INTRODUCTION

This doctoral dissertation is focused on an RNA interference (RNAi) approach directed towards genes involved in a cell cycle. The neuronal cells are terminally differentiated and they are in the G0 phase of the cell cycle. The consequence of re-activation of the cell cycle in those cells (normally not proliferating) is their entering on the apoptotic pathway.

The aim of these studies was to get the proof of concept, that the silencing of the *cdk2*, *cdk4* and *cdk6* genes, that are expressed during the cell proliferation, may have a neuroprotective effect. Inhibition of expression of cyclin-dependent kinases cdk2, cdk4 and cdk6 would allow preventing the induction of apoptosis in neurons. For these studies, 21 siRNA sequences directed towards the human, mouse and rat *cdk2*, *cdk4* and *cdk6* genes were designed, and their interference activity was screened in HeLa, SH-SY5Y, Neuro2a, and PC12 cell lines. The level of the target gene expression was analyzed by the real-time and semi-quantitative RT-PCR and by a Western blot method, and the most active siRNA molecules were selected.

The next step of this work was the generation of the shRNA and its expression in cells. The shRNA inserts were designed based on the sequences of the most active siRNA silencers of *cdk4* and *cdk6* genes (selected in the previous experiments). In total, nine molecules of shRNA were designed and cloned into a pSilencer 2.0-U6 expression plasmid. The interference activity of these plasmids was determined in the selected cell lines and compared to the activity of the corresponding siRNA molecules. Analysis of expression of *cdk4* and *cdk6* genes at the levels of mRNA and proteins has demonstrated that the shRNA molecules show lower interference activity towards the analyzed genes than the corresponding siRNAs.

Next, the level of expression of proteins, which appear in the later stages of the cell cycle, such as cyclin A, cyclin E and PCNA, was analyzed in HeLa and SH-SY5Y cells, in which the *CDK4* gene was silenced by the designed siRNAs. The real-time RT-PCR analysis has shown that cyclin E, cyclin A and *PCNA* genes are down-regulated in comparison to the cells with unchanged expression of cyclin-dependent kinase 4. For the same cell models, the level of expression of the *APP*, *PS1*, and *BACE1* genes, involved in the production of amyloid β, was determined. The study has shown that the cells with reduced expression of the *CDK4* gene also exhibit reduced expression of *BACE1* and *PS1* genes, while the expression level of *APP* gene remained unchanged.

A flow cytometry approach was used to assess cell cycle phases of the cells with lowered expression of the *CDK4* gene. It occurred that the low level of the CDK4 protein affects the HeLa and SH-SY5Y cells proliferation. More cells were in the G0/G1 phase and lower number in the S and G2 / M phases than in the control cells.

The next goal was to establish the cell culture conditions suitable for studies to be carried out under oxidative stress. The hydrogen peroxide (H_2O_2) was chosen as a source of the reactive oxygen species (ROS). The H_2O_2 concentration chosen was sufficient to stimulate model HeLa and SH-SY5Y cells for proliferation while enough safe not to cause the cells death. Then it was checked whether the cells with the lower level of CDK4 are similarly susceptible to oxidative stress as those with the *CDK4* gene non-silenced. The analysis performed by flow cytometry has shown that in the case of cells with the silenced *CDK4* gene and treated with the hydrogen peroxide the number of cells in S and G2 / M phases was lower than in the control group. Furthermore, it was observed that silencing of the *CDK4*

gene in HeLa and SH-SY5Y cells slightly reduced the amount of apoptotic cells.

In the next step, an assessment of the degree of induction of apoptosis during oxidative stress induced by hydrogen peroxide was performed in the Neuro2a cells with the silenced cdk4 gene only or with simultaneously silenced three genes *cdk2*, *cdk4* and *cdk6*. In these studies, the level of caspase 3 and 7 was determined. The results indicate that the silencing of cyclin-dependent kinases, especially of cdk4 kinase, protects the cells against oxidative stress. Cells with the silenced *cdk4* gene were less vulnerable to activation of the cell cycle. In this case, the activity of caspases 3 and 7 was lower comparing the activity in cells exposed to oxidative stress, but without silencing of cyclin-dependent kinases.

The aim of the next stage of the studies was to obtain in eukaryotic cells an overexpression of inhibitors of cyclin-dependent kinases, that is p16(INK4), p18(INK4c) and p21(CDKN1A, WAF1) proteins. For this purpose, two expression plasmids pVax1 and pEGFP-C1 were used, which contained the insert encoding the desired transgenes (*CDKi*) The cells transfected with the plasmids encoding the *CDKi* genes allowed to assess an impact of these inhibitors on the cell cycle of the examined cells. The analysis of the changes of the phases of cell cycle was carried out using a flow cytometer. The results have shown that overexpression of inhibitors of cyclin-dependent kinases, achieved with the pVax1 vector, stopped the cell cycle in HeLa cells. In contrast, overexpression of inhibitors of cyclin-dependent kinases achieved with the pEGFP-C1 plasmid only slightly affected a cell cycle HeLa.

Finally, the efficiency of nucleic acids delivery to the cells was studied by the use of neurospecific RVG-9R peptide and Lipofectamine 2000 (lipofectamine), a is positively charged lipid carrier. The studies have shown that the RVG-9R peptide effectively introduces siRNA into the neuronal cells (e.g. into Neuro2a cells), due to previously demonstrated interaction of the RVG-9R peptide with nicotinic receptors present on the membrane of neuronal cells. However, HeLa cells, which do not express nicotinic receptors, do not uptake siRNAs in the presence of RVG-9R peptide. This result was demonstrated by a real-time RT-PCR analysis of the level of expression of *cdk4* gene in the Neuro2a and HeLa cells transfected with both delivering agents screened. The expression of the *cdk4* gene in Neuro2a was specifically silenced by siRNA delivered with RVG-9R peptide, while no down-regulation of this gene was observed in analogous delivery to the HeLa cells. In contrast, the lipofectamine was not neurospecific and delivered siRNA to both types of cells with similar efficiency.