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# Drug with Neuroprotective Properties Noopept Does Not Stimulate Cell Proliferation

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Effects of Noopept (*N*-phenylacetyl-*L*-prolylglycine ethyl ester) on the relative level of proliferation marker Ki-67 and cell cycle parameters were studied in HEK293 and SH-SY5Y cell lines. The previously established multifactorial mechanism of action of the drug includes enhancement of neurotrophin NGF and BDNF expression and increase in HIF-1 activity. The possible mitogenic action of Noopept was estimated by its effect on cell proliferation. Noopept did not affect cell distribution over G1, S, G2 cell cycle phases and the relative level of proliferation marker Ki-67 in the cell lines under study. These data suggest that Noopept does not stimulate cell growth.

**Key Words:** *Pro-Gly substituted dipeptides; Noopept; proliferation; cell cycle; Ki-67*

Noopept is an original nootropic drug developed at the V. V. Zakusov Research Institute of Pharmacology (MP 015770). Along with the positive effect on cognitive functions, Noopept has anxiolytic and neuroprotective properties [13]. Neuroprotective activity of Noopept has been shown in various *in vivo* models of ischemic, traumatic neurodegenerative brain injury [5]. In the *in vitro* $Ab_{25-35}$-induced neurotoxicity model, the drug exerted a reparative effect, in particular, it restored the number and length of neurites in differentiated PC12 cells [9]. The neuroprotective properties of Noopept include prevention of cell death induced by $H_2O_2$ [1], glutamate [2], β-amyloid protein fragment [9], α-synuclein [8]. The studies of the mechanism of action of Noopept revealed its ability to increase NGF and BDNF level in rat hippocampus [6], to reduce expression of stress-induced and mitogen-dependent pSAPK/JNK and pERK1 kinases, respectively [4], to increase transcription factor HIF-1 activity *in vitro*, both basal and induced by a hypoxia-mimetic drug [3].

Neurotrophins, in particular NGF and BDNF, are involved in the formation, growth, differentiation, maintenance of functions, and survival of mature cells. However, along with pronounced neuroprotective properties, the mitogenic effect of NGF, predominantly mediated by the MAP-dependent signaling cascade, was observed in non-neuronal cells [7]. HIF-1 stabilizers can stimulate proliferation, because, along with hypoxia adaptation gene regulation, HIF-1 also coordinates the activity of genes involved in tumor growth, neoangiogenesis and vascularization during carcinogenesis [10]. In view of the positive effect of Noopept on neurotrophin synthesis, as well as on the components of HIF-1-dependent pathway, it seems interesting to find out whether it affects proliferation of cells of various origin.

The aim of this study was to assess the effect of Noopept on cell cycle parameters and expression of proliferation marker Ki-67 in conditionally normal HEK293 cells and SH-SY5Y neuroblastoma line.

## MATERIALS AND METHODS

SH-SY5Y and HEK293 cells ($4\times10^5$/ml) were cultured in 24-well plates in a complete DMEM culture medium containing 10% fetal calf serum, 2 mM L-glutamine, and 50 μg/ml gentamicin sulfate at 5% $CO_2$ and 95% humidity. Cell cycle and Ki-67 level

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were analyzed by flow cytofluorometry (Novocyte 2060, ACEA Bioscience Inc.), preparations (at least 10,000 cells in each plate well) were stained with PI (Invitrogen) and monoclonal antibodies to Ki-67 (Cell Signaling) according to manufacturers' protocols. Quantitative analysis of fluorescence histograms (proliferation marker Ki-67) was performed using the NovoExpress 1.2.5 software (ACEA Bioscience Inc.); the cell cycle phase distribution by DNA content was performed using cell cycle assessment module of the same software. In this study, Noopept was used in a concentration of 100 μM, because the maximum HIF-1-positive effect of the drug was observed at this concentration [3]; cells not exposed to Noopept were used as the control.

Statistical data processing (fluorescence values from 10000 cells/well; 3 parallel experiments; 3 independent experiments) was performed using a standard set of statistical methods (BioStat Pro 6.2.5.0, AnalystSoft Inc.; GraphPad Prism 5.0 , GraphPad Software), paired Student's *t* test for dependent samples. Distribution normality was assessed using Kolmogorov—Smirnov test. The observed fluorescence level distribution did not differ from the theoretically expected normal distribution for all random samples used in the analysis ($p<0.05$ according to H0: character distribution does not differ from the theoretically expected normal distribution; significance level according to degrees of freedom=1.00 according to Kolmogorov–Smirnov).

**TABLE 1.** Effect of Noopept on the Level of the Proliferation Marker Ki-67 in HEK293 and SH-SY5Y Cell Lines ($n$=3; $M\pm SD$)

| Cell line | Level of Ki-67, fluorescence arb. units | |
|---|---|---|
| | Control | Noopept |
| HEK293 | 2159±47 | 2168±28 |
| SH-SY5Y | 2173±32 | 2152±14 |

**Note.** Noopept (100 μM) was administered every 24 h. After 48 h cells were trypsinized and stained with monoclonal antibodies to Ki-67.

## RESULTS

In order to analyze the effect of Noopept on cell proliferation, the content of the proliferation marker Ki-67 was evaluated. The nuclear protein antigen Ki-67 is expressed in proliferating cells (in G1, S, G2, and M phases of the cell cycle) and reflects proliferation intensity [12]. Noopept did not affect the relative Ki-67 level in normal HEK293 cells and neuroblastoma SH-SY5Y cells (Table 1); therefore, the drug has no cell growth-stimulating ability.

The effect of Noopept on cell cycle parameters was assessed by flow cytometry in HEK293 and SH-SY5Y cells after staining with PI. Detection was carried out every 24 h for 72 h, which covers the doubling time of HEK293 and SH-SY5Y cells. Noopept did not induce changes in the percentage and proportion of

**TABLE 2.** Analysis of Cell Cycle in HEK293 and SH-SY5Y Cell Lines against the Background of Noopept ($n$=3; $M\pm SD$)

| Cell line | | | Proportion of cells, % | | | |
|---|---|---|---|---|---|---|
| | | | subG0 | G1 | S | G2 |
| HEK293 | Control | 24 h | 0.31±0.23 | 56.34±2.04 | 19.43±2.01 | 24.22±1.68 |
| | | 48 h | 0.58±0.13 | 53.83±1.33 | 21.53±3.03 | 18.92±2.76 |
| | | 72 h | 0.96±0.20 | 54.51±2.70 | 23.50±1.12 | 16.40±1.70 |
| | Noopept | 24 h | 0.13±0.09 | 58.76±1.50 | 17.15±1.45 | 24.09±2.72 |
| | | 48 h | 0.26±0.17* | 57.31±3.15 | 20.82±1.98 | 17.66±2.32 |
| | | 72 h | 0.34±0.11* | 59.09±1.79 | 21.82±0.53 | 17.22±0.66 |
| SH-SY5Y | Control | 24 h | 0.72±0.34 | 63.28±3.03 | 29.62±1.87 | 7.10±1.21 |
| | | 48 h | 0.86±0.19 | 57.41±1.23 | 33.23±2.30 | 9.37±1.03 |
| | | 72 h | 0.81±0.17 | 55.36±3.31 | 32.48±2.68 | 8.24±2.39 |
| | Noopept | 24 h | 0.51±0.10 | 60.30±0.87 | 32.28±0.43 | 7.41±0.54 |
| | | 48 h | 0.67±0.14 | 62.84±1.64 | 30.36±0.23 | 6.80±1.61 |
| | | 72 h | 0.72±0.45 | 63.28±3.58 | 29.62±2.09 | 7.10±1.76 |

**Note.** Noopept (100 μM) was administered every 24 h. After incubation was completed, cells were trypsinized, fixed with 70% ethanol for 24 h, then washed with 1× phosphate buffer, incubated with RNase A (100 μg/ml) for 5 min and PI for 15 min (25 μg/ml). *$p<0.05$ in comparison with the control.

HEK293 and SH-SY5Y cells in all cell cycle phases (G1, S, and G2) during the entire observation period (Table 2), therefore the drug does not possess its own mitogenic activity. It is important that Noopept significantly reduced the number of subG0 phase HEK293 cells (in 48 and 72 h) and subG0 phase neuroblastoma cells. As subG0 phase specifies the presence of apoptotic cells, the decrease in the number of cells in the subG0 phase under the influence of Noopept (in comparison with the control) can be considered as apoptosis-suppressing ability.

Thus, obtained data demonstrate the absence of mitogenic properties of Noopept: the drug did not stimulate cell division and did not affect proliferation marker Ki-67 expression. On the other hand, we revealed the ability of Noopept to reduce the number of apoptotic cells, which is consistent with the previously obtained data on the Noopept-induced increase in survival of different cell types exposed to various damaging agents [1,2,8,9]. The studies in cortical neurons of aborted fetuses with Down syndrome showed that Noopept (1 μM) administered to the culture for 7 days increased survival of pathological neurons; in experiments on neurons from healthy fetuses, it enhanced survival of $H_2O_2$-treated neurons [11].

Modern concepts suggest that molecular mechanisms of neuroprotection involve activation of proliferative and/or antiapoptotic programs mediated by various signal transduction pathways [14]. The data obtained in this study seem to rule out the possibility of cell proliferation activation by Noopept. Investigation of the anti-apoptotic action of this proline-containing dipeptide is a subject for further research.

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