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Covalently conjugated DNA aptamer with doxorubicin as *in vitro* model for effective targeted drug delivery to human glioblastoma tumor cells

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ABSTRACT

Targeted delivery of chemotherapeutic agents with aptamers is a very effective method increasing therapeutic index compared to non-targeted drugs.

Objective. To study the effectiveness of in vitro therapeutic effect of covalently conjugated GR20 DNA aptamer with doxorubicin on glioblastoma cells compared to reference culture of human fibroblasts.

Material and methods. A Sus/fP2 cell culture was obtained from glioblastoma tissue sample to analyze the effectiveness of conjugate. A linear culture of human dermal fibroblasts (mesenchymal stem cells) DF1 was used as a control. To assess antiproliferative activity of covalently conjugated GR20 aptamer with doxorubicin, we used the MTS test. The Cell Index was measured using the xCelligence S16 cell analyzer assessing viability of cell cultures by recording changes in real time.

Results. Human glioblastoma Sus/fP2 cells reduce own proliferative potential by 80% when exposed to doxorubicin (0.5 μM, 72 hours, MTS test), by 9% when exposed to GR20 aptamer (10 μM, 72 hours, MTS test) and by 26% when exposed to covalently conjugated DOX-GR20 (0.5 μM, 72 hours, MTS test). A long-term study of proliferative potential of Sus/fP2 cells on the xCelligence S16 analyzer revealed a significant decrease in the number of cells under the effect of doxorubicin and covalently conjugated DOX-GR20. Effectiveness of covalently conjugated DOX-GR20 is halved. GR20 aptamer at a concentration of 10 μM and its conjugate with doxorubicin DOX-GR20 at a concentration of 1 μM have no negative effect on cells of the control culture of DF1 fibroblasts, while doxorubicin is toxic for these cells. MTS test and xCelligence S16 cell analyzer found no decrease in metabolic activity of DF1 cells and their ability to proliferate.

Conclusion. We established obvious antiproliferative effect of covalent conjugate DOX-GR20 on continuous human glioblastoma cell culture Sus/fP2 without toxic effect on the reference culture (dermal fibroblasts DF1).

Keywords: *glioblastoma, doxorubicin, DNA aptamer, covalent conjugate of DNA aptamer with doxorubicin.*

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Abbreviations

GB — glioblastoma
EGFR — epidermal growth factor receptor
BBB — blood-brain barrier
DOX — doxorubicin
DOX-GR20 — doxorubicin-conjugated GR20 aptamer
MTS test — colorimetric test for assessing the metabolic activity of cells
HPAC — high performance affinity chromatography

Introduction

Gliomas are heterogeneous primary malignant brain tumors. Glioblastoma (GB) is the most aggressive glioma accounting for 70—75% of all gliomas, and the median overall survival without treatment is 14—17 months [1]. Despite significant advances in knowledge about tumor and treatment over the past few years, prognosis is still poor due difficult target delivery of chemotherapeutic drugs or potential antitumor molecules to the tumor. Understanding the pathogenesis of this disease is necessary to develop more effective treatment. Epidermal growth factor receptor (EGFR) is one of the essential mediators. This is a transmembrane protein (receptor tyrosine kinase) overexpressed in many cells with high proliferative activity, in particular, aggressive cancers including non-small cell lung cancer and glioma [2]. A cascade following ligand interaction with EGFR modulates cell growth, survival, adhesion, migration and differentiation. This makes this molecule a promising target for antitumor therapy [3].

Thus, one of the most important directions is development of target drug delivery system overcoming the blood-brain barrier and specifically affecting the tumor. Molecules such as aptamers can effectively recognize various proteins on cell membrane or in blood, modulate their interactions with receptors and impact on the target biological pathways for various diseases [4—5]. Aptamers have many characteristics that make them ideal therapeutic agents for the treatment of GB. These are short single-stranded nucleic acids (RNA or DNA) capable of binding to molecular target with high affinity and specificity. Several target aptamers for GB have been developed for imaging, isolation of tumor cells from specimens and delivery of drugs/antitumor molecules cells. Numerous aptamers have been selected against GB biomarkers and tested on GB cell lines due to their properties (low immunogenicity, long-term stability and no toxicity). Some of them have also been tested in GB models in vivo. These ones are potentially considered as new diagnostic and/or therapeutic tools for the treatment of GB [6]. The number of therapeutic aptamers in various stages of clinical trials has increased over the past few decades [5—7]. In addition to being therapeutic agents, aptamers have been widely explored as targeted carriers for delivery of other drugs. Aptamer-drug conjugates have been developed for various cancer-related biomarkers for different therapeutic modalities such as chemotherapy, immunotherapy, etc. [5—8]. Chemotherapy is one of the most common traditional approaches for cancer. At the same time, there are various side and negative effects on intact tissues due to high toxicity of modern drugs. Target drug delivery via aptamer-drug conjugates is being developed to increase therapeutic efficacy and reduce side effects.

We chose doxorubicin for pilot studies as a molecule causing death of tumor cells. Doxorubicin (DOX) is an anthracycline antibiotic harvested from Streptomyces peucetius species. This agent is widely used for the treatment of various extracerebral malignancies [9]. However, it is not used for gliomas due to poor delivery. Doxorubicin can be incorporated into DNA (dsDNA) and also bind to DNA-degrading enzymes. This leads to DNA damage and cancer cell apoptosis via inhibition of cellular cycle. Like other chemotherapeutic drugs, doxorubicin has various side effects including reduced white and red blood cell counts or dose-dependent cardiac toxicity [9]. Doxorubicin can be directly incorporated into double-stranded DNA due to flat aromatic rings. According to fluorescence quenching properties, aptamers and doxorubicin can form a physical complex after incubation. Several aptamer-DOX conjugates have been recently synthesized using this method. For example, H. Liu et al. and H. Hu et al. designed aptamer-DOX conjugates that could specifically recognize and deliver doxorubicin to HER-2- and MUC-1-positive tumor cells with their death [10]. A. Zhu et al. [11] synthesized a bispecific aptamer SD from two modified monovalent aptamers sgc8c and sgd5a. Conjugate of this aptamer with doxorubicin is able to recognize different cells in cellular mixture. Thus, aptamers can effectively deliver cytotoxic agents to different cell populations in heterogeneous tumors. Thus, the conjugate/complex can solve the problem of target delivery to brain tumor tissue.

We examined in vitro therapeutic effectiveness of a covalent DNA aptamer conjugate GR20 (EGFR as a target [12, 13]) and doxorubicin on glioblastoma cells and human fibroblasts.

Results

DOX-GR20 conjugate

We obtained DOX-GR20 conjugate via a copper-catalyzed azide-alkyne cycloaddition **(fig. 1)**.

We assessed the purity of DOX-GR20 compounds using high-performance affinity chromatography and analytical denaturing 14% polyacrylamide gel electrophoresis. HPAC was performed using the Agilent 1100 device and Waters xBridge 4.6×250 mm column (linear gradient 5—50% MeCN) in triethylammonium acetate gradient 0.1—0.02 M over 30 min **(fig. 2)**.

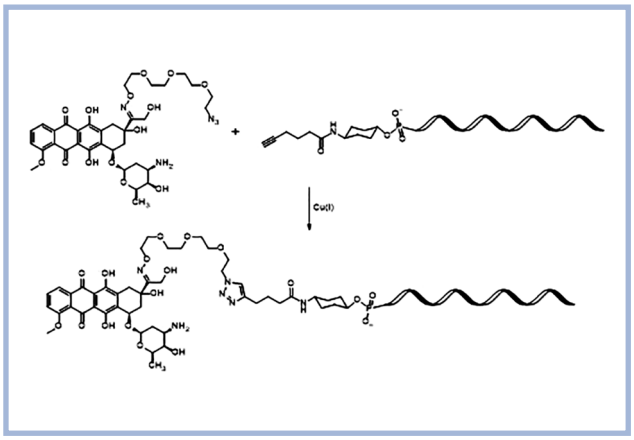


Fig. 1. **Scheme of obtaining a covalent aptamer-doxorubicin conjugate.**

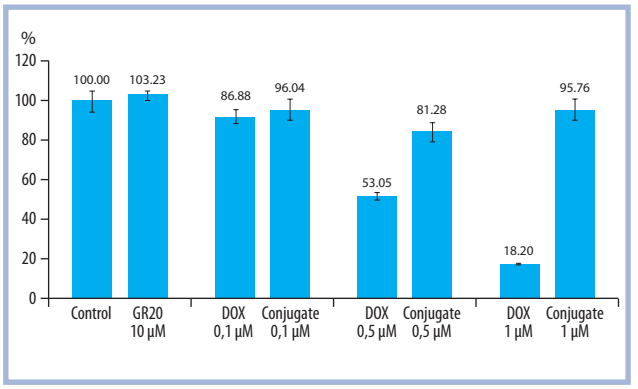


Fig. 3. **Effect of doxorubicin (DOX), GR20 aptamer and DOX-GR20 conjugate on proliferative activity of human dermal fibroblast cells DF1 compared to the control group (intact cells). MTS test.**

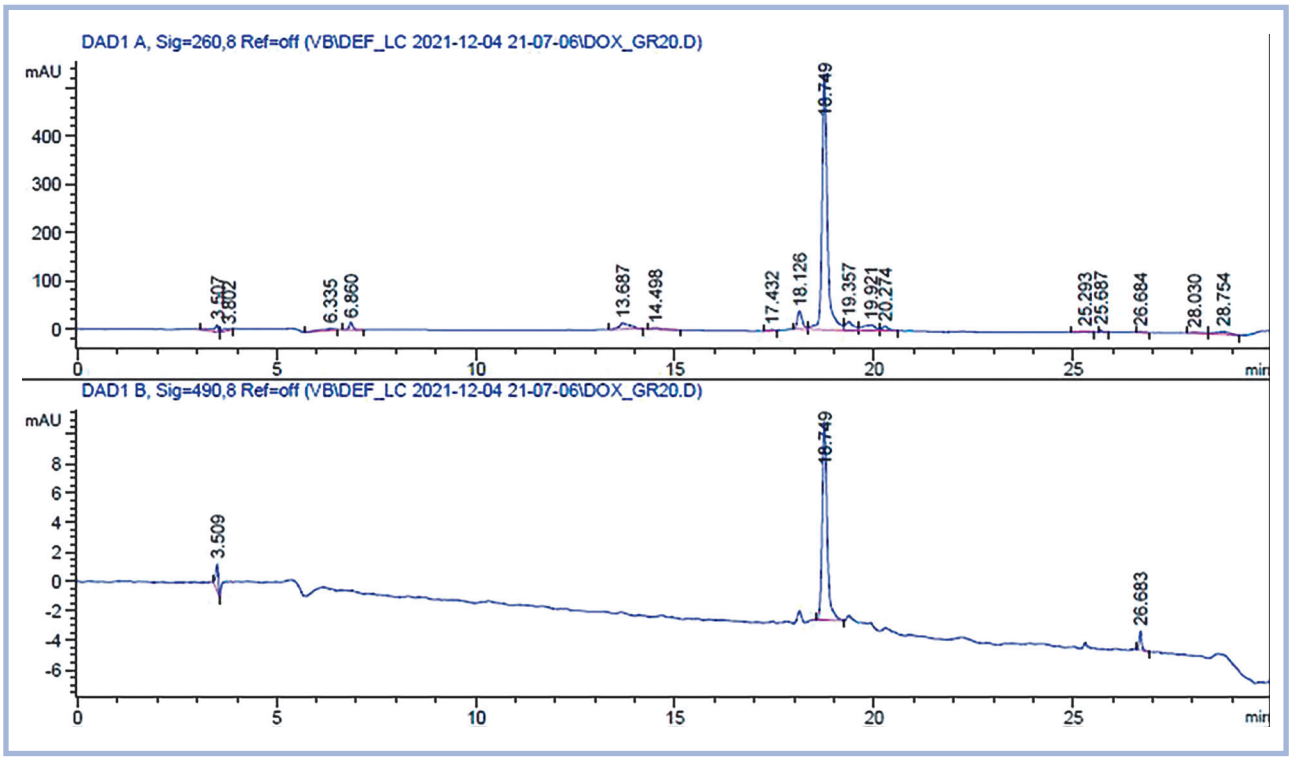


Fig. 2. **HPAC profile of GR20 conjugate with doxorubicin (detection at 260 and 490 nm).**

MTS test

We assessed the results of exposure of human dermal fibroblasts DF1 and continuous GB cell culture Sus/fP2 to doxorubicin (DOX), GR20 aptamer and DOX-GR20 conjugate using MTS test. These results are presented in histograms as changes of cellular metabolic activity and proliferation. Statistical analysis was carried out using the nonparametric Mann—Whitney test.

We observed no effect of GR20 aptamer at a concentration of 10 μM on metabolic activity of human dermal fibroblasts DF1 **(fig. 3)**. Pure doxorubicin led to significant dose-dependent decrease in metabolic activity by 13, 47 and 82% for concentrations of 0.1 μM, 0.5 μM and 1 μM, respectively. The aptamer-doxorubicin conjugate DOX-GR20 significantly reduced metabolic activity of cells by 19% only at a concentration of 0.5 μM ($p \leqslant 0.01$). There were no differences with the control group at concentrations of 0.1 μM and 1 μM.

Analysis of Sus/fP2 culture **(fig. 4)** revealed trivial (about 9%) decrease in cellular metabolic activity under the influence of GR20 aptamer with significant differences with the control group ($p \leqslant 0.01$). Addition of doxorubicin resulted in significant dose-dependent decrease in metabolic activity by 27, 79 and 91% for concentrations of 0.1 μM, 0.5 μM and 1 μM, respectively. Meta-

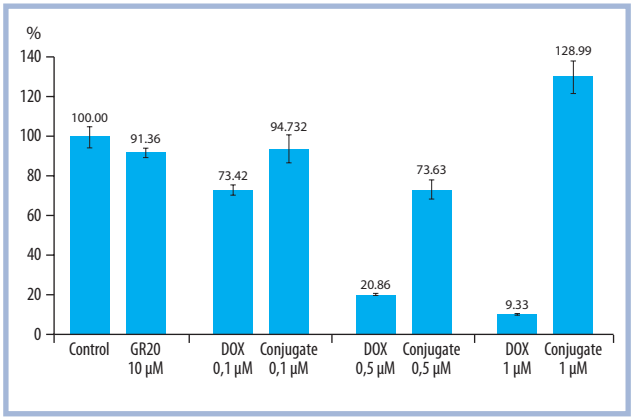


Fig. 4. **Exposure of human glioblastoma Sus/fP2 cell culture to doxorubicin (DOX), GR20 aptamer and DOX-GR20 conjugate (conjugate). MTS test.**

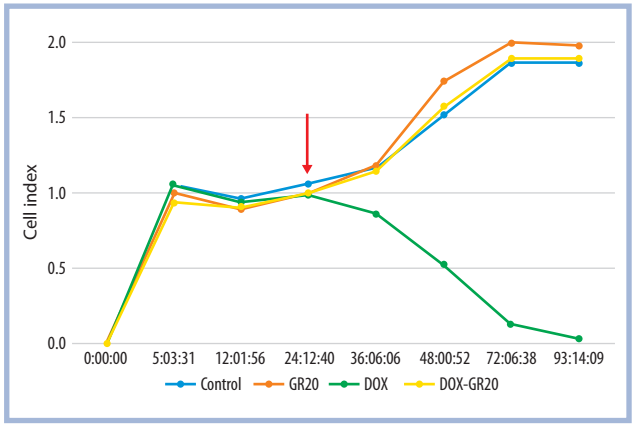


Fig. 5. **Dynamics of cell index in human dermal fibroblasts DF1 under the influence of doxorubicin (DOX), GR20 aptamer and DOX-GR20 conjugate. The red arrow indicates the time of addition of the test compounds to the cells.**

bolic activity of cells did not differ from the control group under the influence of aptamer-doxorubicin conjugate DOX-GR20 at a concentration of 0.1 μM. At the same time, conjugate at a concentration of 0.5 μM decreased this parameter by 26% ($p \leqslant 0.01$). However, the opposite effect was observed at a concentration of 1 μM. Metabolic activity of cells increased by almost 30% compared to the control group.

xCelligence S16 cell analyzer

MTS test was carried out in 72 hours after exposure. Importantly, MTS test data usually correlate with the number of viable cells growing under standard culture conditions. Nevertheless, tetrazolium reduction rate characterizes overall metabolic activity in cell culture and makes this method not very accurate. In this regard, we applied an additional method using the xCelligence S16 cell analyzer. The last one allows us to evaluate proliferative activity of cells over a long period of time after exposure to antiproliferative molecules.

Dynamics of cell index in DF1 fibroblast culture from the moment of cell seeding onto the plate until the end of experiment is presented in **fig. 5**. Cell growth in all groups was the same within 24 hours before adding the test compounds (red arrow). This trend in the control group and groups of GR20 aptamer and its conjugate with doxorubicin throughout the entire registration indicates no negative effect of these compounds on culture cells. At the same time, the number of cells rapidly decreased under the influence of doxorubicin. Cell index decrease to almost zero within 3 days indicates almost complete cell death.

In addition, the data processing program provides the histograms showing necessary time for cell population to double or halve in experimental groups. Thus, cell index is presented in specific units of measurement rather a conditional parameter including many characteristics of cul-

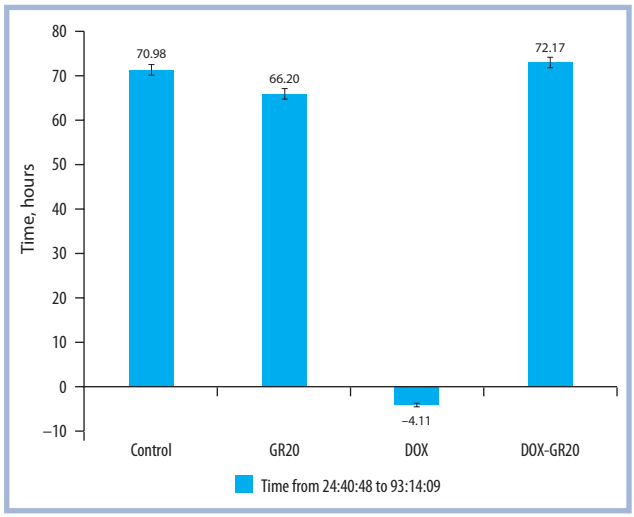


Fig. 6. **Time to human dermal fibroblasts DF1 duplication/halving after addition of doxorubicin (DOX), GR20 aptamer and DOX-GR20 conjugate.**

ture. In the control group, cells require about 70 hours to double their number from the moment the test compounds are added to the end of experiment **(fig. 6)**. Similar values in the GR20 and DOX-GR20 groups once again confirm no negative effect of aptamer and its conjugate. Native doxorubicin induced rapid cell death. Time for a twofold decrease in their number was 4.11 hours.

Dynamics of cell index in human GB cell culture Sus/fP2 is presented in **fig. 7**. As in DF1 culture, proliferation proceeded at the same rate in all groups before addition of test substances (doxorubicin (DOX)/GR20 aptamer/DOX-GR20 conjugate) into culture medium (red arrow). Then, we observe a gradual increase reaching a plateau in the control group. Addition of GR20 aptamer initially leads to mild acceleration of proliferation with subsequent decrease of this process after two days. DOX-GR20 conjugate also first activates proliferation, but the number

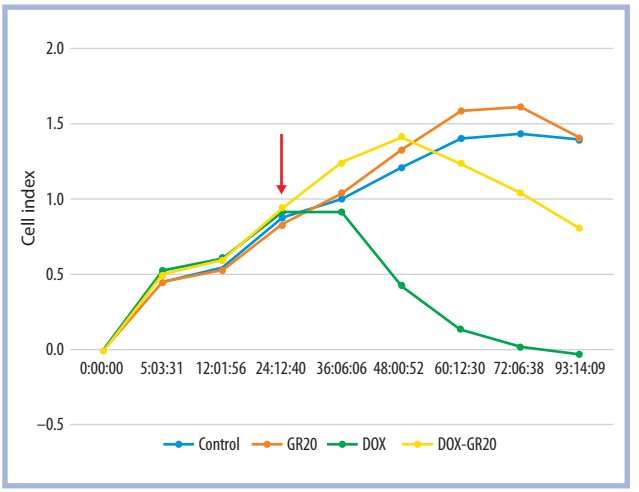


Fig. 7. **Cell index in human glioblastoma cell culture Sus/fP2 under the influence of doxorubicin (DOX), GR20 aptamer and DOX-GR20 conjugate. The red arrow indicates the time of addition of the test compounds to the cells.**

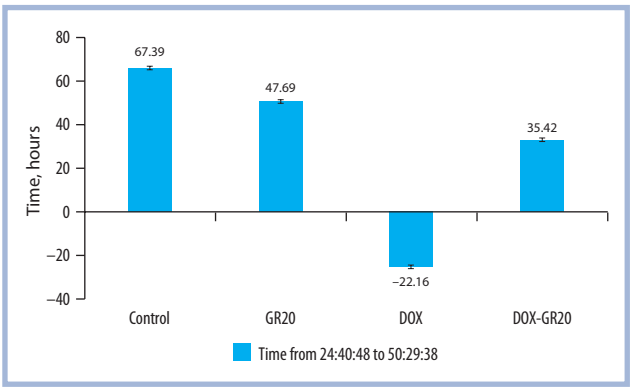


Fig. 8. **Time to human glioblastoma cell culture Sus/fP2 duplication/halving after addition of doxorubicin (DOX), GR20 aptamer and DOX-GR20 conjugate (0—25 hours after exposure).**

of cells decreased a day later. Pure doxorubicin at a concentration of 1 μM leads to fast cell death almost immediately after addition. This process completed throughout the experiment.

It is interesting to analyze changes in cell culture proliferation rate at different stages after addition of compounds. In the control group, cells required about 67 hours to double their number on the first day after adding compounds **(fig. 8)**. In case of GR20 aptamer and its conjugate, growth is somewhat faster at this stage. Cells require 1.4 and 1.9 times shorter period for this process in each group, respectively. Doxorubicin decreased the number of cells immediately after addition. The time for a two-fold decrease is less than a day.

We observed a significant slowdown of proliferation in the control group in the last two days of experiment **(fig. 9)**. Doubling the number of cells now takes 757 hours. At the same time, very slow decrease in the number of cells begins at this stage under the influence of GR20 aptamer.

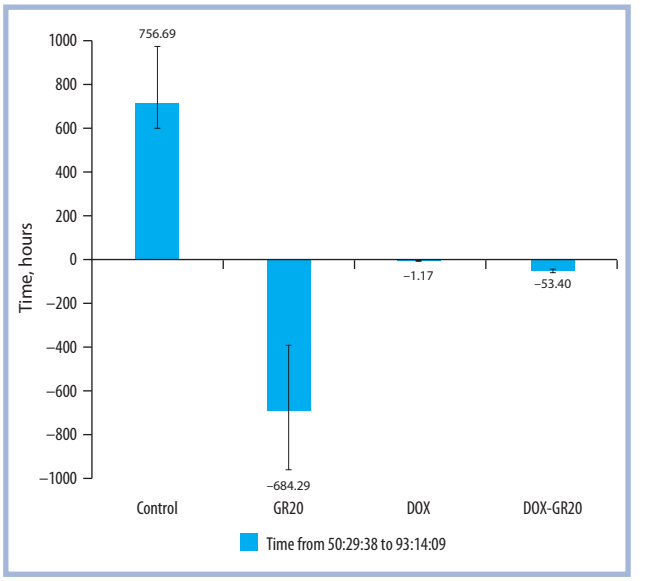


Fig. 9. **Time to human glioblastoma cell culture Sus/fP2 duplication/halving after addition of doxorubicin (DOX), GR20 aptamer and DOX-GR20 conjugate (25—68 hours after exposure).**

It takes 684 hours to reduce by half. The number of cells in the group to which the covalent DOX-GR20 conjugate was added dropped much faster. The halving time is 53 hours. Complete cell death occurs during this time under the influence of pure doxorubicin at a concentration of 1 μM.

Discussion

Various scientists research the aptamer molecules not only as therapeutic agents, but also as effective carriers for target delivery of drugs to tumor cells with minimal damage to intact cells [10, 11].

We can conclude that GR20 aptamer at a concentration of 10 μM and their conjugates with doxorubicin DOX-GR20 at a concentration of 1 μM have no negative effect on fibroblasts DF1. MTS test and xCelligence S16 cell analyzer found no decrease in metabolic activity and proliferation of cells compared to the control group. We observed almost complete cell death under the influence of doxorubicin at a concentration of 1 μM within 3 days after addition.

Results of both methods are not completely consistent on human glioblastoma culture Sus/fP2. The end point of experiment on cell analyzer corresponds to the moment of MTS test. In both cases, we observed complete cell death within 72 hours after adding the doxorubicin at a concentration of 1 μM to the culture. Metabolic activity decrease by 9% under the influence of GR20 aptamer at a concentration of 10 μM in the MTS test coincides with slow decrease in the number of cells observed in cell analyzer on the last day of experiment. As for the effect of covalent DOX-GR20 conjugate at a concentration of 1 μM, fast de-

crease in culture cell count under this conjugate (xCelligence S16 analyzer) does not coincide with the MTS test. Importantly, the last method assesses metabolic activity of cells and only indirectly indicates the effect of compounds on proliferation. Therefore, data of cell analyzer may be more relevant. Thus, we can conclude insignificant effect of GR20 aptamer on human glioblastoma cell culture Sus/fP2 and significant negative effect of its covalent conjugate with doxorubicin.

Conclusion

We found a significant negative effect of the studied compounds on human glioblastoma cell culture Sus/fP2 and almost no effect on dermal fibroblasts DF1. Doxorubicin leads to complete cell death in both cultures. Importantly, covalent conjugate of doxorubicin with GR20 aptamer to EGFR provides effective target delivery of chemotherapeutic agent into tumor cells. This leads to their fast death in contrast to DF1 culture where the conjugate had no any effect on cells compared to the control group.

Author contribution:
Concept and design of the study — Pavlova G.V., Samoilenkova N.S.
Collection and analysis of data — Sliman Y.A., Samoilenkova N.S., Antipova O.A., Brylev V.A., Veryutin D.A., Sapozhnikova K.A., Alekseeva A.I., Pronin I. N., Kopylov A.M., Pavlova G.V.
Statistical analysis — Sliman Ya.A., Samoilenkova N.S.
Writing the text — Sliman Y.A., Samoilenkova N.S., Pavlova G.V.
Editing — Pavlova G.V.
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No conflict of interests to declare.

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