with 100 µl of secondary antibodies (DyLight 488 Donkey anti-rabbit IgG) for 45min in the dark. After incubation, coverslips were washed three times with PBS and stained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen) for 10 min. Slides were labelled and the coverslips were mounted and sealed with transparent nail varnish. Slides were analysed using ZOE Fluorescent Cell Imager (BioRad) microscope in the GFP detection channel.

Statistical analysis

Data were analyzed using the unpaired Student t test with the help of GraphPad Prism 5 software.

RESULTS AND DISCUSSION

Selection of Signal Peptide

Lentiviral transduction of T-lymphocytes is the most effective method of genetic modification of human T-cells [12]. This approach provides successful insertion of a specific sequence into cell genome, stable level of gene expression over a long period of time, and a significantly higher efficiency of cell transformation relatively to methods of chemical transfection [13]. To compare efficiencies of different signal peptides, we constructed lentiviral vectors, containing sequences of two secretion signals, known for their efficacy: HMMsp38 - an artificial secretory peptide, characterized by a high efficacy index (D = 0.932) and the GLuc peptide - *Gaussia princeps* luciferase secretion signal, which has the highest efficiency among natural signals (D = 0.88) [14-16]. Both of constructed vectors: pL-CMV-HMMsp38-Apoptin and pL-CMV-Gaussia-Apoptin (shown in Figure 1) contained sequences of chosen signal peptides fused to the apoptin sequence.

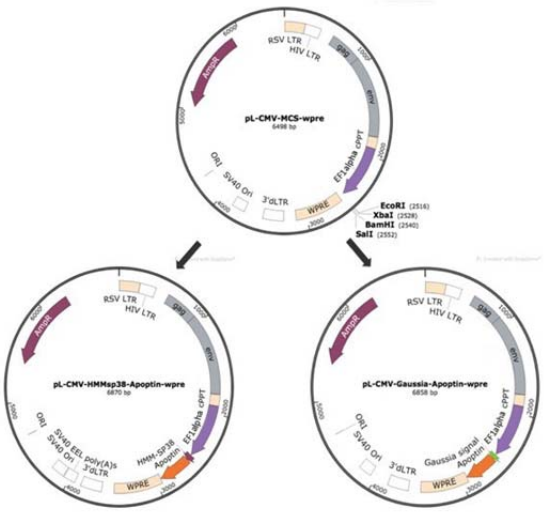

Figure 1. Construction of lentiviral vectors encoding sequences of extracellular localization signals Gaussia and HMMsp38

Created genetic constructs were successfully introduced in a population of T-cells with the help of lentiviral transduction. The efficiency of each studied secretion signal was compared by measuring the amount of the target protein in culture medium 36 hours after the transduction. Presence of the target protein was determined by ELISA (Figure 2A), PAGE-electrophoresis and Western blot analysis (Figure 2B).

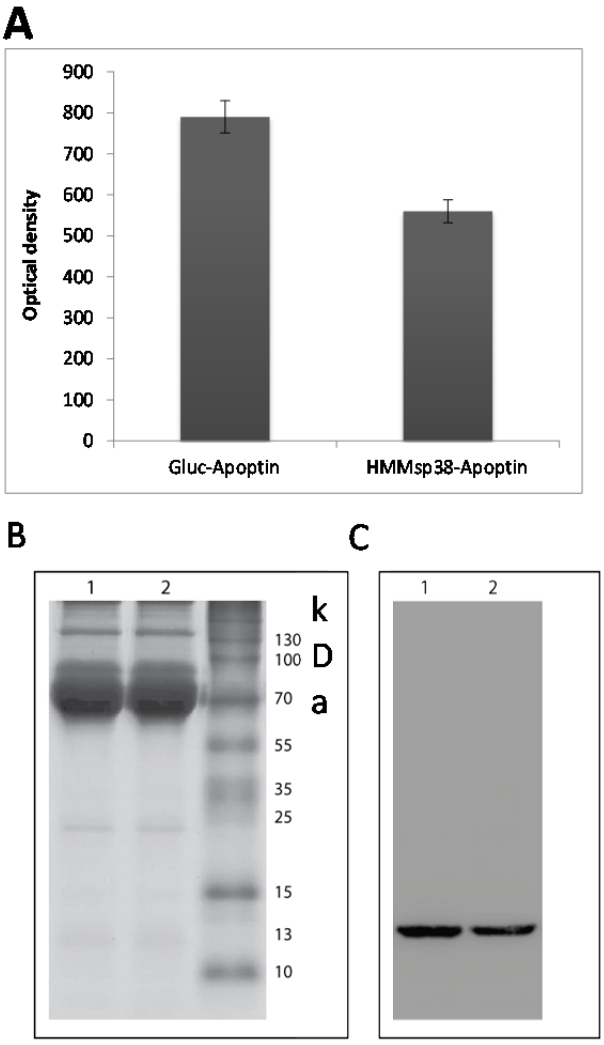

Figure 2. Expression and secretion analysis of recombinant forms of apoptin oncoprotein: HMMsp38-apoptin and GLuc-apoptin; A - the enzyme-linked immunosorbent analysis (ELISA) of T-cells culture medium; B - SDS-PAGE electrophoresis of the T-cells culture medium with a total protein concentration of 100 µg (lane 1 corresponds to the GLuc apoptin sample, lane 2 to HMMsp38 apoptin); C - Western blot analysis of media samples hybridization with anti-apoptin antibodies (lanes 1-2 correspond to the tracks in Figure B)

The results, shown in Figure 2, support the fact, that the *Gaussia princeps* luciferase secretion signal provides a higher level of apoptin expression compared to HMMsp38. This data determined the choice of the GLuc sequence for further use during engineering of the apoptin expression system.

Testing of transport peptide

The next step in creating the system for apoptin secretion and internalization was selection of the most efficient transport peptide. In most cases, these peptides are unique and are responsible for the transport of specific proteins [17]. However, there are also non-specific types of traffic signals, best known of them is the transducing transcription factor of the human immunodeficiency virus (TAT-HIV1). This non-specific transport peptide is used in

most experimental works, related to the transport of various protein structures to target cells [18, 19]. It is worth noting that TAT signaling factor was also used in the research of Guelen (2004) and Lee (2012), who described the possibility of using recombinant apoptin for clinical practice [20, 21]. Thereby, this transport peptide seemed to be an effective choice for the apoptin internalization system, and the sequence of TAT-HIV1 was cloned at the N-terminus of apoptin sequence after GLuc secretion signal (Figure 3),

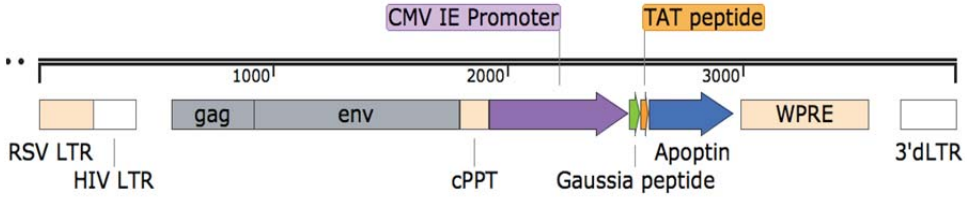

Figure 3. Schematic representation of the pL-CMV-Gluc-TAT-Apoptin-WPRE expression vector

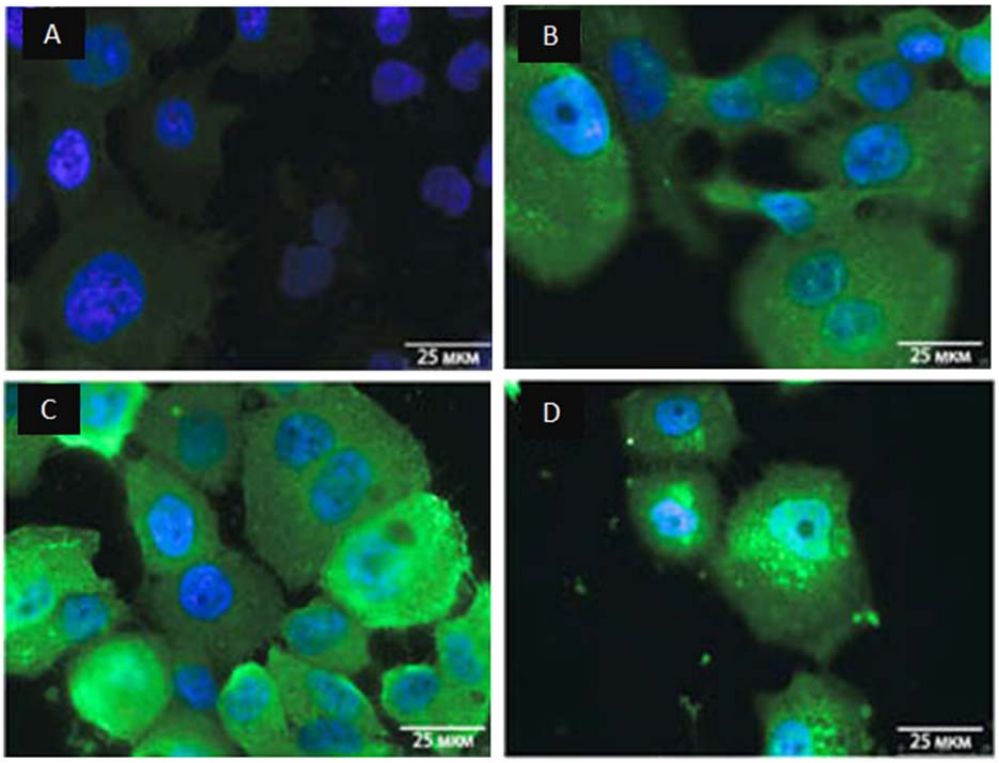

Figure 3. Immunofluorescence analysis of apoptin accumulation in MCF7 cells, treated with the Gluc-TAT-Apoptin-containing medium; photos were made after 12 (A), 24 (B), 48 (C) and 72 (D) hours of incubation.

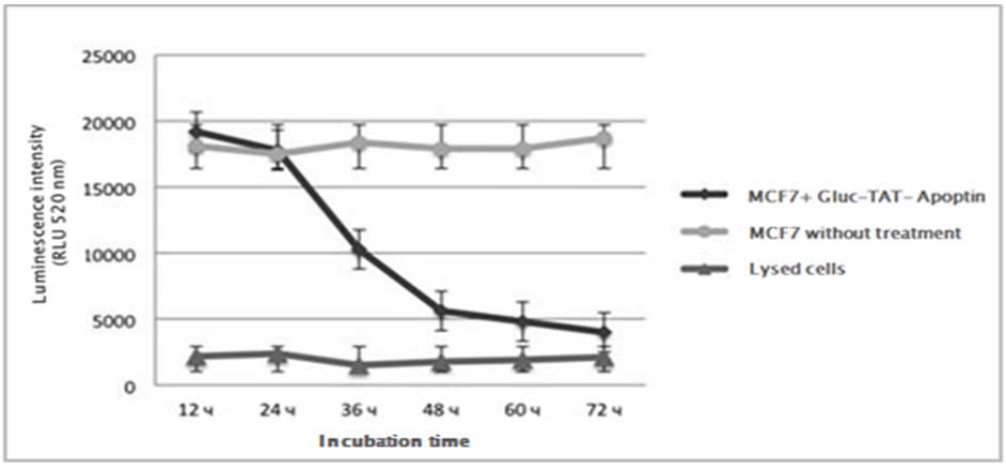

Figure 5. Analysis of MCF7 viability after treatment with the Gluc-TAT-Apoptin-containing medium; untreated cells were used as a positive control; negative control was treated with camptothecin. All measurements were carried out using a CellTiter Glo 2.0 reagent (Promega) and a microplate reader (BioRad).

In vitro analysis of cytotoxic potential of the apoptin intercellular delivery system

Constructed pL-CMV-Gluc-TAT-Apoptin-WPRE expression vector was used for lentiviral transduction of human T-lymphocytes. To estimate efficiency of the apoptin intercellular transport system, we studied an accumulation of the recombinant protein in MCF7 cell line. Twelve hours after transfection of the T-cells, the culture medium was collected and transferred to MCF7 cell culture; changing of culture medium was carried out 2 times per day. Every day after first medium replacement, one of the samples of MCF7 cells was stained and then analyzed by fluorescence microscopy (Figure 4).

According to the presented micrographs, the fluorescence intensity of MCF7 cells increases proportionally to incubation time, which indicates that the intercellular transport system based on Gluc and TAT signal peptides provides an efficient transport of recombinant apoptin from the producer cell line into target cancer cells.

To study the oncotoxic proporties of the apoptin internalization system, we performed an analysis of MCF7 viability after treatment with Gluc-TAT-Apoptin-containing medium; viability was measured with CellTiter Glo 2.0 kit every 12 hours after the beginning of cell treatment (Figure 5).

Results indicate that after 24 hours of incubation, amount of apoptin in treated cells reached a critical level, which leads to a sharp increase in apoptosis intensity. After 72 hours, luminescence level detected for treated cells was comparable to the signal for camptothecin-treated cells used as a negative control, which indicates high cytotoxic properties of apoptin.

CONCLUSION

Summarizing obtained results, we can conclude that developed system of apoptin expression and internalization provides an efficient elimination of cancer cells, which allows us to expect its further application in cancer therapy.

ACKNOWLEDGEMENTS

The work was funded by MESR, contract No. 14.607.21.0140 (RFMEFI60715X0140).

REFERENCES

[1] Ashdown M.L., Robinson A.P., Yatomi-Clarke S.L., Allison A., Abbott D., Markovic S.N., Coventry B.J. 2015. Chemotherapy for Late-Stage Cancer Patients: Meta-Analysis of Complete Response Rates. *F1000Res*. **4,** 232.

[2] Owonikoko T.K., Behera M., Chen Z., Bhimani C., Curran W.J., Khuri F.R., Ramalingam S.S. 2012. A systematic analysis of efficacy of second-line chemotherapy in sensitive and refractory small-cell lung cancer. *J Thorac Oncol*. **7,** 866-872.

[3] Wang M., Yin B., Wang H.Y., Wang R.F. 2014. Current advances in T-cell-based cancer immunotherapy. *Immunotherapy*. **6,** 1265-1278.

[4] Rosenberg S.A., Restifo N.P., Yang J.C., Morgan R.A., Dudley M.E. 2008. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. *Nat Rev Cancer*. **8,** 299-308.

[5] Essand M.,Loskog A.S. 2013. Genetically engineered T cells for the treatment of cancer. *J Intern Med*. **273,** 166-181.

[6] Varela-Rohena A., Carpenito C., Perez E.E., Richardson M., Parry R.V., Milone M., Scholler J., Hao X., Mexas A., Carroll R.G., June C.H., Riley J.L. 2008. Genetic engineering of T cells for adoptive immunotherapy. *Immunol Res*. **42,** 166-181.

[7] Barrett D.M., Grupp S.A., June C.H. 2015. Chimeric Antigen Receptor- and TCR-Modified T Cells Enter Main Street and Wall Street. *J Immunol*. **195,** 755-761.

[8] Sharpe M.,Mount N. 2015. Genetically modified T cells in cancer therapy: opportunities and challenges. *Dis Model Mech*. **8,** 337-350.

[9] Backendorf C.,Noteborn M.H. 2014. Apoptin towards safe and efficient anticancer therapies. *Adv Exp Med Biol*. **818,** 39-59.

[10] Noteborn M.H., Zhang Y.H., van der Eb A.J. 1998. Apoptin specifically causes apoptosis in tumor cells and after UV-treatment in untransformed cells from cancer-prone individuals: a review. *Mutat Res*. **400,** 447-455.

[11] Los M., Panigrahi S., Rashedi I., Mandal S., Stetefeld J., Essmann F., Schulze-Osthoff K. 2009. Apoptin, a tumor-selective killer. *Biochim Biophys Acta*. **1793,** 1335-1342.

[12] Bilal M.Y., Vacaflores A., Houtman J.C. 2015. Optimization of methods for the genetic modification of human T cells. *Immunol Cell Biol*. **93,** 896-908.

[13] Frecha C., Levy C., Cosset F.L., Verhoeyen E. 2010. Advances in the field of lentivector-based transduction of T and B lymphocytes for gene therapy. *Mol Ther*. **18,** 1748-1757.

[14] Luft C., Freeman J., Elliott D., Al-Tamimi N., Kriston-Vizi J., Heintze J., Lindenschmidt I., Seed B., Ketteler R. 2014. Application of Gaussia luciferase in bicistronic and non-conventional secretion reporter constructs. *BMC Biochem*. **15,** 14.

[15] Stern B., Optun A., Liesenfeld M., Gey C., Grafe M., Pryme I.F. 2011. Enhanced protein synthesis and secretion using a rational signal-peptide library approach as a tailored tool. *BMC Proc*. **5 Suppl 8,** O13.

[16] Clerico E.M., Maki J.L., Gierasch L.M. 2008. Use of synthetic signal sequences to explore the protein export machinery. *Biopolymers*. **90,** 307-319.

[17] Yang N.J.,Hinner M.J. 2015. Getting across the cell membrane: an overview for small molecules, peptides, and proteins. *Methods Mol Biol*. **1266,** 29-53.

[18] Rizzuti M., Nizzardo M., Zanetta C., Ramirez A., Corti S. 2015. Therapeutic applications of the cell-penetrating HIV-1 Tat peptide. *Drug Discov Today*. **20,** 76-85.

[19] Vives E., Richard J.P., Rispal C., Lebleu B. 2003. TAT peptide internalization: seeking the mechanism of entry. *Curr Protein Pept Sci*. **4,** 125-132.

[20] Guelen L., Paterson H., Gaken J., Meyers M., Farzaneh F., Tavassoli M. 2004. TAT-apoptin is efficiently delivered and induces apoptosis in cancer cells. *Oncogene*. **23,** 1153-1165.

[21] Lee M.S., Sun F.C., Huang C.H., Lien Y.Y., Feng S.H., Lai G.H., Chao J., Chen H.J., Tzen J.T., Cheng H.Y. 2012. Efficient production of an engineered apoptin from chicken anemia virus in a recombinant E. coli for tumor therapeutic applications. *BMC Biotechnol*. **12,** 27.