosmann was applied triplicate separately for different concentrations of GAL – LEU (1.875 μM – 30 μM). The absobances of the obtained formazan are summarized on Table 2.

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

Table 1. *Absorbances for positive (A(+)) and negative (A(-)) control in MTT – test of Mosmann.*

N:	AK(+)	AK(-)
1.	1.208	0.098
2.	1.222	0.096
3.	1.236	0.090
4.	1.340	0.098
5.	1.210	
6.	1.350	
$\overline{X}$	1.261	0.096
SD	0.066	0.004

Table 2. *Absorbances of formazan produced from 3T3 cells treated with GAL – LEU.*

$C_{GAL-LEU}$ [μM]	Absorbances of formazan [AU]				
	1.	2.	3.	$\overline{X}$	SD
1.875	1.229	1.179	1.192	1.200	0.026
3.75	1.168	1.079	1.146	1.131	0.046
7.5	1.116	1.027	0.968	1.037	0.075
15	0.465	0.329	0.530	0.441	0.103
30	0.097	0.098	0.095	0.097	0.002

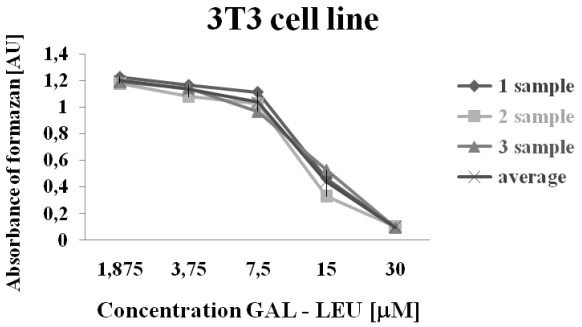

Fig. 2. *Absorbance – concentration relation for GAL – LEU*

In the applied MTT test of Mosmann the absorbance of formazan is proportional to the viability of cells. Index of cell viability V (%) and the inhibition of cell growth (%) are calculated by the following equations:

$$V(\%) = \frac{A(t)-A(-)}{A(+)-A(-)}.100$$

$$I(\%) = 100 - \frac{A(t)-A(-)}{A(+)-A(-)}.100$$

V (%) – index of cell viability
I (%) – inhibition of cell growth

At – mean absorbance derived from a well added with test solution GAL – LEU

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

A(-) – mean absorbance of negative control, derived from a well added with cell culture without test solution and MTT solution.

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

$C_{GAL-LEU}$ [μM]	Index of viability of 3T3 cell line [%]				
	1.	2.	3.	$\overline{X}$	SD
1.875	97.25	92.96	94.08	94.76	2.23
3.75	92.02	84.38	90.13	88.84	3.98
7.5	87.56	79.92	74.86	80.78	6.39
15	31.70	20.03	37.28	29.67	8.80
30	0.13	0.21	-0.04	0.1	0.13
$C_{GAL-LEU}$ [μM]	Inhibition of 3T3 cell growth [%]				
	1.	2.	3.	$\overline{X}$	SD
1.875	2.75	7.04	5.92	5.24	2.23
3.75	7.98	15.62	9.87	11.16	3.98
7.5	12.44	20.08	25.14	19.22	6.39
15	68.30	79.97	62.72	70.33	8.80
30	99.87	99.79	100.04	99.90	0.13
IC_{50}	20.24	18.24	19.10	19.19	1.00

The results from the effect of the examined peptide ester on index of cell viability (%) and inhibition of cell growth (%) are summarized on Table 3.

On Fig.3 is illustrated the index of 3T3 cell viability and cytotoxic effect assessed by an MTT assay following exposure to GAL – LEU.

By using the equations for mean index of cell growth inhibition: $y = 7.603.e^{0.098x}$ the value for the inhibition concentration (IC_{50}) of GAL – LEU: 19 μM is calculated. IC_{50} of used standard Cicloheximide is 0.26 μM.

Discussion.

Survival of cells was evaluated by using of MTT assay, based on the capacity of mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a soluble in dimethylformamide violet formazan product, which absorbance is measured spectrophotometrically at λ = 570 nm [18]. Since reduction of MTT can occur only in metabolically active cells, the increased growth inhibitory effect is in accordance with a reduced absorbance of formazan.

3T3 cell line is established by George Todaro and Howard Green in 1962 from disaggregated Swiss mouse (Mus musculus) embryo tissue. 3T3 cell line is a standard fibroblast cell line used in a wide spectrum of research and industrial biomedical applications. 3T3 cells are not normal cells because they are capable of growing indefinitely [19].

The experimental results demonstrate that the ester treatment results in reduced concentration and absorbance of formazan, which indicates its growth inhibitory activity. While GAL – LEU in low concentration 1.875 μM inhibits 5.24 % of cell growth with index of cell viability 94.76 %, in high concentration 30 μM inhibits 99.9 % of cell growth with index of cell survival 0.1 %.

Treatment of 3T3 cells with the protein kinase C inhibitor Staurosporine induces apoptotic cell death. Experimental results show that the tyrosine kinase inhibitor Herbimycin A possesses an antiproliferative activity on 3T3 cells. Inhibitor of arachidonic acid metabolism Quinacrine inhibits growth rate of 3T3 cells. These results suggest that in immortalized and

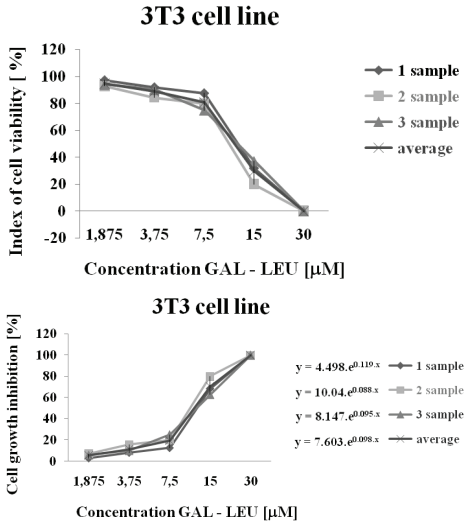

Fig. 3. *Effect of GAL – LEU on 3T3 cell viability (%) and growth inhibition (%).*

transformed 3T3 cells, blocking the cellular signal transduction might trigger the induction of cell apoptosis. In this connection Curcumin has been demonstrated to be an effective inhibitor of tumor promotion In immortalized mouse embryo fibroblast NIH 3T3 erb B2, which are the oncogene transformed 3T3 cells. The cellular and biochemical effects of curcumin in these mouse fibroblast cells are: inhibition the activity of protein kinase C and tyrosine protein kinase and suppression of arachidonic acid metabolism. All of these effects lead to induction of characteristics of apoptosis: cell shrinkage, chromatin condensation and DNA fragmentation [20].

Conclusion

Survival of 3T3 cells was evaluated by using of MTT test of Mosmann. GAL – LEU in concentration 30 μM inhibits 99.9 % of 3T3 cell growth. The experimental results proved that against 3T3 cell line the examined Galantamine ester GAL – LEU exerts cytotoxic activity with IC_{50} = 19 μM, calculated from the equation $y = 7.609.e^{0,098.x}$.

Acknowledgements

Authors acknowledge for experimental support and technical help to prof. Iqbal Choudhary, Sadia Siddiq and Rizwana Malik from Dr Panjwani Center for Molecular Medicine and Drug Research, ICCBS, University of Karachi in Pakistan.

References

1. Sasco AJ, Secretan MB, Straif K. Tobacco smoking and cancer: a brief review of recent epidemiological evidence. Lung Cancer 2004; 45(Suppl 2): S3-S9.
2. Pagano JS, Blaser M, Buendia MA, Damania B, Khalili K, Raab-Traub N, Roizman B. Infectious agents and cancer: criteria for a causal relation. Semin Cancer Biol 2004; 14(6): 453-471.
3. Anand P, Kunnumakkara AB, Kunnumakara AB, Sundaram C, Harikumar KB, Tharakan ST, Lai OS, Sung B, Aggarwal BB. Cancer is a preventable disease that requires major lifestyle changes. Pharm Res 2008; 25(9): 2097-2116.
4. Anisimov VN, Sikora E, Pawelec G. Relationships between cancer and aging: a multilevel approach. Biogerontology 2009; 10(4): 323-338.
5. Kaatsch P, Sikora, E, Pawelec, G. Epidemiology of childhood cancer. Cancer Treat Rev 2010; 36(4): 277-285.
6. Danchev N, Nikolova I. Pharmacological treatment of cognitive impairements in Alzheimer's disease. Autonomic Autocoid Pharmacol 2006; 26(1): 46-49.
7. Villaroya M, Garcia, AG, Marco-Contelles J, Lopez MG. An update on the pharmacology of galantamine. Expert Opin Investig Drugs 2007; 16(12): 1987-1998.
8. Ezoulin MJM, Ombetta J-E, Dutertre-Catella H, Warnet J-M, Massicot F. Antioxidative properties of galantamine on neuronal damage induced by hydrogen peroxide in SK-N-SH cells. Neurotoxicology 2008; 29(2): 270-277.
9. Tsvetkova D, Obreshkova D, Zheleva – Dimitrova D, Saso L. Antioxidant activity of Galanthamine and some of its derivatives. Curr Med Chem 2013; 22(36): 4595-4608.
10. Maelicke A, Samochocki M, Jostock R, Fehrenbacher A, Ludwig J, Albuquerque EX, Zerlin M. Allosteric sensitization of nicotinic receptors by galanthamine, a new treatment strategy for Alzheimer's Disease. Biological Psychiatry 2001; 49(3): 279-288.
11. Lilienfeld S. Galantamine – novel cholinergic drug with a unique dual mode of action for the treatment of patients with Alzheimer's disease. CNS Drug Rev 2002; 8(2): 159-176.
12. Mintzer JE, Kershaw P. The efficacy of galantamine in the treatment of Alzheimer's disease: comparison of patients previously treated with acetylcholinesterase inhibitors to patients with no prior exposure. Int J Geriatr Psychiatry 2003; 18(4): 292-297.
13. Raskind MA Update on Alzheimer drugs (Galantamine). Neurologist 2003; 9(5): 235-240.
14. Vezenkov L, Georgieva M, Danalev D, Ivanov Tch, Ivanova G. Synthesis and characterization of new Galanthamine derivatives comprising peptide moiety. Protein and Peptide Letters 2009; 16(9): 1024-1028.
15. Vezenkov L, Sevalle J, Danalev D, Ivanov T, Bakalova A, Georgieva M, Checler F. Galantamine-based hybrid molecules with acetylcholinesterase, butyrylcholinesterase and γ-secretase inhibition activities. Curr Alzheimer Res 2012; 9(5): 600-605.

16. Tsvetkova D, Zheleva – Dimitrova D, Obreshkova D. Estimation of an antioxidant activity of new peptide esters of Galanthamine by applying of Ferric reducing antioxidant power (FRAP) method. Compt Rend Acad Bulg Sci 2013; 66(3): 445-450.
17. Thiele DL, Lipsky PE. Modulation of human natural killer cell function by L-leucine methyl ester: monocytedependent depletion from human peripheral blood mononuclear cells. J Immunol 1985; 134(2): 786-793.
18. Mosmann T. Rapid colorimetric assay for cellular growth and survival. Application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65(1-2): 55-63.
19. Todaro GJ, Green H. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J Cell Biol 1963; (17): 299-313.
20. Jiang MC, Yang-Yen HF, Yen JJ, Lin JK. Curcumin induces apoptosis in immortalized NIH 3T3 and malignant cancer cell lines. Nutr Cancer 1996; 26(1): 111-120.

✉ **Corresponding author**
Dobrina Tsvetkova
Faculty of Pharmacy, Medical University
2 Dunav Str. 1000 Sofiaa
Tel: +35929236566
Fax: +35929879874
e-mail: dobrinka30@abv.bg