

METHODS

Development of prospects for adjuvant treatment of malignant brain tumors in experiments on C6 glioma

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The treatment of malignant tumors and their postoperative relapses is largely limited by toxic manifestations from a large number of known chemotherapy drugs. Finding a way to reduce the toxicity of drugs while maintaining their antitumor activity is a major challenge. In this work, the highly toxic drug gemcitabine was diluted 100 and 10,000 times with ion-free water and, together with verapamil hydrochloride, at a dilution of 10,000 times, was studied for the ability, when used together, to influence the blood cell aggregation. Our previous studies with low concentrations of verapamil showed a satisfactory effect in the treatment of malignant brain gliomas. In an experiment with C6 glioma in Wistar rats, the blood cell aggregation (SPR indicators) was determined daily *in vitro* under the combined action of gemcitabine and verapamil at low concentrations. The daily effect of these drugs on the level of blood cell aggregation turned out to be very variable, which is an important argument against the use of chemotherapy drugs in doses that are narrowly limited by protocols. The study of the correlation coefficients between the surface plasmon resonance (SPR indicators) and the quantitative composition of cell fractions in rats after transplantation of C6 glioma made it possible to determine the scope of regenerative processes in the tumor tissue and its microenvironment, which can also make changes in the methods of tumor treatment.

Keywords: glioma C6, SPR indicator, gemcitabine, verapamil, low concentrations

Introduction

In our previous studies, satisfactory results were obtained in glioblastoma patients with verapamil hydrochloride in low concentrations and in experiments on rats with transplanted gliomas (strains C6 and 101.8) (1, 2). At low concentrations, verapamil was more effective after glioma removal, significantly lengthening life expectancy as a result of an increase in the time before the recurrence of glioblastomas. Co-administration of low doses of verapamil with the chemotherapeutic drug gemcitabine, which was used at generally accepted doses, did not always increase lifespan *in vivo* experiments on rats with transplanted C6 glioma (3).

Gemcitabine realizes its antitumor potential by destroying the structure of chromosomes in cells (4). In the future, it is planned to use the antitumor effect of gemcitabine with malignant gliomas, which are characterized by high chemoresistance and require a special approach to choosing a treatment regimen.

Verapamil acts as a calcium channel blocker in the structure of cell membranes as part of ionotropic receptors. It is worth considering the fact that traditional doses of chemotherapy drugs are not physiological for the body and they try to suppress the growth of tumors with the highest possible doses, which are quite dangerous for the life of the body.

Verapamil hydrochloride (dilution of 10,000 times) was added to the blood and promoted SPR indicator, which was associated with its antitumor effect. It can be assumed that if the dose of a toxic drug such as gemcitabine is reduced a 1000-fold, its effect on blood cell membranes will change toward an increase in their transmembrane potential mediated through the SPR indicator.

Of interest is the study of the joint effect on the SPR indicators when gemcitabine and verapamil hydrochloride were added in low concentrations and the correlation of blood cell number with cell transmembrane potential (SPR) in experiments on rats.

The aim of this study was to investigate changes in SPR indicators with the combined use of the chemotherapy drugs such as gemcitabine and verapamil at low concentrations on glioma rats experiments daily after transplantation of C6 glioma and correlation of these changes with the cellular composition of the blood of rats.

Research elaboration

The blood of 150 white Wistar rats of 3–4 weeks of age with transplanted C6 glioma, an analog of malignant human gliomas, was studied. Ten intact rats of the same age served as controls. Blood sampling was performed daily, starting from day 2 after inoculation and up to the agonal stage of animal life on day 16 after inoculation. The amount of blood in rats obtained by decapitation, on average, was 3 mL. Before blood sampling, heparin was added and the samples were centrifuged separately. After the removal of blood plasma, blood cells were distributed into three Eppendorf tubes, and ion-free water was added to the first Eppendorf tube. Gemcitabine (dilution of 1:100) and verapamil (dilution of 1:10,000) were added to the second tube, and gemcitabine (dilution of 10,000) and verapamil (dilution of 10,000) were added to the third tube. Changes in the level of blood aggregation were studied using the Plasmon device (5, 6) under the combined action of low concentrations of drugs daily after tumor inoculation up to the death of the animals. Studies were carried out on the blood of five patients with malignant brain tumors in order to determine SPR indicators with gemcitabine dilutions of 10, 100, 1000, and 10,000 times.

The number of cells was determined on a Mindray-3000 hematological analyzer.

The correlation between the number of cells and their membrane charge, indirectly determined SPR indicators, studied with the Spearman coefficient.

Statistical studies

The “Statistics-10 v” package was used for statistical analysis.

Results

With a decrease in the concentration of gemcitabine from 10 to 10,000 times, SPR indicators in glioma patients significantly increase, as shown in [Figure 1](#).

Gemcitabine dilutions of 100 and 10,000 times were used, at which SPR indicator in glioma patients was maximum, together with dilutions of verapamil hydrochloride by 10,000 times.

After inoculation of C6 glioma in rats, the level of SPR indicator was determined on day 2 after inoculation. The data are shown in [Figure 2](#).

It has been shown that, due to the growth of transplantable C6 glioma in rats, the level of aggregation changes under the combined effect of dilutions of gemcitabine in low concentrations of verapamil hydrochloride daily.

As can be seen from [Figure 2](#), at gemcitabine dilutions of 100 and 10,000 times, it also differs. When gemcitabine is diluted 100 times, the level of cell aggregation is lower only on days 4, 7, 8, and 11 after inoculation. On other days, the level of aggregation is lower when combined with verapamil dilution of gemcitabine by 10,000 times.

In our earlier correlation data of the blood cell number in glioma patients with their SPR indicators, we concluded that there were significant changes in the cellular composition under the influence of changes in the level of SPR indicator. In healthy people, this correlation was absent. This result indicates active regenerative processes in the tumor.

Unlike glioma patients, we also found a correlation between cell composition and their transmembrane potential in glioma rats. Before transplantation of the tumor, rats were healthy and there was no correlation between the indicated parameters. However, during the growth of C6 glioma in rats, a correlation appears between these parameters with varying degrees of severity and direction of the processes. Below are the results of determining the correlation coefficient on days 7, 8, and 10–13 after tumor inoculation as the most representative.

On day 7, there is a correlation only between changes in SPR parameters, and the cells are clearly not yet involved in the process of tumor tissue regeneration ([Table 1](#)). On the remaining days after inoculation of the C6 strain, a change in the cellular composition of the blood is manifested under the influence of a change in the transmembrane potential, which indicates their close correlations ([Tables 2–6](#)). In subsequent periods (14–15 days), the correlation disappears and cells interact only with each other ([Tables 7, 8](#)).

These data reveal the daily dynamics of development and completion of regeneration stages in the process of C6 glioma growth from the moment of its inoculation into animals.

Gemcitabine was first used to treat glioblastoma by Rieger et al. in 1999. Gemcitabine inhibits the growth of glioblastomas, has radiosensitizing properties, and penetrates the blood–brain barrier. The negative property of gemcitabine is its high toxicity, which, along with

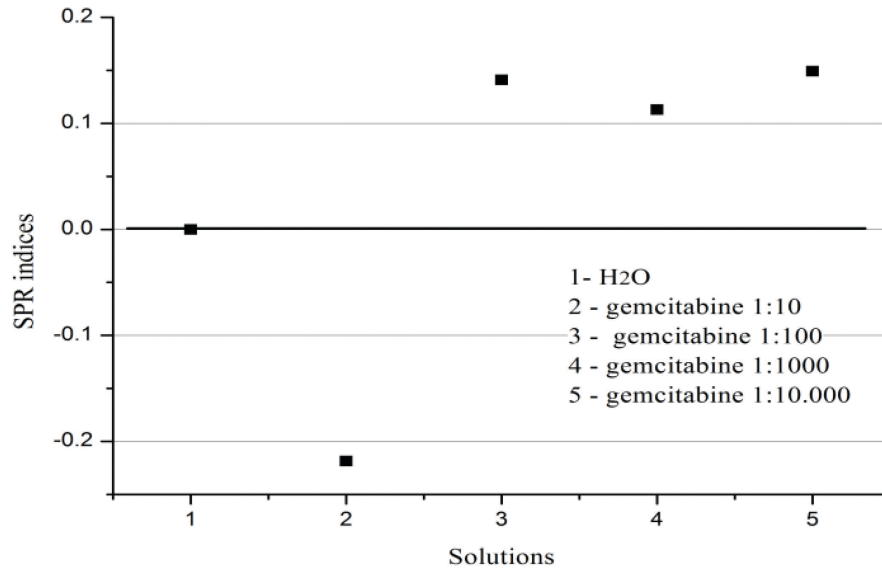


FIGURE 1 | Dependence of the SPR indicators in glioma patients when diluting gemcitabine by 10, 100, 1000, and 10,000 times. Data are presented relative to SPR values in blood samples supplemented with ion-free water.

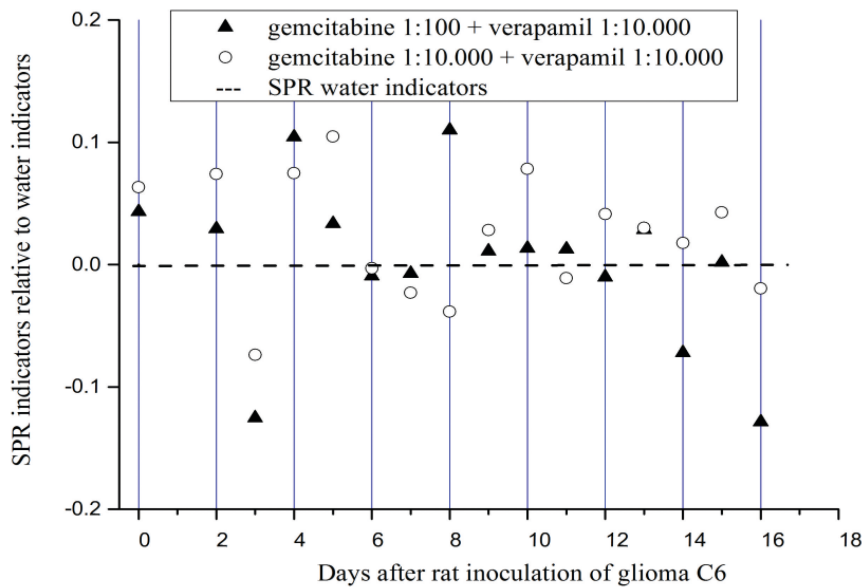


FIGURE 2 | Change in the parameters of blood SPR of rats with C6 glioma, measured from day 2 to day 16 after transplantation. The zero value in the table is the group of healthy intact rats. The data are presented in relation to the indicators of SPR in blood samples with the addition of ion-free water.

TABLE 1 | Correlation coefficient on day 7 after transplantation of C6 glioma.

	H ₂ O	1:100	1:10,000	Leukocytes	Lymphocytes	Monocytes	Granulocytes
H ₂ O	1	0.94286*	0.9*	-0.08571	-0.02899	0	0.32367
1:100	0.94286*	1	0.9*	-0.02857	0.02899	0	0.44137
1:10,000	0.9*	0.9*	1	0	0.0513	0.10541	0.35909

temozolomide, requires careful use in patients on an individual basis.

In clinical practice, gemcitabine is used together with a large number of other drugs for cancer patients

(7). Gemcitabine ranked as the third anticancer agent prescribed worldwide.

In addition, the choice of gemcitabine in our studies is also due to its effect on transformed blood cells (8).

TABLE 2 | Correlation coefficient on day 8 after transplantation of C6 glioma.

	H ₂ O	1:100	1:10,000	Leukocytes	Lymphocytes	Monocytes	Granulocytes
H ₂ O	1	0.85455*	0.21429	-0.21667	0.35909	0.3	0.22361
1:100	0.85455*	1	0.56667	-0.4303	-0.08697	-0.31429	-0.40119
1:10,000	0.21429	0.56667	1	-0.66667	-0.57977	-0.71429	-0.83324*

TABLE 3 | Correlation coefficient on day 10 after transplantation of C6 glioma.

	H ₂ O	1:100	1:10,000	Leukocytes	Lymphocytes	Monocytes	Granulocytes
H ₂ O	1	0.77473	0.79341	0.05947	0.08406	0.24785	0.57593
1:100	0.77473	1	0.86264	-0.09366	-0.26879	0.14388	0.47315
1:10,000	0.79341	0.86264	1	0.26212	0.27321	0.53161	0.64698

TABLE 4 | Correlation coefficient on day 11 after transplantation of C6 glioma.

	H ₂ O	1:100	1:10,000	Leukocytes	Lymphocytes	Monocytes	Granulocytes
H ₂ O	1	0.89427	0.82648	0.39912	0.35281	0.54608	0.52326
1:100	0.89427	1	0.83562	0.33297	0.24035	0.5039	0.5616
1:10,000	0.82648	0.83562	1	0.66819	0.55835	0.79082	0.86211

TABLE 5 | Correlation coefficient on day 12 after transplantation of C6 glioma.

	H ₂ O	1:100	1:10,000	Leukocytes	Lymphocytes	Monocytes	Granulocytes
H ₂ O	1	0.79121	0.84615	0.18981	0.17163	0.14949	0.34483
1:100	0.79121	1	0.9021	-0.03515	0.04904	-0.25983	0.05029
1:10,000	0.84615	0.9021	1	0.20138	0.1549	0.32102	0.60273

TABLE 6 | Correlation coefficient on day 13 after transplantation of C6 glioma.

	H ₂ O	1:100	1:10,000	Leukocytes	Lymphocytes	Monocytes	Granulocytes
H ₂ O	1	0.16364	0.23529	0,2	0.16514	0.66332	0.78182
1:100	0.16364	1	0.82353	0.52727	0.56883	0.66332	0.30909
1:10,000	0.23529	0.82353	1	0.05882	0.05882	0.62622	0.35294

Papers describe the action mechanism of gemcitabine on tumor cells, including disorders in nucleic acids (9, 10).

A promising direction in the gemcitabine brain gliomas is considered (11, 12).

This work shows, for the first time, that low concentrations of gemcitabine can affect the SPR indicator with brain glioma patients and, in combination with verapamil, with transplanted C6 glioma rats. These rates are not stable as they change daily during the growth of C6 graft glioma. These data make us think about the effectiveness of tumor treatment by standard protocols, which do not change not only in different patients but also in different periods of treatment of the tumor process of an individual patient.

The use of low concentrations of gemcitabine in the clinic is of great interest, as it will reduce the toxic effect of this drug, which does not allow it to be used daily in patients with malignant tumors. In addition, due to low concentrations, it acquires a positive effect on the SPR indicator, increasing it, can more effective adjuvant treatment of brain tumors.

The study of the Spearman coefficient shows that, when a tumor is transplanted into an intact animal, correlations are established both with the transmembrane potential (SPR indicators) and with the cell pools of the peripheral blood of rats. You can track the time after transplantation when these correlations appear and disappear.

TABLE 7 | Correlation coefficient on day 14 after transplantation of C6 glioma.

	H ₂ O	1:100	1:10,000	Leukocytes	Lymphocytes	Monocytes	Granulocytes
H ₂ O	1	0.9	0.9	0	-0.1026	-0.0513	0.52705
1:100	0.9	1	1	0.2	0.0513	0.2052	0.73786
1:10,000	0.9	1	1	0.2	0.0513	0.2052	0.73786

TABLE 8 | Correlation coefficient on day 15 after transplantation of C6 glioma.

	H ₂ O	1:100	1:10,000	Leukocytes	Lymphocytes	Monocytes	Granulocytes
H ₂ O	1	0.2	1	0.30779	-0.46169	0.73786	0.8
1:100	0.2	1	0.4	-0.82078	-0.66689	0	0.8
1:10,000	1	0.4	1	-0.10541	-0.63246	0.63246	1

Conclusion

Adjuvant therapies for cancer are developing at a rapid pace, including the use of drugs that have not been used previously in the treatment of tumors and in completely new concentrations. Only those methods that use the pathophysiological mechanisms of growth and progression of malignant tumors can be effective in the treatment or prevention of progressive growth of neoplasms. The data presented in the work can be used in the further development of adjuvant methods for the treatment of malignant brain gliomas.

Author contributions

GN: author of the idea of the work, development of a method for determining SPR indicators, writing an article, and designing an article. KA: transplantation of C6 glioma to rats and intraperitoneal administration of drugs to animals. ZT: search of low concentrations of verapamil hydrochloride and gemcitabine for research. MI: study of verapamil hydrochloride and gemcitabine for cellular toxicity. KR: installation of the working version of the “Plasmon” device and its operation during research work and obtaining SPR data. KL: definition of blood cell number in patients on a hemoanalyzer. BG: blood sampling in animals with C6 glioma. GA: consultation of a neurosurgeon and selection of patients for research. VE: consultations on research on the SPR sensor. All authors contributed to the article and approved the submitted version.

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