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PT (II) AND PD (II) COMPLEXES INFLUENCE ON SPHEROIDS GROWTH OF BREAST CANCER CELLS

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The aim of the research was to examine the changes in multi-cellular tumor spheroid growth, adhesion properties and gamma-glutamintranspeptidasic activity in model systems of human breast cancer multicellular spheroid MCF-7 under the influence of Pt(II) and Pd(II) π-complexes with allyl-containing thioureas. Comparing with cisplatin, Pt(II) and Pd(II) complexes reduce gamma-glutamintranspeptidasic activity, increase adhesive properties in model system of solid tumor and inhibit the multicellular spheroids' growth. All changes prove the importance of further investigation and analysis of these compounds as potential analogues of anticancer drugs that possibly do not cause resistance and reduce the level of metastasis in breast cancer.

Key words: Pt(II) and Pd(II) π-complexes, gamma-glutamintranspeptidase, adhesive properties, multi-cellular tumor spheroids.

The efficiency of neoplasm treatment is known to depend on the target cell phenotype, namely on their sensitivity to the implemented chemotherapy. Recently the problem of neoplasm resistance to chemotherapy is considered to be linked with biochemical anaplasia, the changed activity of glutathione system in particular, the accumulation of lactate in the microenvironment of tumor cells. Thus it is the most important to develop approaches for creating new antitumor compounds which will be able to solve effectively the problem of transformed cells' resistance.

One of the most popular anti-cancer drugs is cisplatin, whose cytotoxic activity has been discovered by Rosenberg in 1969 [1], and which still is effective in treatment of sarcomas, cancer of the soft tissues, bones, muscles and blood vessels [2]. Since the discovery of cisplatin activity, chemists synthesized thousands of Pt complexes and tested their antitumor properties but only two Pt-containing preparations were accepted world-wide and three more in several countries [3, 4]. Even though the new cisplatin analogues have numerous advantages, the issue of neoplasm resistance is not resolved. Our research is concerned with finding approaches to the creation of new anticancer metal-organic complexes to which tumor cells would be more sensitive [5].

Several mechanisms are involved in the formation of tumor resistance to cisplatin:

– decreased intracellular accumulation and/or increased excretion of the medication;

– drug inactivation due to increased levels of cellular thiols;

– changes in compound targets, processing of cisplatin-mediated DNA damage through increased nucleotide excision repair and lower levels of mismatch repair which leads to evasion of apoptosis [6, 7].

One of the aspects of medication resistance formation is the role of gamma-glutamintranspeptidase (GGT), which is a leading enzyme in glutathione antioxidant metabolism, causing detoxification, protection from active forms of oxygen and preventing oxidative stress. Certain chemotherapy medications induce the activity of this enzyme leading to formation of inactive complexes of chemotherapy agents with glutathione and its analogues that cause medication resistance

[8]. The expression of GGT in neoplasms correlates with the resistance to Pt-containing preparations. It occurs because the "soft" ion Pt^{2+} in the cisplatin molecule has a very high affinity to the "soft" sulfur atom of glutathione in the cytoplasm. As a result new chemically inert substances are formed, deactivating the preparation and causing medication resistance. There are several ways for overcoming that resistance: inhibition of intracellular protection systems or creating analogues of cisplatin which would be less sensitive to glutathione.

In order to surmount the resistance we used the phenomenon of "anti-symbiosis" in trans-effect: two "soft" ligands in mutual trans- position destabilize each other [10]. In other words, using "soft" bearing ligands can prevent the deactivating of platinum compounds by binding with thiol-containing compounds (glutathione and its derivatives) in the cytoplasm enabling them to reach the DNA, the main target of their pharmacological action.

This idea was implemented using N-allyl thiourea derivatives, such as N-allylmorpholine-4-carbothioamide (HL^1) and N-allyl-N′-tert-butyl thiourea (HL^2) with incredibly soft C=S and C=C groups as "soft ligands" for antitumor complexes.

The π-complexes, used in research, are: $[Pd(HL^1)Cl_2]$ **(I)**, $[Pt(HL^1)Cl_2]$ **(II)**, $[Pd(HL^2)Cl_2]$ **(III)**, $[Pt(HL^2)Cl_2]$ **(IV)**, synthesized in reaction of $[PtCl_4]^{2-}$ and $[PdCl_4]^{2-}$ anions with HL^{1-2} in aqueous alcoholic solution in the presence of HCl. The **I–IV** compounds are quite similar in structure to cisplatin, enabling their antitumor activity. The combination of transition metals and biologically active molecules induces the activity through their unique ability to interact with various biological targets [6]. In comparison with cisplatin, ligands of N-allyl-containing thioureas do not have nitrogen donor atoms and therefore can form DNA crosslinks of different chemical nature. This difference reduces the possibility of recognizing the crosslinks in DNA repair and prevent the cross-resistance with already developed Pt preparations.

The studied compounds exhibited cytotoxic activity with IC_{50} in the range from $2 \cdot 10^{-6}$ to $1.5 \cdot 10^{-4}$ M and higher pro-apoptotic and cytostatic effect than cisplatin [5]. Similarly to cisplatin new synthesized complexes bind with DNA, mostly interacting with N7 guanine. Later this interaction prohibits the normal DNA replication and transcription due to crosslinks which are not repaired and may cause apoptosis [8].

Multicellular neoplasm spheroids are used in cell biology as the model system for neoplasms. It is formed *in vitro* through the conversion of adherent cells which grow in 2D-culture into spheroid cell aggregates [9, 10]. These spheroids have better respective growth characteristics of solid cells *in vivo* that are locally characterized by hypoxia, acidosis and lack of nutrients which lead to genetic and adaptive tumor changes [11]. The changing of cell aggregation followed by formation of multicellular spheroids in the cells of hepatocellular carcinoma HepG2 [12] and breast cancer MCF-7 [13] can also be caused by three factors: addition of cytokines to culture medium, simple hypoxia or mechanic stimulation causing epithelial-mesenchymal transition due to the overexpression of vimentin and loss of E-cadherin [11]. Hence, multicellular microspheroids are used as organotypic models of normal and neoplasm tissues [14, 15]. During studies of different types of tumor cells, the breast adenocarcinoma MCF-7 was found to be the most appropriate model [16, 17]. Microspheroids resemble the initial, non-vascular stage of tumor development. The concentric structure of heterogeneous cell population of the spheroid is divided on several zones that correspond to the tumor model with proliferating cells at the periphery, sleeping cells at the middle zone and necrotic nucleus. It is shown that the spheroid growth characterizes the metastatic cell potential [14]. At the first stages of spheroid formation their mass increases exponentially, and linearly as soon as it becomes 200–1 500 μm at the diameter. It is supposed to believe that the main factor of spheroid formation is hyper-expression of surface receptors. These receptors might be produced autocratically by tumor cells or can be activated by specific mitogenes which circulate in the tumor microenvironment. The formation of microneoplasms depends also on adhesive molecules [16].

Thus, the main aim of the research was to determine the influence of new synthesized compounds **I–IV** and cisplatin on the MCF-7 spheroids' growth, levels of GGT activity and adhesive properties.

Materials and Methods

Obtaining multicellular microspheroids

The effect of cisplatin and Pt(II) and Pd(II) π-complexes on multicellular cancer spheroids was studied. The experimental model was used as MCF-7 cell lines, long-term cultured

under standard conditions (37 °C, 100% of humidity, 5% of CO_2) in RPMI medium with the addition of 10% fetal bovine serum (FBS), 2 mmol L-glutamine, and 40 μg/ml gentamicin. Spheroids were generated using carboxymethylcellulose. The medium was not changed during the long-term culturing. The multicellular spheroids were generated and their sizes were measured daily (after addition of studied compounds) by measuring their areas to observe the growth dynamics.

Determination of gamma-glutamintranspeptidasic activity

The GGT activity was determined using the standard kit "Filicit-Diagnostics LLC" at the 7^{th} day after addition of studied compounds, at the final stage of research. The levels of GGT activity were determined at the incubation medium after 7 days incubation period with the studied compounds in concentration of $IC_{50}/5$, which has been estimated from previously obtained experimental IC_{50} values for Hela cells [8]. For evaluation of total gamma-glutamintranspeptidasic activity the test kit (Filicit-Diagnostics LLC, Ukraine) was used. The method was based on the GGT-induced conversion of glutamine from y-L(+)-glutamine *n*-4-nitroanilide to the acceptor dipeptide glycineglycine. It was accompanied with release of the chromogen п-nitroaniline, concentration of which was determined photometrically after the enzymatic reaction was stopped. Thus, 50 μl of working substrate solution were added in microtubes for experimental, negative control, calibrating, and comparative samples, and incubated for 5 min at 37 °C. Then 50 μl of culture medium were added to the experimental samples and incubated for 15 minutes at 37 °C. 2.5 μl of ready calibrating solution (*n*-nitroaniline, 5.4 mmol/l) were added to the calibrating sample. To stop the reaction, 300 μl of 10% acetic acid were added to all samples. 50 μl of culture medium were added to the negative control sample, 47.5 μl of distilled water to the calibrating sample, 50 μl of distilled water to the comparative sample. The samples were incubated for 5 min at room temperature; the optic density of experimental samples (E_e) was evaluated at 405 nm using multi-well spectrophotometer (BioTek) against negative control samples, optic density of calibrating sample (E_{cal}) was evaluated against comparative sample. The GGT activity was calculated as follows:

$$C = E_e/E_{cal} \cdot 3{,}0 \quad [\mu kat/l],$$

where C is GGT activity, μkat/l; 3 — conversion factor, μkat/l; E_e is optic density of the experimental sample in optic density units; E_{cal} is optic density of calibrating sample in optic density units.

Determination of adhesive properties of cells

To evaluate adhesive *properties* of MCF-7 cells under the influence of studied compounds, the method of measuring adhesive abilities of macrophages was adapted. After incubation of cells with tested compounds, the culture medium was removed, and the layer of adherent cells was washed thrice with normal saline solution (pH 7.4). The cells were fixed for 30 min with addition of 100 μl 96% ethanol in each well. After fixation, ethanol was removed and the plate was thoroughly dried. The staining solution (crystal violet) was added to the fixed cells, 100 μl per well, and incubated for 15 min at 20 °C. After incubation, the staining solution was removed and the cells were thrice washed with normal saline solution. To the layer of stained cells, dimethyl sulfoxide was added per each well 100 μl and incubated for 15 min at 37 °C. After full dissolution of the stain, the optic density (OD) of solution was determined in each well using the spectrophotometer at 570 nm. The results were given in adhesion index (AI) units, calculated as follows:

$$AI = E_e/E_c \cdot 100\% \quad [\%],$$

where E_e is extinction of fluid, measured in the presence of studied compound, E_c is extinction of fluid in control wells.

Statistical analysis of the results was carried out using the statistical software Microsoft Excel 2010. To assess the statistical significance of the detected changes, Student *t*-test was applied; significance of the values was controlled by the $P < 0.05$.

Results and Discussion

At the first phase of the study, the changes in spheroid area depending on the time of incubation with the active compounds were determined. This parameter was expressed as percentages of spheroid given in five ranges of sizes: 20–170 μm^2, 170–500 μm^2, 500–850 μm^2, 850–1350 μm^2, and 1350–1700 μm^2 and more (Fig. 1).

Compound I insignificantly stimulated spheroid growth, since the ratio of spheroids of the area 170–500 μm^2 increased and the ratio of the smallest spheroids (20–170 μm^2)

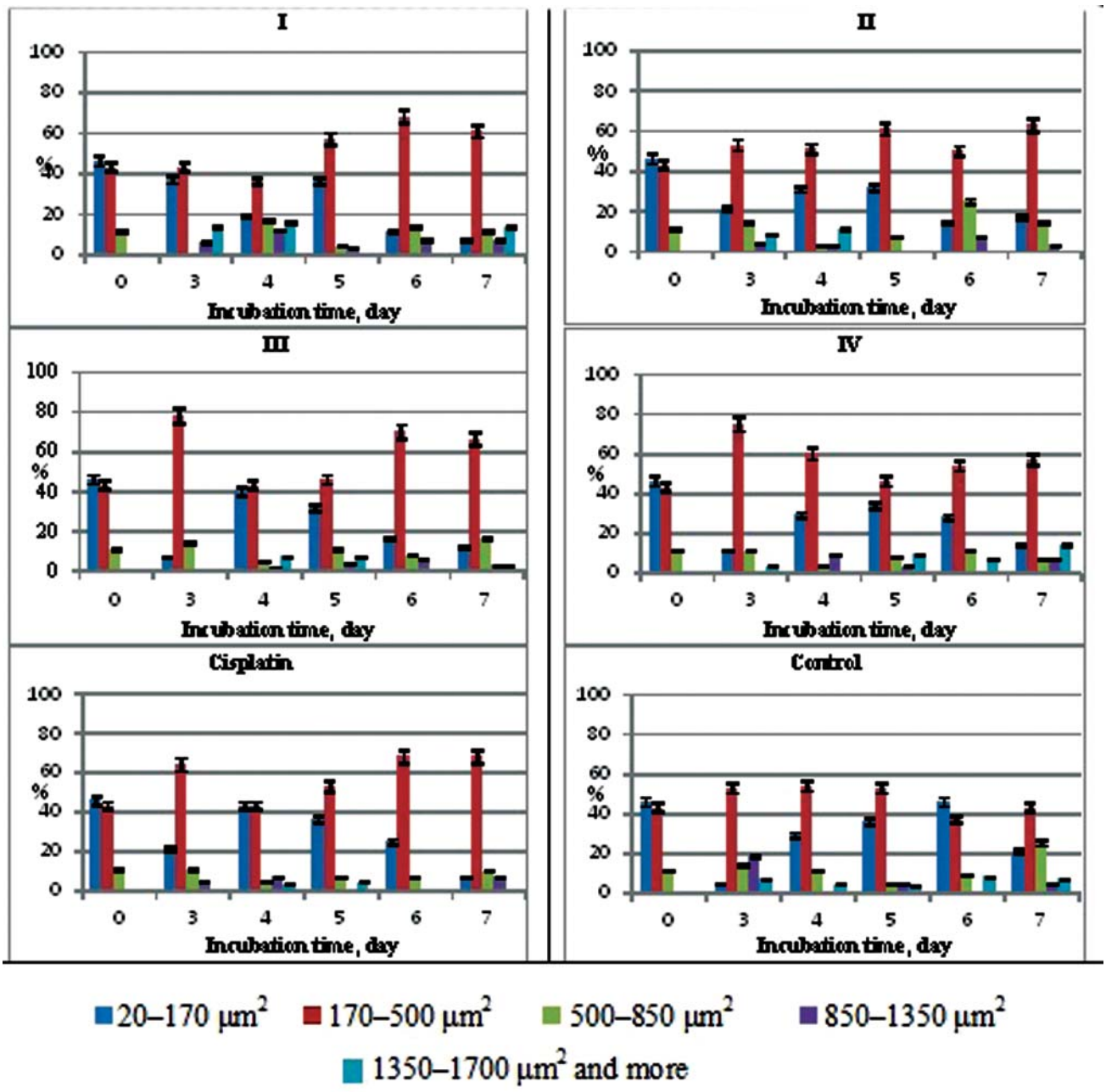

Fig. 1. **MCF-7 spheroid area distribution under the effect of $[Pd(HL^1)Cl_2]$-H_2O (I), $[Pt(HL^1)Cl_2]$ (II), $[Pd(HL^2)Cl_2]$ (III), $[Pt(HL^2)Cl_2]$ (IV) compounds and cisplatin**
(hereinafter: $M \pm m$, $n = 5$)

gradually decreased. This also indicated that single cells did not separate from the spheroids, thus the metastatic ability could be reduced in perspective.

Adding the compound II also resulted in gradually increased percentage of spheroids of the 500–850 μm² area after 6 and 7 days of incubation, and decreased percentage of spheroids of the 20–170 μm² area. But no significant stimulation of spheroid growth was observed.

The compound III decreased percentage of spheroids of the 20–170 μm² area. Hence it was possible that this compound slightly stimulated spheroid growth.

The compound IV did not seem to stimulate spheroid growth, similarly to cisplatin. But the percentage of spheroids of the 20–170 μm² area steadily decreased. The spheroid area stayed relatively stable in control, too. Hence, we could conclude that the studied complexes **I–IV** and cisplatin

reduced percentage of the smallest spheroids (of the 20–170 μm^2 area), and stimulated spheroid growth. The spheroid growth was the most prominent under the action of compound **I** or cisplatin. In control, the spheroid sizes remained relatively stable and their growth was not observed.

The next phase of research was to determine the adhesive ability of MCF-7 spheroids according to the adhesion index. Fig. 2 shows the increased adhesive ability of the spheroids by 22.5 % (in 1.25 times) compared to control under the influence of complex **I**. The complex **II** did not affect the adhesive ability of the spheroids, while the compounds **III** and **IV** increased adhesive ability by 13 % (in 1.13 times). Cisplatin decreased adhesive ability by 14 % (in 1.14 times). It can be theorized that higher adhesive ability of the cells promoted the spheroid stability and reduced metastatic ability. In contrast, lower adhesive ability can indicate the possibility of separation of single cells from a spheroid and as a result subsequent metastasis.

It was determined that unlike cisplatin (which increased the level of GGT activity in 1.3 times compared to control), ϖ-complexes did not stimulate the GGT activity (Fig. 3). The compounds **III** and **IV** did not influence this enzyme's activity, and the complexes **I** and **II** decreased in 1.25 and 1.4 times respectively. Thus, GGT activity under the effect of compounds **I** and **II** was almost twice lower than after incubation with cisplatin. Hence, according to the results it might be concluded that the studied π-complexes did not induce GGT activity-based medication resistance in the transformed cells.

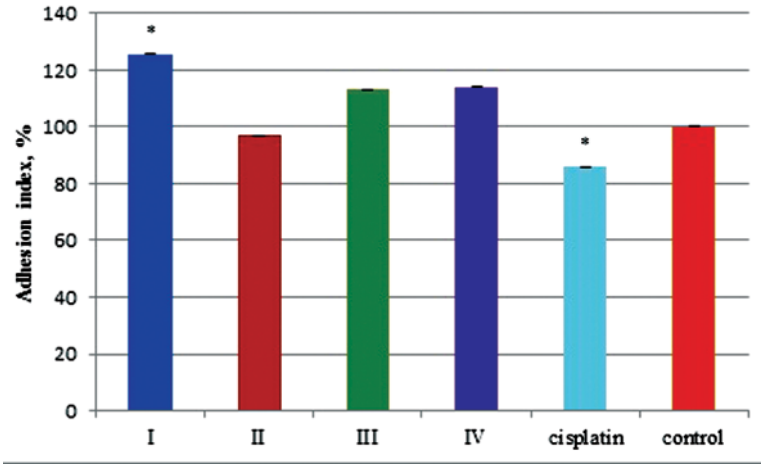

Fig. 2. **Adhesive ability of MCF-7 spheroids under the influence of [Pd(HL^1)Cl$_2$]-H$_2$O (I), [Pt(HL^1)Cl$_2$] (II), [Pd(HL^2)Cl$_2$] (III), [Pt(HL^2)Cl$_2$] (IV) complexes and cisplatin**
(hereafter: * — $P < 0.05$ compared to control)

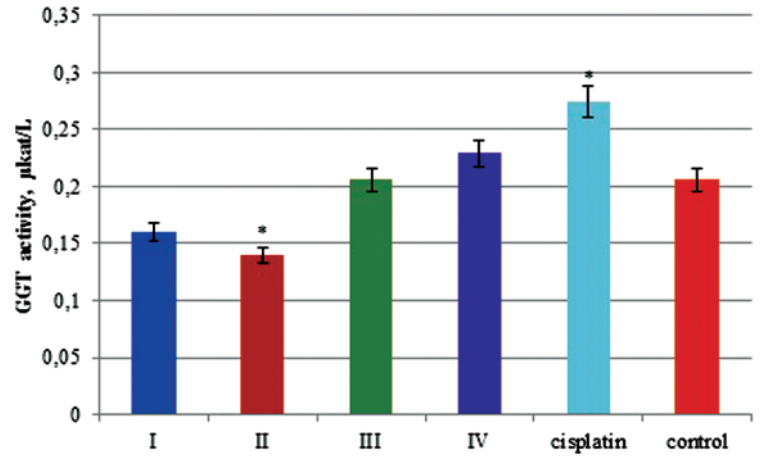

Fig. 3. **GGT activity under the influence of [Pd(HL^1)Cl$_2$]-H$_2$O (I), [Pt(HL^1)Cl$_2$] (II), [Pd(HL^2)Cl$_2$] (III), [Pt(HL^2)Cl$_2$] (IV) complexes and cisplatin**

Thus, it was determined that in comparison with cisplatin, the studied π-complexes of Pt and Pd did not stimulate the GGT activity; therefore they probably would not induce medication resistance. But the tested compounds **I, II, III, IV** insignificantly stimulated the growth of MCF-7 spheroids, similarly to cisplatin. The percentage of the smallest spheroids decreased under the influence of the complexes, leading to the conclusion that these compounds would be able to reduce the cell's ability to migrate and therefore to metastasis. The compounds I, III and IV increased the percentage of cells, adherent to the substrate, at the 7^{th} day of culturing in 1.13–1.25 times, while the compound II did not influence this parameter. In contrast, cisplatin reduced the cellular adhesive ability in 1.14 times. Further investigation using *in vivo* model systems is necessary for understanding other possible mechanisms of effect of these Pt and Pd π-complexes on progression of tumor growth.

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ВПЛИВ КОМПЛЕКСІВ Pt(II) І Pd(II), АНАЛОГІВ ЦИСПЛАТИНУ НА ОСОБЛИВОСТІ РОСТУ СФЕРОЇДІВ РАКУ МОЛОЧНОЇ ЗАЛОЗИ ЗА УМОВ ТРИВАЛОГО КУЛЬТИВУВАННЯ

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Метою роботи було дослідити зміни показників сфероїдного росту, адгезивних властивостей та гамма-глутамінтранспептидазної активності на модельній системі багатоклітинних мікросфероїдів аденокарциноми молочної залози MCF-7 під впливом координаційних π-комплексів Pt(II) і Pd(II) з аліл-тіосечовиною. Порівняно із цисплатином досліджувані сполуки знижували активність гамма-глутамінтранспептидази, підвищували адгезивні властивості та пригнічували ріст багатоклітинних сфероїдів на модельній системі солідної пухлини, що свідчить про доцільність подальших їх досліджень в якості потенційних аналогів протипухлинних препаратів, що не викликають резистентності та знижують рівень метастазування за раку молочної залози.

Ключові слова: π-комплекси Pt(II) і Pd(II), гамма-глутамінтранспептидаза, адгезивні властивості, багатоклітинні пухлинні сфероїди.

ВЛИЯНИЕ КОМПЛЕКСОВ Pt(II) И Pd(II) НА РОСТ СФЕРОИДОВ КЛЕТОК РАКА МОЛОЧНОЙ ЖЕЛЕЗЫ

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Целью работы было исследовать изменения показателей сфероидного роста, адгезивных свойств и гамма-глутаминтранспептидазной активности на модельной системе многоклеточных микросфероидов аденокарциномы молочной железы MCF-7 под влиянием π-комплексов Pt(II) и Pd(II), содержащих аллил-тиомочевину. По сравнению с цисплатином исследуемые комплексы снижали активность гамма-глутаминтранспептидазы, повышали адгезивные свойства и подавляли рост многоклеточных сфероидов на модельной системе солидной опухоли, что свидетельствует о целесообразности дальнейших исследований их в качестве потенциальных аналогов противоопухолевых препаратов, не вызывающих резистентности и снижающих уровень метастазирования при раке молочной железы.

Ключевые слова: π-комплексы Pt(II) и Pd(II), гамма-глутаминтранспептидаза, адгезивные свойства, многоклеточные опухолевые сфероиды.