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Evaluation of Cytotoxic Effects Induced by Galantamine-Linked Peptide Esters on HeLa Cell Line via MTT Assay

Dobrina Tsvetkova[1*], Lyubomir Vezenkov[2], Tchavdar Ivanov[2], Dancho Danalev[3], Ivanka Kostadinova[4]

[1] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Medical University – Sofia, Sofia, Bulgaria.
[2] Department of Organic chemistry, University of Chemical Technology and Metallurgy, 1756 Sofia, Bulgaria.
[3] Department of Biotechnology, University of Chemical Technology and Metallurgy, 1797 Sofia, Bulgaria.
[4] Department of Pharmacology, Pharmacotherapy and Toxicology, Faculty of Pharmacy, Medical University of Sofia, Sofia 1000, Bulgaria.

***E-mail** ✉ dtsvetkova@pharmfac.mu-spfia.bg

Abstract

In this study, the cytotoxic effect of newly developed peptide esters of galantamine—specifically GAL-VAL and GAL-LEU—was assessed against the HeLa cell line, a model for human cervical adenocarcinoma, using a concentration gradient ranging from 1.875 µM to 30 µM. Among the two, GAL-LEU showed a significantly enhanced ability to impair HeLa cell viability, achieving an 86.81% suppression at the highest concentration tested (30 µM), with cell viability reduced to 13.19%. Comparative evaluation of their IC50 values revealed a greater potency for GAL-LEU, which required only 23.63 µM to reduce viability by half, while GAL-VAL showed a less potent IC50 of 31.95 µM. These results demonstrate the strong cytotoxic action of both peptide esters on the HeLa cell line, with GAL-LEU standing out as the more effective agent. The data point toward the promising therapeutic potential of GAL-VAL and GAL-LEU for further development in the treatment of cervical adenocarcinoma.

Keywords: HeLa cell line, Galantamine, Peptide esters, MTT

Introduction

Galantamine is recognized as a long-acting, centrally acting, and reversible inhibitor that competes with acetylcholine at the active site of the enzyme acetylcholinesterase [1, 2]; in addition to this cholinergic activity, it exhibits antioxidant behavior and functions as a positive allosteric modulator of α7-subtype nicotinic acetylcholine receptors [3–6]. Prior research has shown that L-Leucyl-L-Leucine methyl ester can trigger programmed cell death in various cellular models [7], while other compounds such as N-phosphoryl dipeptide methyl esters have been associated with the induction of apoptosis in HCT-15 cells [8]. Furthermore, a compound like (di-isopropyloxyphoryl-Trp)2-Lys-OCH3 has demonstrated inhibitory effects on the proliferation of HeLa cells [9]. Building upon these findings, the present study focused on assessing the cytotoxic behavior of newly synthesized Galantamine-based peptide esters—namely, 6-O-N-[N-(3,4-chlorophenyl)-D, L-Alanyl]-L-Valil-Glycil-Galantamine (GAL-VAL) and 6-O-N-[N-(3,4-chlorophenyl)-D, L-Alanyl]-L-Leucyl-Glycil-Galantamine (GAL-LEU)—against the HeLa cell line, which models human cervical adenocarcinoma [10]. Previous evaluations using the ferric reducing/antioxidant power (FRAP) method revealed that these compounds exhibit dual inhibitory activity against acetylcholinesterase and γ-secretase enzymes [11, 12].

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In this investigation, the cytotoxic impact of newly developed peptide esters of galantamine—specifically GAL-VAL and GAL-LEU—was assessed against the HeLa cell line, a model for human cervical adenocarcinoma, using a concentration gradient ranging from 1.875 µM to 30 µM.

Materials and Methods

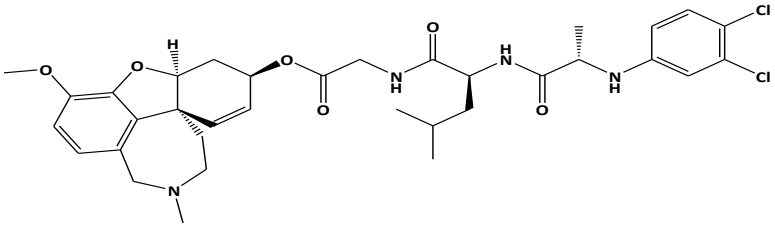
Materials

1. In Vitro Cancer Cell Model – HeLa Cell Line

To evaluate the cytotoxic potential of the peptide esters, experiments were conducted using the HeLa cell line derived from cervical adenocarcinoma, maintained in Minimum Essential Medium Eagle as the culture medium.

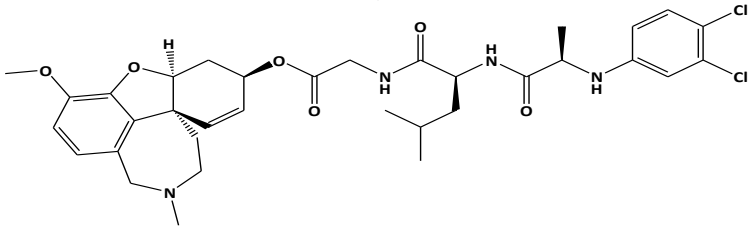
2. Tested Peptide Esters: GAL-LEU and GAL-VAL

The peptide esters GAL-LEU and GAL-VAL utilized in this study were synthesized by Vezenkov *et al*. [10] and are depicted in **Figure 1**.



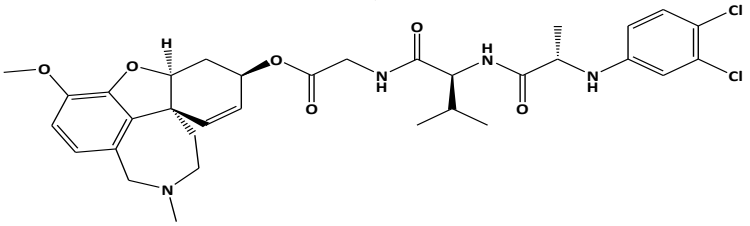
(4a*S*,6*R*,8a*S*)-3-methoxy-11-methyl-4a,5,9,10,11,12-hexahydro-6*H*-benzo[2,3]benzofuro[4,3-*cd*]azepin-6-yl (3,4-dichlorophenyl)-*L*-alanyl-*L*-leucylglycinate

a)



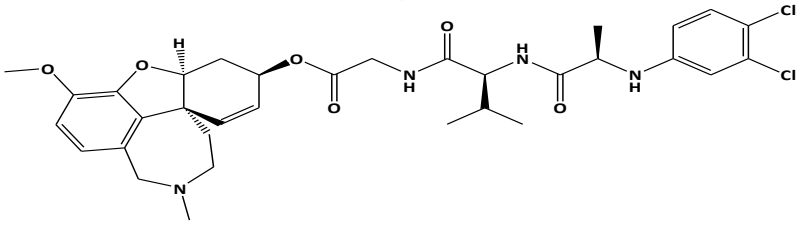
(4a*S*,6*R*,8a*S*)-3-methoxy-11-methyl-4a,5,9,10,11,12-hexahydro-6*H*-benzo[2,3]benzofuro[4,3-*cd*]azepin-6-yl (3,4-dichlorophenyl)-*D*-alanyl-*L*-leucylglycinate

b)



(4a*S*,6*R*,8a*S*)-3-methoxy-11-methyl-4a,5,9,10,11,12-hexahydro-6*H*-benzo[2,3]benzofuro[4,3-*cd*]azepin-6-yl (3,4-dichlorophenyl)-*L*-alanyl-*L*-valylglycinate

c)



(4a*S*,6*R*,8a*S*)-3-methoxy-11-methyl-4a,5,9,10,11,12-hexahydro-6*H*-benzo[2,3]benzofuro[4,3-*cd*]azepin-6-yl (3,4-dichlorophenyl)-*D*-alanyl-*L*-valylglycinate

d)

Figure 1. Chemical structures of GAL-LEU and GAL-VAL

3. Analytical-Grade reagents

The study employed high-purity reagents including fetal bovine serum (FBS), penicillin at a concentration of 100 IU/ml, streptomycin at 100 µg/ml, and standard MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide). Additional materials included dimethylsulfoxide, as well as 0.25 percent Trypsin EDTA 1X for enzymatic cell detachment.

4. Stock solution preparation of peptide esters

The peptide esters of Galantamine, GAL-VAL, and GAL-LEU, were individually solubilized in dimethylsulfoxide to generate stock solutions, each at a final concentration of twenty mM.

5. MTT solution formulation

To prepare the MTT solution, the compound was dissolved in phosphate-buffered saline (PBS) to yield a 5 mg/ml solution. When stored at 4 °C, this preparation remains stable for up to one month.

Methods

1. Cell culture and seeding procedure

To evaluate cytotoxic responses, assays were conducted using 96-well microplates. HeLa cervical cancer cells were grown in minimum essential medium eagle enriched with 5% fetal bovine serum, 100 IU/ml of penicillin, and 100 µg/ml of streptomycin. Cultures were maintained in 75 cm^2 flasks under standard conditions: 37 °C, 5% CO_2, and high humidity. Actively dividing cells were enzymatically detached using trypsin, collected via centrifugation, and counted using a hemocytometer. The cell density was adjusted with fresh culture medium to reach 6×10^4 cells per ml. Subsequently, 100 µl of this suspension was dispensed into each well and incubated for 24 hours at 37 °C in a humidified CO_2 atmosphere. After incubation, the medium was aspirated from the wells to prepare for treatment.

2. MTT assay protocol

The cytotoxic effects of the peptide esters were determined following the MTT assay developed by Mosmann [13]. Solutions of GAL-VAL and GAL-LEU were prepared in varying concentrations from 1.875 µM to 30 µM, and 200 µl of each concentration was added to individual wells. Each condition was tested in triplicate, and the mean values were recorded. After a 48-hour exposure period, 200 µl of MTT solution (0.5 mg/ml) was added to each well, and plates were incubated for an additional 4 hours at 37 °C in a CO_2-enriched environment. Post-incubation, the supernatant was removed, and 100 µl of dimethylsulfoxide was added to dissolve the resulting formazan crystals. Absorbance was then measured at 570 nm. The IC_{50} value, indicating the concentration at which cell proliferation was inhibited by 50%, was calculated. Additionally, cell viability index (V%) and inhibition of cell growth (I%) were determined using standard formulae.

$$V(\%) = \left[\frac{A(t) - A(-)}{A(+) - A(-)}\right] \times 100 \quad (1)$$

$$I(\%) = 100 - V(\%) = 100 - \left\{\left[\frac{A(t) - A(-)}{A(+) - A(-)}\right] \times 100\right\} \quad (2)$$

V (%): cell viability index

Represents the percentage of viable HeLa cells after treatment with the tested compound.

I (%): percentage of cell growth inhibition

Reflects the degree to which cell proliferation is suppressed by the peptide esters.

At: This is the average absorbance measured from formazan crystals in wells containing HeLa cells treated with the test compound.

A(+): Denotes the average formazan absorbance from the positive control group, which includes HeLa cells treated with MTT but without exposure to the peptide esters.

A(−): Refers to the mean absorbance recorded from the negative control setup, where only MTT was added, excluding both cells and test compounds.

Results and Discussion

To calculate the IC_{50} values for GAL-LEU and GAL-VAL against the HeLa cell line, the absorbance data for the formazan formed was plotted against the corresponding concentrations of the peptide esters. Doxorubicin was employed as the reference standard. The absorbance readings from the positive control (A(+)) and negative control (A(−)), as well as the formazan absorbance levels resulting from the treatment of HeLa cells with the test compounds, are summarized in **Table 1.**

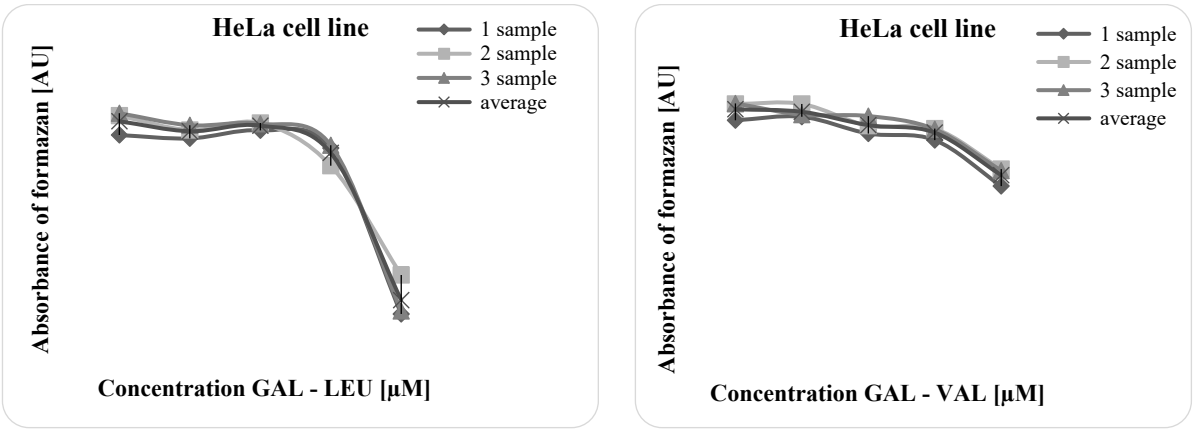
Table 1. Absorbances controls and formazan produced from the GAL-LEU- and GAL-VAL-treated HeLa cell line

N	A (+)	A (–)
1	1.649	0.085
2	1.571	0.084
3	1.672	0.081
4	1.630	0.084
5	1.792	
6	1.827	
$\bar{X}$	1.690	0.084
SD	0.099	0.002

$C_{GAL-LEU}$ [µM]	Formazan absorbance [AU]				
	1.	2.	3.	$\bar{X}$	SD
1.875	1.421	1.553	1.570	1.515	0.081
3.75	1.398	1.455	1.488	1.447	0.046
7.5	1.453	1.503	1.498	1.485	0.028
15	1.332	1.212	1.352	1.299	0.076
30	0.200	0.466	0.220	0.295	0.148

$C_{GAL-VAL}$ [µM]	Formazan absorbance [AU]				
	1	2	3	$\bar{X}$	SD
1.875	1.573	1.684	1.688	1.648	0.065
3.75	1.597	1.684	1.611	1.631	0.047
7.5	1.483	1.536	1.602	1.540	0.060
15	1.438	1.516	1.512	1.489	0.044
30	1.125	1.241	1.23	1.199	0.064

Figure 2 demonstrates the relationship between the peptide ester concentrations and the absorbance levels of formazan.



a) b)

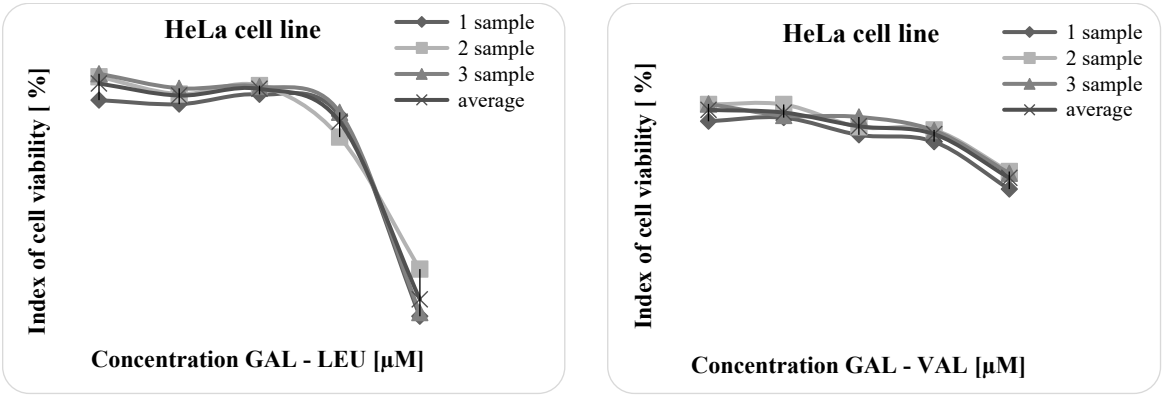
Figure 2. Relation between the absorbance of formazan and concentration of GAL-LEU and GAL-VAL

The impact of varying concentrations (1.875 µM – 30 µM) of GAL-LEU and GAL-VAL on HeLa cell viability is presented in **Table 2**.

Table 2. Index of HeLa cell viability after treatment with GAL-LEU and GAL-VAL

$C_{GAL-LEU}$ [µM]	Index of HeLa cell viability [%]				
	1	2	3	$\bar{X}$	SD
1.875	83.25	91.46	92.52	89.08	5.07
3.75	81.82	85.36	87.42	84.87	2.83
7.5	85.24	88.35	88.04	87.21	1.71
15	77.71	70.24	78.95	75.63	4.71
30	7.25	23.81	8.5	13.19	9.22
$C_{GAL-VAL}$ [µM]	Index of HeLa cell viability [%]				
	1	2	3	$\bar{X}$	SD
1.875	92.71	99.62	99.87	97.4	4.06
3.75	94.2	99.62	95.07	96.3	2.91
7.5	87.11	90.4	94.51	90.67	3.71
15	84.3	89.16	88.91	87.46	2.74
30	64.82	72.04	71.36	69.41	3.99

Figure 3 shows the correlation between the peptide ester concentrations and the HeLa cell viability index.



a) b)

Figure 3. HeLa cells survival after treatment with GAL-LEU and GAL-VAL

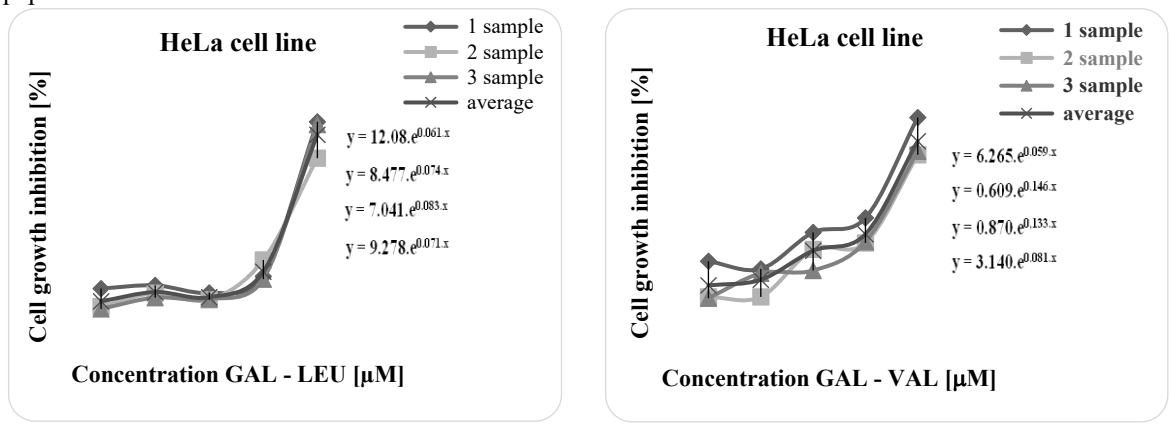
Table 3 summarizes the experimental findings from the evaluation of the cytotoxic effects of peptide esters on HeLa cells.

Table 3. Effect of GAL-LEU and GAL-VAL on HeLa cell line proliferation

$C_{GAL-LEU}$ [µM]	Inhibition of Hela cell growth [%] treated with GAL-LEU				
	1	2	3	$\bar{X}$	SD
1.875	16.75	8.54	7.48	10.92	5.07
3.75	18.18	14.64	12.58	15.13	2.83
7.5	14.76	11.65	11.96	12.79	1.71
15	22.29	29.76	21.05	24.37	4.71
30	92.75	76.19	91.50	86.81	9.22
IC_{50}	23.29	23.98	23.62	23.63	0.35
$C_{GAL-VAL}$ [µM]	Inhibition of Hela cell growth [%] treated with GAL-VAL				
	1	2	3	$\bar{X}$	SD

1.875	7.29	0.38	0.13	2.60	4.06
3.75	5.80	0.38	4.93	3.70	2.91
7.5	12.89	9.60	5.49	9.33	3.71
15	15.70	10.84	11.09	12.54	2.74
30	35.18	27.96	28.64	30.59	3.99
IC_{50}	35.20	30.19	30.46	31.95	2.82

Figure 4 displays the inhibition of HeLa cell growth by the peptide esters.



a) b)

Figure 4. Cytotoxic effect of GAL-LEU and GAL-VAL against Hela cell lines

The results for the 50% inhibitory concentration (IC_{50}) were as follows: GAL-LEU at 23.63 µM, GAL-VAL at 31.95 µM, and the standard Doxorubicin at 3.1 µM. The MTT assay developed by Mosmann has become a widely accepted method for assessing the cytotoxic effects of various compounds due to its numerous advantages, including speed, adaptability, quantitative nature, reproducibility, and sensitivity. This assay measures the ability of viable cells to convert the yellow tetrazolium salt MTT [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide] into insoluble violet formazan crystals, which can be dissolved in an organic solvent and detected spectrophotometrically at a wavelength of λ = 570 nm. The reduction of MTT is driven by the electron transfer from oxidized substrates or enzymes, primarily facilitated by succinate dehydrogenase at the ubiquinone, cytochrome B, and cytochrome C sites of the mitochondrial electron transport chain. This conversion is directly linked to cell viability, as mitochondrial reductase enzymes are required for the reduction to occur. As the number of live cells increases, the amount and absorbance of formazan also increase. A dose-response curve can be constructed by comparing the formazan produced by treated cells with the amount produced by untreated control cells, thereby assessing the cytotoxic potential of the compound [13].

The MTT assay has been utilized to evaluate the cytotoxic effects of various extracts, including *Acalypha wilkesiana* against MCF-7 breast cancer cells [14] and HeLa human cervical cancer cells [15], as well as *Rheum ribes* L. against KB and A-549 cell lines [16]. Cancer, characterized by malignant tumors that invade surrounding tissues, is often a result of mutations in stem cells [17, 18]. In Saudi Arabia, cervical cancer, along with sickle-cell anemia and thalassemia, is prevalent [19].

The HeLa cell line, originating from a cervical cancer biopsy in 1951, was the first human cancer cell line to be continuously cultured. It has since served as a model for studying various cellular processes [20]. In cell culture, cells from different lines are grown in specific media [21]. For the present study, HeLa cells were cultured in Minimum Essential Medium Eagle, with Doxorubicin used as the standard control, known for inhibiting phosphatidylserine decarboxylase and altering mitochondrial membranes in HeLa cells [22, 23].

HeLa cells were treated with each of the peptide esters at varying concentrations (1.875 µM – 30 µM), and the cytotoxicity was assessed using the MTT assay. The experimental data revealed a decrease in formazan production, indicating the growth-inhibitory properties of the peptide esters. Notably, GAL-LEU at 30 µM exhibited 86.81% cytotoxicity, with a cell viability index of 13.19%. In contrast, GAL-VAL at the same concentration showed a lower antiproliferative effect, reducing cell survival to 69.41% and inhibiting 30.59% of cell proliferation. Cytotoxicity was determined by the IC_{50} value, which represents the concentration needed to inhibit cell growth by 50%. It was reported that 0.1 µM of Doxorubicin reduced HeLa cell survival to 40% [24].

The inhibition of cell growth was analyzed using non-linear regression in Microsoft Excel, with IC_{50} values calculated from the respective equations: GAL-LEU (IC_{50} = 23.63 µM ± 0.35 µM) (y = 9.278.e0.071.x) and GAL-VAL (IC_{50} = 31.95 µM ± 2.82 µM) (y = 3.140.e0.081.x). These IC_{50} values were found to be higher compared to the reference Doxorubicin (IC_{50} = 3.1 µM), indicating that the peptide esters exhibit lower antiproliferative activity than Doxorubicin.

Several studies have shown that Ledakrin inhibits DNA replication and synthesis in a dose-dependent manner in HeLa S3 cells by preventing thymidine incorporation [25]. Moreover, inhibition of phosphatidylinositol 3-kinases has been linked to the induction of mitotic cell death in HeLa cells [26]. Anticancer effects have also been noted for coumarin derivative Calanone, isolated from the *Calophyllum* genus, which exhibited an IC_{50} of 22.887 µg/ml in HeLa cells [27], as well as an ethanolic extract of *Canthium parviflorum* Lam., which showed an IC_{50} of 43.15 µg/ml [28].

Additionally, the triterpenoid methyl boswellates demonstrated significant cytotoxicity, with an IC_{50} value of 0.27 µM and a 78.5% reduction in HeLa cell viability at 1 µM [29]. DNA methyltransferase inhibitors such as Zebularine [30], and synthetic antioxidants like butylated hydroxyanisole [31] and propylgallate [32], have been shown to suppress HeLa cell proliferation through caspase-dependent apoptosis, with IC_{50} values of 130 µM, 150 µM, and 800 µM, respectively. Lower IC_{50} values indicate stronger cytotoxic effects, with the triterpenoid demonstrating the highest potency, followed by Zebularine, butylated hydroxyanisole, and propylgallate.

(DIPP-Trp)2-Lys-OCH3 also displayed dose-dependent inhibition of HeLa cell growth, with an IC_{50} of 42.23 µM at a concentration of 100 µM, which inhibited 85% of the HeLa cells [9].

When comparing our experimental findings, it is clear that GAL-LEU demonstrates similar activity to Calanone, while GAL-LEU (IC_{50} = 23.63 µM) and GAL-VAL (IC_{50} = 31.95 µM) are more effective in inhibiting HeLa cell proliferation than Zebularine, butylated hydroxyanisole, and propylgallate.

Conclusion

The observed decrease in formazan production and absorbance following treatment with the peptide esters indicates their potential to inhibit cell growth. The calculated IC_{50} values for GAL-LEU and GAL-VAL were 23.63 µM and 31.95 µM, respectively, showing that both esters have cytotoxic effects on HeLa cells. GAL-LEU demonstrated a more potent antiproliferative effect compared to GAL-VAL, as evidenced by its lower IC_{50}.

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Conflict of Interest: None

Financial Support: None

Ethics Statement: None

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