National University of La Plata and University of Algarve

# DECIPHERING THE EFFECTS OF METAL-BASED DRUGS ON CELL SIGNALING PATHWAYS IN CANCER CELLS

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"A journey of thousand miles begins with a single step"

Kateryna Babych, 2019.

**Abstract (ENG)**

The term ‘cancer’ encompasses a large group of diseases that are characterized by the development of abnormal cells, which divide, grow and spread without control in any part of the organism, spreading through blood or lymph vessels into surrounding tissues. Chemotherapy is a type of cancer treatment that uses drugs to kill cancer cells and is focused on stopping or slowing the growth of cancer cells, which divide abnormally and causes tumors. In most of the cases of chemotherapy prescription and selection of the specific drug, the more effective is a combination of two or more medications that, preferentially, should not interact with each other, either on their mechanism of action and/or their metabolism and elimination. Second and third generations of the existing drugs show increased bioactivity against cancer, but side effects are still a matter of concern. Recent studies show a significant interest in metal-based drugs, with some existing drugs being used already as antitumor agents, with proven effectiveness and fewer side effects, comparing to other drug treatments.

Copper(II) as a metal and its complexes with various organic compounds have been reported to show cytotoxic activity at low concentrations. The aim of this work is to examine the effects of newly synthesized copper complexes in human cancer cell lines, both in terms of cytotoxicity and of mechanism of action. In this research work, two copper(II) based compounds were (copper(II)-tropolone and copper(II)-hinokitiol complexes), for their cytotoxic properties and tested in the human mammary breast cancer cell line MCF7 and MDA-MB-231 for their effect on viability, oxidative stress, apoptosis and interaction with DNA. Additionally, as a model for *in vivo* studies, the 3D model testing on cell viability was conducted and showed a positive result against MCF7 cell line survival. Together with that, the comparative analysis of complexes, its ligands and copper salt was performed. These compounds showed quite promising results in terms of their potential effect as antitumor drugs.

**Keywords**: breast cancer, MCF7, MDA-MD-231, complex, $Cu(trp)_2$, $Cu(hin)_2$.

**Resumen (ES)**

El término ‘cáncer’ comprende un largo grupo de enfermedades que están caracterizadas por el desarrollo de células anormales, las cuales se dividen, crecen y proliferan sin control en cualquier parte del organismo, proliferándose por medio de la sangre o vasos linfáticos dentro de los tejidos cercanos. Quimioterapia es un tipo de tratamiento para el cáncer que usa medicamentos (también conocidas como drogas) para matar las células cancerígenas y que está enfocado en detener o alentar el crecimiento de dichas células, las cuales se dividen de manera anormal llevando a la formación de tumores. En la mayoría de los casos de quimioterapia, la selección del medicamento específico, siendo la combinación más efectiva la de uno o más medicamentos que, preferencialmente, no interactúen entre ellos, ni tampoco con sus mecanismos de acción yo su metabolismo o eliminación. Los medicamentos existentes de segunda y tercera generación muestran bioactividad incrementada contra el cáncer, pero los efectos secundarios son todavía un tema de preocupación. Estudios recientes muestran un interés significativo por medicamentos con base-metálica, con algunos de los medicamentos existentes siendo usados ya como agentes anti-tumores, con efectividad probada y menos efectos secundarios, en comparación con el resto de los tratamientos.

Cu(II) como metal, junto con los complejos que forma con vatios compuestos orgánicos, ha sido reportado por mostrar citotoxicidad cuando está presente en bajas concentraciones. El objetivo del presente trabajo es examinar los efectos de nuevos complejos metálicos de cobre en líneas celulares cancerígenas humanas, lo anterior, tanto en términos de citotoxicidad y de mecanismo de acción. En este trabajo de investigación, dos compuestos fueron considerados para observación y evaluación (complejos Cobre(II) y Cobre (II)-tropolone y Cobre(II)-hinokitiol), por  y sus propiedades de citotoxicidad y estudios en las líneas celulares humanas de cáncer de mama MCF y MDA-MB-231 por su efecto en disponibilidad, estrés oxidativo, apoptosis e interacción con el ADN. Adicionalmente, para un modelo de estudios *in vivo,* el modelo 3D fue realizado para evaluar su disponibilidad, y demostró un resultado positivo contra la línea celular MCF7 sobreviviente. Aunado a esto, el análisis comparativo de los complejos, sus ligantes y las sales de cobre fue realizada. Estos compuestos mostraron resultados prometedores en términos de su efecto como medicamentos antitumorales.

**Palabras clave:** cancer de mama, MFC7, MDA-MD-231, complejos metálicos, $Cu(trp)_2$, $Cu(hin)_2$.

**Resumen (PT)**

O termo ‘cancro’ abrange um largo número de doenças, que são caracterizadas pelo desenvolvimento anormal de células que se dividem, crescem e propagam-se de forma descontrolada por todo o organismo, propagando-se através do sangue ou dos vasos linfáticos para os tecidos vizinhos. A quimioterapia é um tipo de tratamento anticancerígeno, que tem por base o uso de fármacos que atuam por parar ou por diminuir o crescimento de células cancerígenas, células que se dividem anormalmente causando tumores. Na maioria dos casos, ao prescrever um fármaco específico, o uso combinado de dois ou mais fármacos é preferencial, sendo que não deve haver nenhum tipo de interação entre os mesmos, quer no mecanismo de ação e/ou metabolismo, quer na eliminação. A segunda e a terceira geração de fármacos existentes, mostraram um aumento da bioatividade contra o cancro, contudo, os efeitos secundários continuam a ser uma grande preocupação. Estudos recentes têm demonstrado um interesse significativo em fármacos com compostos metálicos, sendo que alguns destes fármacos já são usados como agentes antitumorais, com eficácia e menos efeitos secundários comprovados, comparativamente com outros fármacos.

O Cobre (II), tanto como metal, tanto complexado com vários compostos orgânicos, mostrou ter atividade citotóxica a baixas concentrações. O objetivo desde trabalho é analisar, em linhas celulares humanas, os efeitos de complexos de cobre recentemente sintetizados, tanto em termos de citotoxicidade como em termos de mecanismo de ação. Neste trabalho de investigação, dois compostos com base no cobre (II), (cobre(II)-tropolone) e cobra(II)-hinokitiol complexos), foram observados e avaliados quanto às suas propriedade citotóxicas, e testados na linha celular humana de cancro da mama MCF7 e MDA-MB-231, quanto ao seu efeito na viabilidade, no stress oxidativo, na apoptose e na interação com o DNA. Adicionalmente, como modelo para estudos *in vivo,* foi conduzido um teste modelo 3D quanto à viabilidade, onde foram observados resultados positivos contra a linha celular MCF7 sobrevivente. Simultaneamente, foi realizada uma análise comparativa dos complexos sintetizados, dos seus ligandos e do sal de cobre. Estes compostos mostraram resultados promissores com efeitos potenciais como fármacos antitumorais.

**Palavras chave**: cancro da mama, MCF7, MDA-MD-231, complexo, $Cu(trp)_2$, $Cu(hin)_2$.

## List of abbreviations and acronyms

| | |
|---|---|
| ACS | American Cancer Society |
| BC | Before Christ |
| ctDNA | Calf thymus DNA |
| DMSO | Dimethyl sulfoxide |
| EB | Ethidium bromide |
| EGFR | Epidermal growth factor receptor |
| ER | Estrogen receptor |
| HER2 | Human epidermal growth factor receptor-2 |
| IARC | International Agency for Research on Cancer |
| MTS | Multicellular tumor spheroid |
| PBS | Phosphate buffer saline |
| PI | Propidium iodide |
| PR | Progesterone receptor |
| PS | Phosphatidylserine |
| PXR | Pregnane X receptor |
| ROS | Reactive oxygen species |
| RT | Room temperature |
| SD | Standard deviation |
| UV-vis | UV-visible |
| VEGF | Vascular endothelial growth factor |

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# 1. Introduction

## 1.1. Cancer

Among different sources, related to the research of cancer, such as the American Cancer Society (ACS), Cancer Research UK, National Cancer Institute and International Agency for Research for Cancer (IARC), most of them define the term ‘cancer’ as a combination of diseases, related to abnormal cell change, which leads to uncontrolled cell growth, multiplicity and spreading in tissues, bones, blood, lymph system. Cell proliferation, controlled by different signaling pathways, is a mechanism that is responsible for the normal cell life cycle: from cell growth to cell division and death. While this mechanism undergoes changes, due to cell damage and/or genetic mutations, cells not anymore divide in the normal way, at the same time the cell elimination pathway is disturbed. Therefore, cancer is based on the dysfunction of the cell proliferation mechanism and unmanageable multiplicity of cells, abnormal cell mutations and migration throughout the organism. Cell mutations cause the appearance of tumors, the aggregated form of numerous cells, which could behave independently and differently. Depending on the cell´s origin, distinction is made between noncancerous, precancerous and cancerous tumors.[1] In general terms, tumors are divided into two main groups[2]:

- **Benign tumors**. Tumors that develop slowly and don’t spread to other parts of the body. In general, they are not harmful, but due to their location in the body they could be life-threatening;
- **Malignant tumors**. These are made up of cancer cells, growing faster, spreading into other tissues, causing damage and metastasis.

Currently, there are more than 100 different types of cancer with specific characteristics. They are differentiated mainly by location in the body, but also there are some types, which are distinguished by age, such as childhood and youth cancers. Some cancers are located in a specific part of the body and affect the organs only in those regions while some varieties are highly metastatic, meaning that cells move from their origin and tumors may appear out of the place of initial localization.

According to the cell type, tumors are classified in 6 different types:

- **Carcinoma**. Cancer starts in the skin surface or in tissues that cover internal organs. It has several subtypes, such as adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, and transitional cell carcinoma;
- **Sarcoma**. Cancer that begins in the connective and supportive tissues, such as muscle, fat, blood vessels, lymph vessels, and fibrous tissue (such as tendons and ligaments);
- **Leukemia**. Cancer that forms in blood tissue, such as bone marrow with a further transition into the blood and does not cause solid tumors, but is associated with abnormal growth of white blood cells.
- **Lymphoma and myeloma**. Cancer affects immune system cells (T-, B- or plasma cells) and does not form solid tumors;
- **Melanoma**. Cancer beginning in melanocytes, where melanin is produced. Mainly, it appears in the skin surface, but melanomas could also form in other pigmented tissues, such as the eye.
- **Brain and spinal cord cancers**. Cancer that affects the central nervous system.

The reasons for its occurrence, as a disease itself, are a combination of factors, which are related to genetics, environmental factors, and lifestyle factors, such as smoking or physical activity.

Among genetic changes, which contribute to the appearance and development of cancer, there are three main genetic "drivers": proto-oncogenes, tumor suppressor genes, and DNA repair genes. Proto-oncogenes are genes taking part in the normal cell growth cycle. Nonetheless, if these genes are modified in certain ways, mutated or demonstrate unusual activity, they may become oncogenes, which directly cause cancer. In this case, they enable cells to grow, divide, and proliferate. Tumor suppressor genes are the type of genes that make a protein that is responsible for the negative control of cell growth and division. Different types of changes in their tumor suppressor genes lead cells to divide in an uncontrolled way. In the event of damaged DNA, DNA repair genes are responsible for reversing genetic changes. In the case of failing, cells with such damages get mutated genes, which during division and formation of new cells are conveyed to newly formed cells. Those tend to develop additional mutations and, as a result, may cause the new cells to be potentially cancerous.[3]

From 1990 to 2015, environmental risks shifted from the second largest risk factor of mortality to the 8th largest risk factor, giving way to mostly metabolic and behavioral factors for mortality. Tobacco and alcohol use, unhealthy diet, and lack of physical activity are the highest risk factors of cancer morbidity and are responsible for more than 22% of deaths.[4] And only near 15% of cancers diagnosed in 2012 were due to infectious agents.[5]

| 2012 | | 2018 | |
|---|---|---|---|
| I | M | I | M |
| Breast | | Breast | |
| 25.16% | 14.71% | 24.20% | 15.00% |
| Colorectal | | Colorectal | |
| 9.22% | 9.03% | 9.50% | 9.50% |
| Lung | | Lung | |
| 8.75% | 13.84% | 8.40% | 13.80% |
| Cervix uteri | | Cervix uteri | |
| 7.92% | 7.49% | 6.60% | 7.50% |
| Stomach | | Stomach | |
| 4.81% | 7.16% | 4.10% | 6.50% |
| Others | | Others | |
| 44.14% | 47.77% | 47.20% | 47.70% |



| 2012 | | 2018 | |
|---|---|---|---|
| I | M | I | M |
| Lung | | Lung | |
| 16.72% | 23.61% | 14.50% | 22.00% |
| Prostate | | Prostate | |
| 14.97% | 6.61% | 13.50% | 6.70% |
| Colorectal | | Colorectal | |
| 10.05% | 8.03% | 10.90% | 9.00% |
| Stomach | | Stomach | |
| 8.50% | 10.09% | 7.20% | 9.50% |
| Liver | | Liver | |
| 7.46% | 11.20% | 6.30% | 10.20% |
| Others | | Others | |
| 42.30% | 40.47% | 47.60% | 42.60% |

**Figure 1.1. Distribution data, presented in percentage values, for the 5 most widespread cancer types in women (left) and men (right). The data represents the proportion of the total number of cancer cases, in terms of I (I=Incidence cases) and M (M=Mortality), age-standardized in 2012 to 2018 for its comparative analysis. The values were collected worldwide from 185 countries and estimated by IARC.** [6,7]

According to the World Health Organization (WHO), cancer is the second leading cause of mortality worldwide and 1 of 6 deaths is caused by cancer, accounting for approximately 9.6 million deaths in 2018. The most common are the following five types: lung (2.09 million cases, 1.76 million deaths), breast (2.09 million cases, 627 000 million deaths), colorectal (1.8 million cases, 862 000 million deaths), stomach (1.03 million cases, 783 000 million cases) and liver (782 000 million deaths). The ratio in different types of cancer in men and women is shown in Figure 1.1.

### 1.1.1. Breast Cancer

In 2018 breast cancer took second place among all other types of cancer for both sexes in age-standardized statistics, presented by IARC, and first place in the mortality rate for women. Breast cancer is the most common and leading cause of morbidity and mortality of women in Africa, North and South America, Asia, Europe, and Oceania. The highest rate of female patients with breast cancer is found in Belgium, Luxembourg and the Netherlands.[8]

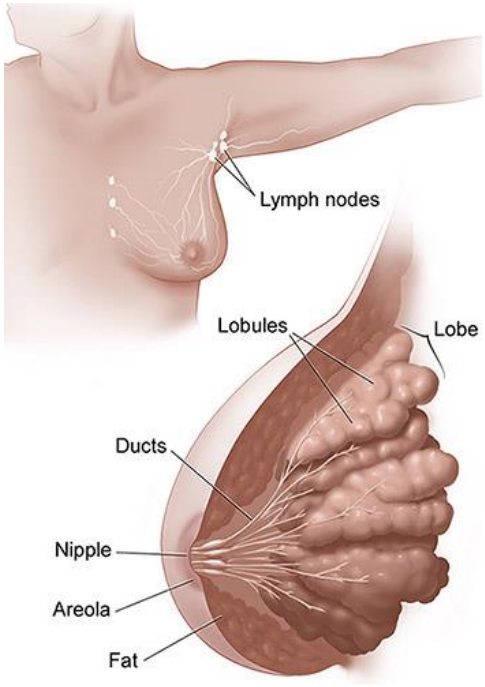


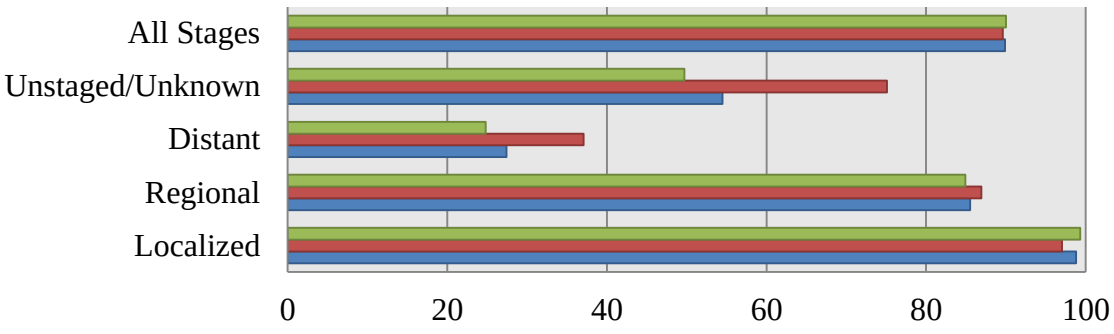

**Figure 1.2. The female breast and adjacent lymph nodes and vessels.**[9]

In most of the cases, the formation of breast cancer tumors starts in the ducts, which carry milk during lactation from the lobules to the nipple. But in some cases, tumors may occur in other tissues of the breast, and these tumors are typically sarcomas or lymphomas. The majority of tumors cause lumps inside the breast, but not all do, and in this case, early detection plays the largest role in treating breast cancer and increasing the patient´s chances of survival. The anatomy of breast cancer schematically represented in Figure 1.2.

The stages of breast cancer development refer to the place, severity of mutation and size of the tumor. The ability of cancer cells to spread is another critical point in determining the stage of cancer. Lately, in 2018, the American Joint Committee on Cancer (AJCC) has proposed guidance on a categorization of breast cancer stages. If a malignant tumor was found in its place of origin, it's named as localized cancer and attributed to the stage I. This means that the area of abnormal cell production is situated only in one part of the breast. It is also considered non-invasive and the common treatment against the tumor is surgery, radiation or a combination of both. It is highly responsive to the treatments and depending on the type of cancer, hormone therapy may be considered effective. The spread to the lymph nodes varies this stage from IA to IB. Regional or distant are called the stages of cancer, when metastases are outside the place of origin, either in breasts or in other parts of the body. Stage II also divides in stage IIA and stage IIB, but like stage I, it has different variations. Stage IIA is diagnosed when tumor reached significant size (usually it's between 2 and 5 cm) and has not spread to lymph nodes, or tumor has the size of less than 2 cm and has spread to lymph nodes, or the tumor has moved from the ducts or lobules and appear only in the lymph nodes. Stage IIB means either the patient has been diagnosed with a tumor larger than 5 cm with no spreading to the lymph nodes, or the tumor is about 2-5 cm and cancer appears in the lymph nodes. Stage III is an invasive type and classifies more widely since it is correlated with migrational tumors spreading beyond the direct region of appearance and shifting to the lymph nodes, skin or bones, but not to nearby organs. Differentiation is determined by the size of the tumor and the location of the spreading of cancer, either to lymph nodes or surrounding tissues. Stage IIIA is diagnosed when the size of the tumor is less than 2 cm and cancer affects 4-9 lymph nodes, or the tumor is more than 5 cm and it is shown to have cancer clusters in a lymph node or the tumor is bigger than 5 cm and cancer has spread to lymph nodes, nearby bones or underarms. Stage IIIB is very likely to behave as an inflammatory type of cancer, which is determined by biopsy and distinguished by the following:

that tumor could be any size and cancer spread to 9 lymph nodes, and chest wall or breast skin. The stage IIIC characterizes itself by leaving the breast and migrating to the lymph nodes. It means that either cancer spread to more than 10 lymph nodes, or to lymph nodes near the collar bone, or to lymph nodes near underarms or the breastbone are affected. Stage IV is the most advanced stage of cancer, invasive and highly metastatic. It means there are metastases in other organs of the body, more likely in the brain, bones, lungs and/or liver. This stage is considered much more difficult to cure and in some cases incurable. However, depending on cancer type, stage IV could be treated as a chronic condition by numerous combined treatments.[10]

The 5-year survival rate of breast cancer between 2009 and 2015 was estimated at 89.9%. This value varies by individual case and health of patients, depending on several groups of factors, including treatment response and diagnosed cancer stage, which strongly correlates with the length of survival and the therapy method. Early detection has a crucial influence on survival and convalescence after the patient is diagnosed with cancer. Figure 1.3 represents the 5-year survival rate by the localization diagnosed, for all ages and between all races (both black and white) of breast cancer patients.[11]




| | Localized | Regional | Distant | Unstaged/Unknown | All Stages |
|---|---|---|---|---|---|
| 50+ years | 99,3 | 84,9 | 24,8 | 49,7 | 90 |
| <50 years | 97 | 86,9 | 37,1 | 75,1 | 89,6 |
| All | 98,8 | 85,5 | 27,4 | 54,5 | 89,9 |

**Figure 1.3. Bar of 5-Year Relative Survival values in percentages for the period of 2009 – 2015 by the diagnosed localization of breast cancer in all races and categorized by ages. The stage at diagnosis is classified using SEER Summary Stage 2000**

A distinction of breast cancer types is made by subtype, depending on characteristic receptors for each subtype. Across years of research, it's been determined that there are 4 to 6 various subtypes of breast cancer. From the receptors, different subtypes have different responses, defined as luminal A, luminal B, HER2 enriched, basal-like and claudin-low, and normal breast-like and molecular apocrine subtypes. The adapted and interpreted table in Annex 2 shows the classification of these subtypes. [12–14]

Each breast cancer subtype varies either by invasive, migrational characteristics or on prognosis and treatment response. Moreover, several breast cancer subtypes have shown migratory behavior, its cells cause metastases in other parts of the body outside the original location. The most metastatic site among all subtypes was the bones, except for the basal-like subtype. For luminal A and HER2-enriched tumor subtypes, there are greater frequencies of metastasis in the brain, lung, and liver. The basal-like subtype has higher rates of lung, brain and distant nodal metastases.[15]

### 1.1.2. Treatment

Each diagnosed tumor type requires a specific treatment type. The most common treatments for cancer are surgery, chemotherapy, and radiation. Surgery is used by doctors to manually remove tumors and sometimes the nearby tissue as a whole, including organs or even an entire body part; and is a preferred treatment to prevent metastasis in other parts of the body. Radiation is the treatment type for cancer targets suppressing and preventing tumor or cell growth. Chemotherapy (or shortly “chemo”) is a common type of therapy used for killing cancer cells, inhibiting their growth, and preventing migration of cancer cells. Additionally, in most cases of chemotherapy prescription, a more effective treatment is a combination of two and more drugs and, in this way, the drugs should not influence the mechanism of action and/or interact between each other. As an additional or supportive therapy, or as a substitute for standard chemo, hormonal and targeted therapy, immunotherapy, stem cell transplantation or a combination of these treatment types could be used. The selection of a specific drug for treating a specific cancer

subtype is usually a complex issue and it should be based on several factors, which include: type and stage of cancer, patient's age and overall health, patient's and tumor's genetic characteristics, other serious health problems and past medical history.

For the last decades, the field of research and development of chemotherapy, which uses drugs to target and kill cancer cells and is focused on stopping or slowing the growth of cancer cells, brought a significant positive effect of the survival percentage of patients, as an example for breast cancer patients.[16] However, to obtain this outcome a considerable price has been paid regarding different side effects, which influence not only the general health condition of the patient, but also the mental and psychological health of the patient, or could have a significant negative effect on other organs and systems. Specific nature-based compounds, which can be found in plants or animals, are gaining attention not only for their anti-inflammatory and antifungal properties, but their antitumor effects have been proved.[17] These compounds and their complexes with other organic and inorganic substituents have much potential as anticancer drugs. In this regard, copper compounds, compared to other metal-based compounds, might take a priority over other metal-based drugs in minimizing the influence of its side effects on the body during cancer treatments.[18]

Among different types of chemotherapeutic drugs, alone or in combination, the main goal is either to kill existing cells, control and suppress multiplication, or not to allow cells to migrate to any other tissues in the body. Together with this, developing a drug that is able to distinguish the target between abnormal cancer cells and normal cells is still one of the most important issues in cancer treatment research today.

### 1.2. Metal Complexes in Cancer Treatment

Since discovery anticancer properties of cisplatin in 1967, broad attention was brought to platinum and metal compounds, in general. Nowadays, cisplatin is one of the most potent drug and still using for prescription in 50-70% cases alone or in combination with other drugs for treatment such types of cancer, as bladder, ovarian, cervical, testicular, ovarian, oropharyngeal, bronchogenic carcinomas, lymphoma, osteosarcoma, melanoma and neuroblastoma.[19] Although, despite the successful positive anticancer activity, cisplatin raised the issue of high general toxicity in the body during treatment and also has less effective activity against many migrational

cell lines and metastatic cancers. Severe side effects are another concern in using cisplatin as an anticancer drug. During treatment, it was observed nephrotoxicity, cardiotoxicity, hepatotoxicity and hear loss, related to using cisplatin.[20] In addition, some types of cell lines are resistant to cisplatin and some cancer types can acquire resistance.[21] New generations of platinum-based compounds aim to increase efficacy on the treatment of many types of cancer, decrease toxicity and side effects influence, and become more effective anticancer drugs. All these factors lay the foundation of using metal ions in a complex with different other ligands, develop next generations of metal-based drugs, but also refocus from platinum to other metals and metal compounds as, potent chemicals for drug design. Due to exhibited characteristics of transition metals, like redox activity, different coordination modes and metal-ligand interaction, the interest in the potential activity of metal compounds against cancer increased.[22]

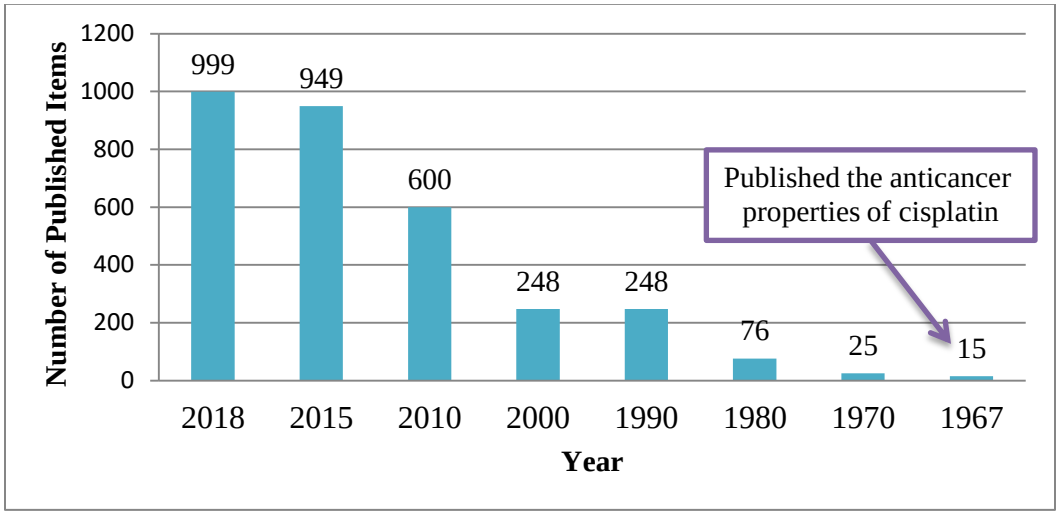



**Figure 1.4. Numbers of articles, presented in PubMed search on the request 'metal compounds cancer' from 1967 to 2018.**

Apart from platinum, the highest interest on research anticancer activity expresses interest to ruthenium, gold, titanium, vanadium, molybdenum and copper compounds. Complexes with these metals were synthesized and showed a certain success in cytotoxic activity and, even more, some of them have been already approved or currently undergoing clinical trials for drugs against many types of cancer. Among others, copper has been investigated mainly in a prospective of being less toxic metal for normal cells, but also effective in the treatment of cancer cells. For more than a decade, mixed chelate copper complexes were synthesized and reported. The group

of over 100 substances, patented under the name Casiopeinas®, substituted with bidentate anionic ligands, like diimine (N=C-C=N), amino acids (N-O) or O-O donors, has exhibited antineoplastic properties and evaluated as a possible anticancer agent. The design of these molecules used a structure of cisplatin as a model and was focused on containing essential metal, preferably *cis*-configuration of chelates around metal ion and mixed chelated may have different hydrophobicity. Experiments have shown that these compounds are related to the generation of ROS, inducing apoptosis, produce DNA fragmentation and, in terms of cytotoxicity, HeLa cells were more sensitive to the presence of copper compounds, than normal cells.[23,24] DNA intercalation and ROS production have been proposed as two main mechanisms of action. Nevertheless, this group of compounds has shown respiratory and cardiovascular toxicity and reducing $O_2$ consumption in *in vivo* studies. The second and third generations of this group of drugs induced a significant higher effect on human lung cancer cells, higher selectivity among cancer and normal cells and caused overproduction of ROS in the mitochondria. Selection of more suitable ligands, control the thermodynamics of the reaction or kinetics of ligand substitution may lead to a more accurate design of new successful anticancer drug.[25]

The complex of Cu(II) with mixed cyano- and cyano-dithiolate ligands has been studied for its antiproliferative activity. Copper complexes with Schiff base ligand exhibit tumor-induced changes in mice and showed higher antitumor activity, compared to cisplatin. Few other copper complexes of ligands together with N-, O- donors and phosphine donors have proved their antitumor activity, being highly selective at the same time.[26] Thereby the further research and studies on new copper-based compounds are highly potential.

### 1.3. General Concerns on Copper Chemistry

The main characteristics of data on copper are presented in Table 1.1.

| Chemical Name | Copper |
|---|---|
| **Molecular formula** | Cu |
| **Molecular weight, g/mol** | 63.55 |
| **CAS number** | 7440-50-8 |
| **Physical and Chemical Properties** | |

| | |
|---|---|
| **Color** | Reddish |
| **Physical state** | Metal |
| **Boiling/melting point, ºC*** | 1083/2595 |
| **Density, g/cm$^3$** | 8.96 |
| **Oxidation states** | I, II |
| | **Environmental Properties** |
| **Vapor pressure at 25ºC** | - |
| **Water Solubility at 25ºC** | Insoluble |
| **Log octanol-water $k_{0w}$*** | -0.57 |
| **Half-Life*** | - |
| **Ozone Reaction*** | No Reaction |
| **Soil Adsorption Coefficient*** | -0.495 |
| **Total Removal In Wastewater Treatment*** | 90.51% |
| **Total Biodegradation in Wastewater*** | 0.02% |
| | **Safety, Hazards, and Toxicity** |
| **Pictograms** |  |
| **GHS Hazard Statements**** | Among reported 56 notifications:<br>H302: Harmful if swallowed;<br>H319: Causes serious eye irritation;<br>H331: Toxic if inhaled;<br>H400: Very toxic to aquatic life;<br>H410: Very toxic to aquatic life with long-lasting effects<br>H411: Toxic to aquatic life with long-lasting effects;<br>H412: Harmful to aquatic life with long-lasting effects; |
| **Precautionary Statement** | P261, P264, P270, P271, P273, P280, P301+P312, |

| | |
|---|---|
| **Codes**** | P304+P340, P305+P351+P338, P311, P321, P330, P337+P313, P391, P403+P233, P405, and P501 |
| **Flammability, Fire Hazard** | In solid state non-combustible, in powdered form may ignite |
| **Acute Toxicity** | $LD_{50}$ (mice) = 110 mg/kg[27] |

**Table 1.1. Overview on some physical, chemical, environmental properties, and hazards, related to copper. *Data calculated by EPISuite; **Data from European Chemicals Agency (ECHA)**

### 1.3.1. Chemical and Physical Properties

Copper is reddish color, soft, ductile and malleable metal with high electrical and heat conductivity. Also, it is a transitional metal, with an atomic number of 29 and an atomic weight of 63.546, takes place the period IV and group 11 in the periodic table. Copper is insoluble in the water but dissolves in most acids and alkalines. It reacts with the oxygen in the air, darkens and converts metal into its oxidative form. Together with oxygen from the air, water and carbon dioxide forms copper (II) carbonate hydroxide with its blue-green color, the product of exposure the metal to the air.

First discoveries of copper date in 9000 BC in the Middle East and taking its name from Latin (*Cuprum*) after the island of Cyprus, which was the mining area for Romans.

### 1.3.2. Copper in Biological and Environmental Systems

Copper is a trace element, prevalent in nature in elemental forms, forms of salts, complexes in the soil, water, plant and animals and essential nutritional element, for living organisms. It is one of the most common elements, which naturally occurs, spreads throughout the environment by natural processes.

Copper is an elemental part of enzymes and proteins with various biological functions. The metal ion is highly redox-active, being donor and acceptor of electrons while changing from one oxidative state to another ($Cu^{+} \leftrightarrow Cu^{2+}$). Copper is an important element in reduction-oxidative processes in organisms as a part of several critical enzymes. One of the main roles of copper is its essential function in being a cofactor in various proteins. It has been reported about at least 10 proteins in prokaryotes that need copper for their function; among them are

cytochrome c oxidase (COX), NADH dehydrogenase-2 (ND-2) and others. Therefore, it takes irreplaceable place in enzymatic productivity.[28] Copper is also involved in such biological processes in the body as pigment formation, free radical scavenging, iron metabolism, connective tissue synthesis, cell signaling, etc. The absorption of copper starts in the digestive system and then transfers to the liver, where enterocytes control the level of copper in the body by distributing copper to blood serum and other tissues. The suggested daily intake for adults stands for 19.10 mg, for adolescents, pregnant and lactating women this amount is a bit less, 8 mg. The excessive amount of copper is eliminated in the urine. [29]

The deficiency of copper in the organism is associated with lots of diseases and critical states. Instability of copper level, its excess, and deficiency presented in metabolic disturbances. Copper is required for the body to metabolize iron and form red blood cells. Anemia is a common symptom of copper and iron deficiencies. In contrast, a high level of zinc leads to a decreased amount of copper. Several studies showed correlation between copper concentration in the body and amount of vitamin C and fat-soluble vitamins, like vitamin A and E. It is known about two genetic conditions, Menkes syndrome and Wilson's disease, where the mechanism of transportation of copper to the organs is disturbed and lead to excessive amount in the first case and toxic accumulation the metal in liver and brain in the second.[29]

### 1.3.3. Toxicity and Carcinogenicity Studies

As a trace nutrient, copper is vital to human health. It plays a role in iron metabolism, regulation of heart rate and blood pressure, activation of the immune system, and development and maintenance of bones, connective tissue, and organs. When the body is exposed to excessive amounts though, copper can produce reactive oxygen species (ROS), causing copper toxicity.

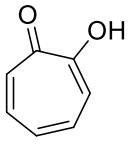
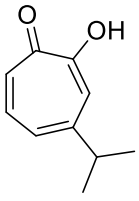
In general, the reported incidences of acute and chronic copper toxicity are quite rare, mainly related to accidents and/or contamination of drinking water. Chronic toxicity is a result of copper accumulation in the liver and brain, contributing to liver and brain damage, and develops into cirrhosis.[30] In 1979-1980, a study was performed on the pro-angiogenic properties of copper salts, collected from tumors, cause the early step of angiogenesis by motility of endothelial cells.[31] High level of copper in blood serum was studied as a possible cause of a variety of cancers; the increased copper level is incidental to progress some types of colorectal and breast

cancer. During a remission of patients with chronic lymphoid leukemia, non-Hodgkin's lymphoma, multiple myeloma and Hodgkin's lymphoma, the level of copper in serum has shown as below or normal levels. Excessive in 130-160% copper level in patients with advanced stages of cancer was related to drug resistance during chemotherapy.[32]

Despite its toxicity, copper was also investigated as a potential anticancer agent in the form of metal-coordinated compounds. Copper-coordinated compounds, both alone and in combination with other chemicals, have been observed in preclinical and clinical studies as effective agents against various cancer types in several different stages. Years of research have shown copper's angiogenetic properties, including binding to angiogenic growth, controlling the secretion of cytokines and inducing vascular endothelial growth factor (VEGF) expression. The mechanism of copper coordinated compounds targeting cancer cells was clearly described. Ionophores of copper force it to enter the cell, while chelators don't allow the metal to contact with the cell.[30]

### 1.4. General Concerns on Chemistry of Tropolone and Hinokitiol

The main characteristics data on tropolone and hinokitiol briefly present in Table 1.2.

| | Tropolone | Hinokitiol |
|---|---|---|
| **Chemical Name** | 2-Hydroxy-2,4,6-cycloheptatrien-1-one; 2-Hydroxytropone; Purpurocatechol | β-Thujaplicin; 4-Isopropyltropolone; 2-Hydroxy-6-propan-2-ylcyclohepta-2,4,6-trien-1-one |
| **Structure** |  |  |
| **Molecular formula** | $C_7H_6O_2$ | $C_{10}H_{12}O_2$ |
| **Molecular weight, g/mol** | 122.12 | 164.204 |
| **CAS number** | 533-75-5 | 499-44-5 |
| **Physical and Chemical Properties** | | |

| | | |
|---|---|---|
| **Color** | From white-beige to yellow | Colorless to pale yellow |
| **Physical state** | Solid | Solid, crystals |
| **Boiling/melting point, ºC*** | 80-84/50.8 | 140/50-52 |
| **Water solubility at 25ºC** | | 1.2 mg/ml |
| | **Environmental Properties** | |
| **Log octanol-water $k_{0w}$*** | 0.53 | 1.82 |
| **Ozone Reaction*** | $0.985\times10^{-17}$cm3/molecule-sec (Half-life: 1.881 h) | Not specified |
| **Soil Adsorption Coefficient*** | 10.06 | Not specified |
| **Total Removal In Wastewater Treatment*** | 2.45% | Not specified |
| **Total Biodegradation in Wastewater*** | 0.09% | Not specified |
| | **Safety, Hazards, and Toxicity** | |
| **Pictograms** |   |   |
| **GHS Hazard Statements**** | H314: Causes severe skin burns and eye damage<br>H315: Causes skin irritation<br>H317: May cause an allergic skin reaction<br>H318: Causes serious eye damage<br>H319: Causes serious eye irritation<br>H335: May cause respiratory irritation<br>H400: Very toxic to aquatic life<br>H410: Very toxic to aquatic life with long-lasting effects | H302: Harmful if swallowed |

| | | |
|---|---|---|
| **Precautionary Statement Codes**** | P260, P261, P264, P271, P272, P273, P280, P301+P330+P331, P302+P352, P303+P361+P353, P304+P340, P305+P351+P338, P310, P312, P321, P332+P313, P333+P313, P337+P313, P362, P363, P391, P403+P233, P405, P501 | P264, P270, P301+P312, P330, P501 |
| **Flash Point, ºC** | 112 | 140 |
| **Acute Toxicity** | LD50 (mouse, intraperitoneal) = 212 mg/kg<br>LD50 (mouse, intravenous) = 106 mg/kg | LD50 (mouse, intraperitoneal) = 85 mg/kg<br>LD50 (mouse, intravenous) = 128 mg/kg |

**Table 1.2. Overview on some physical, chemical, environmental properties, and hazards, related to tropolone and hinokitiol. *Data, calculated by EPISuite; **Data from European Chemicals Agency (ECHA)**

### 1.4.1. Chemical and Physical Properties

Tropolone is a 7-carbon aromatic organic compound of the terpenoid group. It has properties of both phenols and acids and weak ketone. Tropolone is weakly acidic, soluble in organic solvents and water. Easily react with metal ions, widely used as ligand precursors in chemical synthesis.

Hinokitiol is a tropolone derivative compound with pale yellow color crystals. It is moderately soluble in water, well soluble in alcohol and organic solvents; dissolves in concentrated sulfuric acid with further recover by neutralization. The sodium salts of hinokitiol are very stable in alkaline; acetate and methyl ether are easily hydrolyzed. Easily form complexes while contact with a metal ion.

### 1.4.2. Occurrence in the Environment

After it was discovered stipitatic acid as a metabolite *Penicillium stipitatum*, tropolone, which is produced by *Pseudomonas* bacteria with strong antimicrobial activity, was firstly isolated in 1980. At the same time, it was proven its antibacterial activity.[33]

In 1935 Tetsuo Nozoe extracted from the essential oil of *Caldimonas taiwanensis* the compound and gave its name 'hinokitiol', which showed itself as a quite stable compound. [34] Since then it was found in various is trees and its essential oils of *Thuja plicata* (western red cedar), Cupressaceous family, such as *Chamaecyparis obtuse* (Japanese cypress).

### 1.4.3. Cytotoxic Properties

Tropolone due to its interesting chemical properties found significant application in coordination reaction with metals. Most of the naturally-occurred tropolones were isolated from plants and fungi. Many of tropolone derivatives and complexes with tropolone as a ligand, thanks to its interesting chemical structure and ability to bind with metals, found significant biological activities of tropolone such as antibacterial[35], antifungal[36], and antiviral activities. Some of the published recent data showed that tropolones could act as potent and selective inhibitors for enzymes.[37] Already in the 1980s was studied the antitumor activity of both tropolone, hinokitiol, and their derivatives as well *in vitro* and *in vivo*.[38]

Hinokitiol accounts for various properties, similar to tropolone, including inhibition of apoptosis, antifungal[36], anti-tumor, antibacterial[39], anti-inflammatory, and cytotoxic activities. β-Thujaplicin promotes inhibition of migration of lung cancer cell lines through raising cytochrome c, activation of protease enzymes, caspase-3 and caspase-9, and antioxidants CAT and SOD.[40] The complex of hinokitiol with copper inhibits apoptosis and replication of human influenza virus and studied as potential anti-influenza viral drug for treatment and prevention of spreading of influenza infection.[41]

## 1.5. Objectives

The aim of the work is to evaluate the cytotoxicity and the mechanism of action of two Copper (II) complexes with tropolone and hinokitiol ligands, $Cu(trp)_2$ and $Cu(hin)_2$, on breast cancer cell lines. This study is focused on observing *in vitro* the cytotoxic effect of $Cu(trp)_2$ and

$Cu(hin)_2$ complexes on MCF7 and MDA-MB-231 cell lines. Together with that, one of the concerns is to estimate its mechanism of action on the cells together with an evaluation of its potential antitumor properties. The intention is to contribute to the design of new promising metal coordinated compounds for succeeding clinical investigations of new drugs for treating different cancer types.

## 2. Experimental part

### 2.1. Chemical part

#### 2.1.1. Synthesis

The complexes of testing were synthesized and characterized by Prof. Dr. Enrique J. Baran and kindly provided by Prof. Ignacio E. León.

##### 2.1.1.1. Complex $Cu(trp)_2$

The $Cu(trp)_2$ complex was synthesized and characterized by as it was reported and shown in Figure 2.1.[42]

Tropolone was dissolved in methanol and after introduced in the flask by slowly mixing with the solution of copper acetate in methanol : water solution (1 : 6). Tropolone and copper acetate calculated in a 2:1 molar ratio. The obtained mixture refluxed while stirring for 2 hours. The occurred crystals were filtered off, washed several times with cold methanol and dried in vacuum over $H_2SO_4$.

The results of elemental analysis are following (%): Cu: 20.9, C: 55.2, H: 3.49; (calculated for $Cu(trp)_2$ (%): Cu: 20.8, C: 55.0, H: 3.28)).[42]

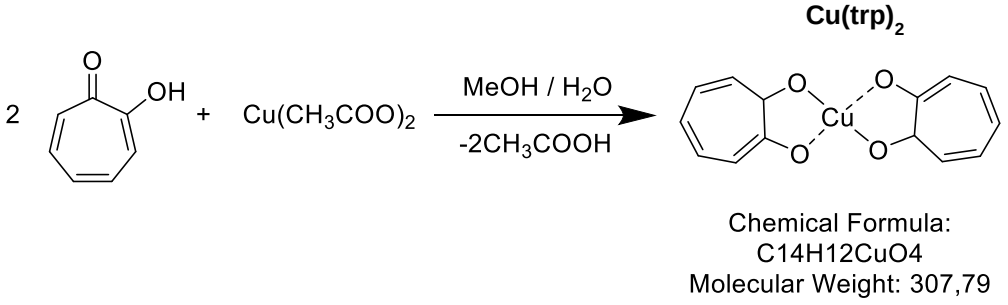



**Figure 2.1. The scheme of synthesis and structure of the copper-tropolone complex.**

### 2.1.1.2. Complex $Cu(hin)_2$

The complex of testing was synthesized and characterized as previously reported.[43] The scheme of synthesis is shown in Figure 2.2.

After dissolving and during the continuous stirring of hinokitiol in ethanol : water solution (50 : 50), a solution of copper acetate was introduced. The molar ratio of concentrations of hinokitiol and copper acetate is 2:1 respectively. The blue-green mixture was stirring and gently heated for 2 hours. After that, the solution was standing for 1 week in order to crystals allow forming and growing from the product. The occurred crystals were filtered off, washed several times with cold methanol and dried in vacuum.

The results of elemental analysis are the following (%): (Calc. for $Cu(hin)_2$): C: 61.6, H: 5.69. Selected IR data ($cm^{-1}$): ν (C=O): 1600, 1574; ν (C=C): 1512; ν (C–O): 1286-1228.

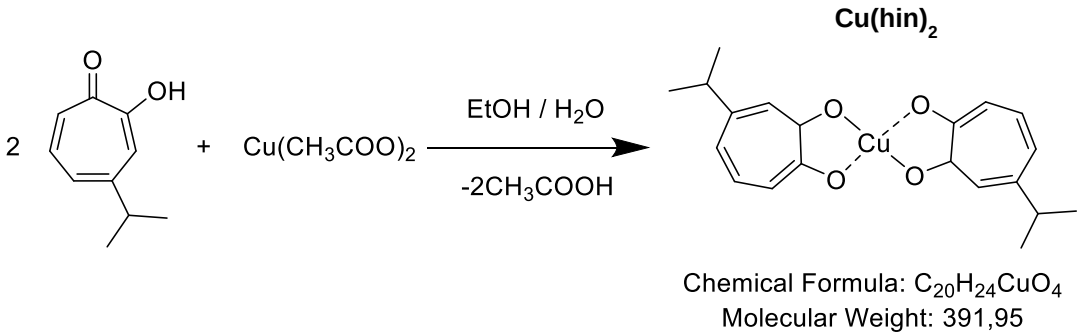



**Figure 2.2. The scheme of synthesis and structure of copper-hinokitiol complex.**

### 2.1.2. Dilutions

The dilution of $Cu(trp)_2$ and $Cu(hin)_2$ complexes, hinokitiol, and tropolone typically were done from 2 stock solutions. The first stock solution was prepared from the dissolving weighted powders using analytical weights (up to 2.00 mg), in dimethyl sulfoxide (DMSO) to reach the concentration of chemicals to 0.02M (20 000 μM). The first stock solution was used for preparing the second stock of 100 μM concentration in the DMEM medium by using aliquot from the first stock solution. The solutions of complexes of concentrations of testing were prepared by using the second stock solution and diluted to the desired concentration. The studies never exceeded 0.5% of DMSO in the solutions and control samples were treated with the lowest concentration of DMSO in order to standardize conditions of experiments.

## 2.2. Biological part

### 2.2.1. Cell Lines

Through years of research, the implication of cell lines tended to become the key element as *in vitro* models and in diagnostics and widely use in laboratory research. Moreover, it can imagine the drug development process without testing a potential drug on the targeted object, as for cancer research is mammalian cancer cells.

#### 2.2.1.1. MCF7

First established by Dr. Soule in 1973 at the Michigan Cancer Foundation, where the name of the cell line comes, MCF7 becomes the most recognizable cell line among others in many aspects of laboratory research, and *in vitro* particularly. The MCF7 cells were derived from the pleural effusion, taken from 69-years old Caucasian female patient, who undergo right mastectomy from a benign tumor and radical left mastectomy for a malignant adenocarcinoma. Also, according to Dr. Soule reports, the patient was treated with a high dosage of synthetic estrogen diethylstilbestrol and after took a tamoxifen medication for cancer treatment. Later was reported that the influence of antiestrogen tamoxifen by inhibiting the growth of MCF7 cells could be reversed by estrogen activity. This fact and further investigations made the MCF7 the hormone-responsive breast cancer cell line[44]

This cell line becomes commonly used in breast cancer research, mainly because of being a proven suitable and sustainable model for testing. With the passage of time, it has been produced more data in molecular profiles through using MCF7 cells in experiments. It has been proven MCF7 cell line as an ER(estrogen receptor)-positive and PR(progesterone receptor)-positive cancer line and relates to the luminal A molecular subtype. The growth of these cells is controlled either by ER and PR and by plasma-associated growth factor receptors, such as epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor-2 (HER2). It is also low aggressive and showed low tendency to migration and invasion, due to low level of vascular endothelial growth factor (VEGF), a key mediator of angiogenesis in mechanisms of formation of new blood vessels and involves in growth and development of cancer. Without estrogen supplementation, this cell line doesn't tend to become tumorigenic and in mice do not induce metastasis.[45] Among observed features of MCF7 cells, it was estimated its

ability for the formation of multicellular aggregated forms, which further growing into lumen-containing spheroids. [46] MCF7 reported to express the WNT7B oncogene. This oncogene was expressed at the same level in normal and benign tumors, but in the case of the second, it was found in approximately 30 times higher in 10% of tumors.[47] MCF7 cell line is suitable to be investigated for anti-hormone therapy resistance, inasmuch as during cultivation, they keep the ER expression. Once the estrogen is removed, the rate of proliferation stays near a month after ER removal.[48]

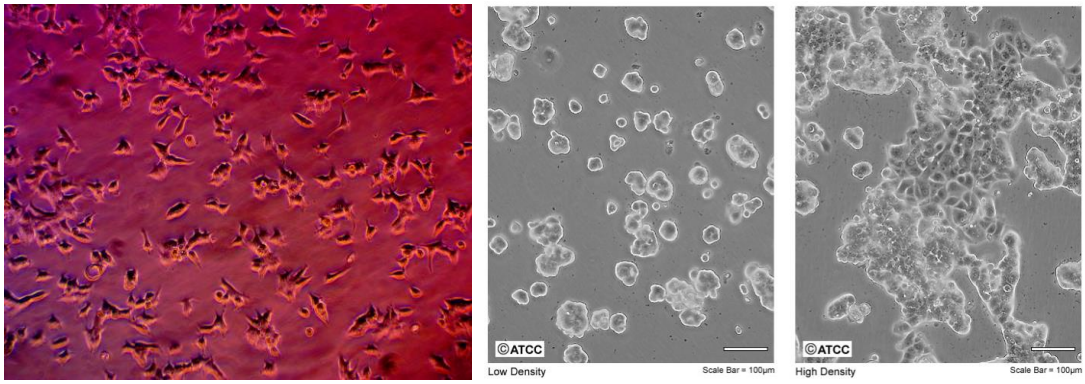



**Figure 2.3. Microscope photo of MCF7 cells in a presence of DMEM (left) and in a low and high density of cells (right).**[49]

#### 2.2.1.2. MDA-MB-231

Breast cancer adenocarcinoma cell line MDA-MB-231 was firstly established in 1973 and derived from single pleural effusion of the metastatic site was introduced from 51-years old Caucasian female patient with stage III adenocarcinoma, who went through a radical mastectomy and earlier received treatment of prednisone and fluorouracil, which apparently was ineffective.[50] MDA-MB-231 cells belong to the basal subtype of breast cancer and have no expression of the ER and PR receptors. However, cells express the epidermal growth factor (EGF) and transforming growth factor-α (TGF-α).

In contrary to the MCF7 cell line, MDA-MB-231 is an ER-negative breast cancer cell line and does not respond to the anti-hormone treatment.[48] This cell line has proved to be highly invasive *in vitro*. At the same time, MDA-MB-231 cells showed relatively low metastatic activity *in vivo*, but despite this, introduction cells into cultivation has been shown sufficient, which makes this cell line the useful model in experimental models.[12] It has been expressed the

correlation between VEGF and aggressiveness in the behavior of the cells.[45] MDA-MB-231 reported to express the WNT7B oncogene.[47]

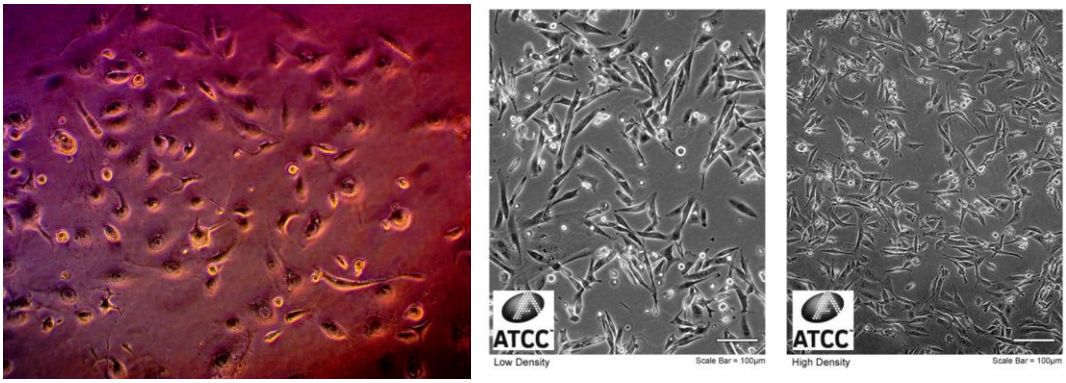



**Figure 2.4. Microscope photos of MDA-MB-231 cells in the presence of DMEM (left) and in a low and high density of cells (right).**[51]

### 2.2.2. 2D Cell System

#### 2.2.2.1. Cell Viability. MTT assay

The main purpose of this method is the evaluation of the effect of compounds with variable concentrations on cell viability and possible therapeutic effects. The assay is performing on the basis of reported by Mosmann's methodology with slight changes.[52]

The assay is based on the conversion of the water-soluble yellow dye of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to insoluble purple formazan, which is the product of the action of mitochondrial reductase. Due to the interaction of MTT with the capacity dehydrogenase and its following reduction, it causes the change of the solution color from yellow with MTT to the appearing of purple crystals of formazan. After formazan solubilizes, it is determined by optical density to be at 570 nm.

In order to evaluate the effect of two compounds on cell viability, the cells were grown and cultured for 24 hours in a $CO_2$ incubator with the level of $CO_2$ of 5% and 37ºC in a plate of 96 wells per 250 000 cells in each. This creates a confluent monolayer. After it was placed in the culture medium (DMEM or DMEM/F12) with dilutions of various concentrations of the drugs and well plate was kept in a humidified atmosphere with 5% $CO_2$ during 48 hours and 37ºC. In the next step, the medium was discarded and 100 µl of PBS put into each well for washing the

cells from the used medium. After PBS was eliminated from the plate, it was incubated with 100 µl per well of 0.5 mg/ml (10% v/v) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in DMEM solution in a humidified atmosphere with 5% $CO_2$ during 3 hours and 37ºC. The change of color of the solution in the wells indicates the formazan creation. Subsequently, the formazan generated by mitochondrial enzymes was dissolved and extracted with DMSO. The plate was filled with 100 µl of DMSO into each well and put into the plate reader for the absorbance determination in the wavelength of 570 nm. The percentages of viable cells were calculated, according to the formula:

$$Cell\ viability, \% = \frac{A}{\overline{A_{ctrl}}} \times 100,$$

$A$ – absorption value of each of probation well value

$\overline{A_{ctrl}}$ – average value of absorption for control samples.

#### 2.2.2.2. Cell Migration

The wound-healing assay provides a reliable, valuable and accessible cell migration *in vitro* study of combined cell traveling-like behavior or migration and proliferation in a monolayer. Also, among those processes, cell interaction in the presence of a chemical has taken place. The approach of the assay is the observation of the behavior of cells in a confluent monolayer after making a gap or scratch (wound) and incubate it free (control) and in the presence of the drugs. The cell-free gap is created in the monolayer of cells by either direct manipulation or physical exclusion. Each manipulation could be performed and destroy the area of the monolayer by using mechanical, electrical, chemical or thermal tools.[53]

Cells were grown and cultured in a 24 wells cell culture plates of (300 000 – 350 000 cells/ml) for 24 hours in $CO_2$ incubator with the level of $CO_2$ of 5%, humidified atmosphere and temperature of 37ºC with complete medium DMEM for MCF7 cells or DMEM/F12 for MDA-MB-231 cells, including 10% FBS, until 90-100% of confluence. After removing the medium, the monolayer was scratched with a sterile 200 µl pipette tip and washed after with PBS in order to remove non-adherent cells. At this point, it was taken digital images by an inverted microscope for the wound by using AmScope software. Then, the cells were treated with copper complexes for 48 hours in a $CO_2$ incubator with the level of $CO_2$ of 5%, a humidified atmosphere and

temperature of 37ºC, dissolved in DMEM for MCF7 cells or DMEM/F12 for MDA-MB-231 cells, including 5% FBS. After treatment time and discarding the medium, the monolayer was washed with PBS and stained with Giemsa solution (5%) for 10 min. Digital images were taken using an Olympus BX51 inverted microscope with a digital camera. The inhibition of cell migration was analyzed with ImageJ software. The percentage (%) of migration was calculated using the following formula:

$$Cell\ migration, \% = \frac{average\ value\ of\ gap\ initial\ area - value\ of\ final\ treated\ area}{average\ value\ of\ gap\ initial\ area} \times 100$$

#### 2.2.2.3. Mechanism of Action

The exact mechanism of action is unknown for any of Cu(trp)2 and Cu(hin)2 complexes. Moreover, copper complexes with similar chemical structure and its influence on cancer cells have not been reported in the literature, thus it was proposed to observe the occurrence of oxidative stress, calf thymus interaction and apoptosis studies for evaluation of possible way of mechanism action. In this study, the ROS production and calf thymus interaction were taken into the scope of the drug-cell and drug-DNA assays to determine the effect and influence of these drugs on mammalian cells.

##### 2.2.2.3.1. Oxidative Stress. Determination of Reactive Oxygen Species (ROS Production)

The appearance and formation of ROS is an aerobic process, which could be caused either by genetic factors, nutritional intake, hormonal or environmental influence. Even if it is an invalid process, the overproduction of ROS leads to oxidative and nitrosative stress, damage of DNA and attack cellular components by production of reactive species after the interaction with proteins and lipids in the living organisms. Therefore, the generation of ROS is a mutagenic process and is used as a potential carcinogenic marker.[54]

Cells were grown and cultured in a 24 wells cell culture plates of (150 000 cells/ml) for 24 hours in a $CO_2$ incubator with the level of $CO_2$ of 5%, humidified atmosphere and temperature of 37ºC with complete medium DMEM, including 10% FBS, until 90-100% of confluence. After the medium was discarded, complexes were introduced into wells with an increasing amount of the concentration of the drug and incubated for 48 hours in the incubator with the level of $CO_2$ of 5%, humidified atmosphere, and temperature of 37ºC. After incubation, the cells were washed

with Hank's buffered saline (HBS). The solution of dihydrorhodamine-123 (DHR-123) in HBS with concentration of 1 μg/ml was prepared and introduced into each well with the following incubation for 30 min and temperature of 37ºC in place without a source of light, in order to allow the fluorescent probe to enter the cytoplasm for its following oxidation. The cells were washed with PBS and the solution of Triton-X100 in the concentration of 0.1% was poured into wells, incubated for 30 min in the place without a source of light. The fluorescence intensity of the cell extract was measured on a Shimadzu RF 6000 spectrofluorometer at a wavelength of 536 nm.

#### 2.2.2.3.2. Calf-Thymus Interaction (ctDNA)

The affinity of both complexes to ctDNA was studied by using both fluorescence and UV spectroscopy in order to confirm the results of binding to the DNA. In current DNA binding research, several studies are dedicated to using calf thymus DNA as a relevant model and mostly for the drug binding mechanism.

##### 2.2.2.3.2.1.1. Fluorescence studies

Fluorescence spectroscopy experiments were performed on various concentrations of complexes and were used for the detection of the interaction with ctDNA. The measurement was done on a constant concentration of ctDNA (20 μM) and variable concentrations of $Cu(trp)_2$ and $Cu(hin)_2$. The aqueous solution of sodium chloride (0.9% w/w) was used as a solvent and ethidium bromide (EB) probe (25 μM) was used as a fluorescent marker in DNA intercalation studies. After adding components to each of the probes before transferring to a cuvette for measurement in order to avoid the influence of light on fluorescence, probes were kept in a place without any source of light. Each probe was prepared in the same order at room temperature, equilibrated and kept for 10 minutes. The fluorescence spectrum was recorded in a range of wavelengths from 550 nm to 800 nm with the excitation wavelength of 540 nm on the RF 6000 Shimadzu fluorometer. The obtained results were processed by LabSolutions RF Shimadzu software.

##### 2.2.2.3.2.1.2. UV-vis studies

The potential interaction of complexes was investigated in two ways with the constant concentration of ctDNA (104.24 μM) and increasing concentrations of complexes (2.5 – 50 μM)

for the first method, and the second method used the constant concentrations of compounds (10 μM for both $Cu(trp)_2$ and $Cu(hin)_2$) an increasing amount of ctDNA (1 – 250 μM). The probes for observation were prepared at room temperature and equilibrated and kept for 10 min. The UV-visible absorption spectrum was taken in a broad spectrum of wavelengths from 190 to 600 nm with a characteristic peak for ctDNA at 260 nm. The results of the emissions in the corresponding wavelength for each probe were analyzed with software UVProbe 2.43. The sensitivity to structural changes and external factors in the π-π* band of the DNA β-form causes the perturbation of non-covalent interaction and leads to the increased shift in absorption of the DNA at 260 nm, becoming hyperchromic.[55]

The ratio of absorbances in wavelengths of 260 and 280 nm shows the result of 1.88, which corresponds to not samples of ctDNA not contaminated by proteins and could be used for the measurement. The electrostatic binding mode between the compound and DNA induces the shift of absorbance lines to the up, comparing to the baseline of absorbance for a control sample of ctDNA and makes the hyperchromic effect occurs. The corresponding changes confirm the increase of absorbance of DNA and influence of the drug on DNA structure through its denaturation. [56] Mainly, the electrostatic attraction promotes charged cations of compounds to bind to the phosphate group of DNA structure causing the overall damage of the secondary structure of DNA. [57]

Counting another possibility of shift, the hypochromic effect refers to the intercalative binding mode, which involves in interaction of chromophore with the base pair of DNA. Both the distance and strength, of the bond between DNA and compound decreasing contribute to the hypochromic effect. [56]

#### 2.2.2.3.3. Apoptosis Study

The MCF7 cells were grown and cultured in a 12 wells cell culture plates of (200 000 cells/ml) for 24 hours in $CO_2$ incubator with the 5% $CO_2$ level, in humidified atmosphere and temperature of 37ºC with complete medium DMEM, including 10% FBS, until 90-100% of confluence. After removing the medium, cells were incubated with the different concentrations of the drug for 48 h at 37ºC. The medium was removed from each well and transferred into Eppendorf tubes and the content centrifuged in order to recover the deattached (dead) cells. The

cells were washed with PBS, centrifuged for better homogenization and discarded the PBS. The cells, which are adhered to the bottom surface of the well, were raised with TrypLE and further washed with the medium in order to transfer all the volume of cells into the tube. Each tube was washed with PBS, centrifuged and discarded. After that, the Annexin buffer in water (0.1 μg/ml) was introduced into each tube together with Annexin V (1 μg/ml) and propidium bromide (5 μg/ml). The apoptosis of cells was measured using the BD FACSCalibur™ flow cytometer with 10 000 events was taken and the FlowJo V10 software for processing the obtained results.

### 2.2.3. 3D Cell Systems

#### 2.2.3.1. Formation of spheroids

In order to form, grow, transfer and operate the spheroid formations, two techniques were used in combination but in the following order. These combined techniques are widely used for the formation of MTS (multicellular tumor spheroids) and were implemented as described.[58]

The *hanging drop* technique accounts for the formation of the spheroid form from the spread of the surface of the drop cells into a conjugated form of cell aggregation, which is revealed in a spheroid form. Cell spheroids develop under the effect of gravitational force and create a solid-like structure. Firstly, for seeding the spheroids, small drops (~25 μl) of cells suspension in the DMEM medium with FBS (10% v/v) with the number of cells 80 000 cells/ml put into the upper lid of a sterile Petri dish. Drops should be separate and placed accurately with a distance from each other in order to obtain a similar amount of cells in each, which leads to the formation of spheroids of the same size under the same conditions. The upper lid after fulfilling with drops of cells inverted and placed up to the lower lid, which filled with PBS to avoid evaporation of the liquid during the incubation. Due to surface tension, drops stay attached to the upper lid. Under the gravitational force, cells tend to settle and concentrate in the top of the drop, reversed to the lower lid, and lead to the formation of spheroids in each of drops. The Petri dish is placed and incubated for 48 hours at 37 ºC in a humidified atmosphere with a 5% $CO_2$ level.

After the spheroids were formed the second technique was used. The *liquid overlay* is a technique, which allows shaped spheroids to produce more cells, inhibit cell-to-cell aggregation and, subsequently, promote enlargement into tumor-like spheroids. Therefore, the drop with the spheroid is transferred into the low adhesive surface, in the presence of DMEM medium with

double-filtered FBS (10% v/v) to facilitate the cell production. After the incubation, 50 µl of the sterile agarose was introduced into each of the well of the 96-wells plate and kept until solidification. The DMEM medium with filtered FBS of 200 µl placed into each well on the top of the agarose layer. Each of the drops with a formed spheroid was transferred from the Petri dish into each well of the plate and incubated for 10-12 days at 37 ºC in a humidified atmosphere with 5% $CO_2$. Every 2 days, the DMEM medium with FBS (10%) was changed in each well and carried out the medium renewal. To record its growth, every 2-3 days pictures of the spheroids were taken to compare and reach them the specifically desirable size of the spheroid. The photos of spheroids were collected by AmScope software and microscope. For size measurement, monitoring and comparative analysis the Image J software was used.

#### 2.2.3.2. Cell viability of spheroids

The resazurin reduction assay is a longstanding analysis initially proposed for use in milk quality testing, which is focused on the ability of living cells to reduce non-fluorescent blue, purple-blue color dye resazurin into a fluorescent red to pink color resorufin.[59] Figure 2.5 represents the irreversible reduction mechanism of resazurin. It has been not clearly defined where specifically the reduction takes place, whether in the inner side the cell or in the outer layer of the cell. It has been suggested that resazurin is being introduced into the cell from the surrounding medium, where it is transformed and metabolized in mitochondria into fluorescent resorufin.[60] On the other hand, the reduction mechanism has been proposed to occur outside the cell in the medium with the following diffusion of resorufin into the cytoplasm and nucleus.[61] It has been suggested that intracellular coenzymes, such as NADH, are entangled in reaction and by using a catalyst promote the reduction reaction.[62]

Talking about areas of application, resazurin was used in various assays, mainly for determination of the biological activity of bacterias, bacterial cell number, cell cytotoxicity in the fields of food, feed, soil quality and contamination as a simple, cost-effective and non-invasive method *in vitro* studies. Furthermore, resazurin assay is considered as non-toxic compounds for cells at low concentrations, which makes it applicable for a large scale of viability studies.[63] In this study, the resazurin assay is used for evaluation of the viability of multicellular tumor spheroids (MTS) formation.

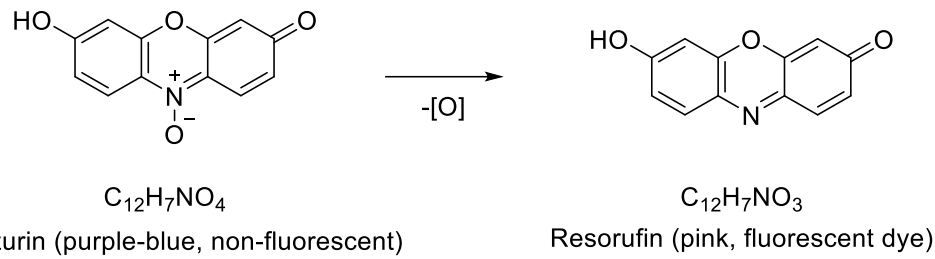



**Figure 2.5. The irreversible reduction of weakly or non-fluorescent resazurin (Alamar blue) into pink highly fluorescent resorufin, the last one indicates by fluorescent spectrophotometry with the emission at 590 nm and excitation at 570 nm.**

The multicellular spheroids were grown as it was described before for 10-12 days in 96–well plate and incubated with the different concentrations of $Cu(trp)_2$ and $Cu(hin)_2$ in DMEM for 48 hours at 37ºC in a humidified atmosphere with 5% of $CO_2$. After the incubation time, the photos were taken of the formed and treated MTS by AmScope software and microscope and the probe of resazurin was added into each well and incubated overnight in the incubator at 37ºC in a humidified atmosphere with 5% $CO_2$ level with a reduced source of the light, since resazurin is able to absorb the light. The plate was read on the Shimadzu spectrofluorometer with the excitation of 560 nm and an emission of 590 nm.

### 2.3. Statistical Analysis

The statistical analysis was performed and expressed as mean value ± standard deviation (SD) of independent experiments, using a one-way analysis (ANOVA) of variance. All of the experiments were performed twice with its repetition for confirming the results, except for ROS production, which was performed only one time as qualitative analysis on the presence of ROS in the presence of drugs. Obtained data were processed and analyzed via GraphPad Prism v6 software.

## 3. Results and Discussion

### 3.1. Results

#### 3.1.1. Chemical part

Cu(II) tropolone and Cu(II) hinokitiol were synthesized by Prof. Dr. Enrique J. Baran and provided by Prof. Ignacio E. León. The crystals of both of the complexes seemed to be

anhydrous. Thus, the dissolving of compounds was performed in DMSO in order to reach the concentration of 20 mM of the stock solution.

The common copper (II) derivatives of α-hydroxyketones are four-coordinate, square-planar geometry compounds. The isopropyl groups in $Cu(hin)_2$ complex are placed in a *cis*-position to each other, whereas in contrast the similar complexes with hinokitiol and maltol with Zn are shown to be placed in *trans*- to each other.[64]

The stability constant of Cu(II) chelate with tropolone has shown the highest value among other tropolone-metal complexes.[65]

### 3.1.2. Biological Part

#### 3.1.2.1. 2D Observation System

##### 3.1.2.1.1. Cell Viability

The cell viability study, based on using 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide salt (MTT study), is a relevant instrument and the most sensitive assay, among the cell viability assays, for an evaluation of drug efficacy and toxicity.[66] In order to observe the influence of the compounds $Cu(trp)_2$ and $Cu(hin)_2$ on the cell viability, it used the reduction of the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide salt and was measured colorimetrically. During the interaction with water-soluble MTT salt with the alive cells, thanks to the mitochondrial complex of succinate-tetrazolium-reductase, present only in viable cells, the salt transforms into water-insoluble formazan.

Figure 3.1[1] shows the results, obtained by Lucia Santa Maria de la Parra in cooperation with Prof. Ignacio E. León. The values of Figure 3.1 and Figure 3.2 represent the results obtained in cell viability assay in both breast cancer cell lines MCF7 and MDA-MB-231, obtained after MTT assay. There was taking into consideration not only complexes of observation but also separately ligands and metals. This was done in order to investigate the impact of complexes, comparing to its elements and demonstrating the beneficial effect of the complexation.

[1] These results were obtained by Lucia Santa Maria de la Parra and kindly provided by Prof. Ignacio E. León
[2] This IC50 value was obtained by Lucia Santa Maria de la Parra and kindly provided by Prof. Ignacio E. León

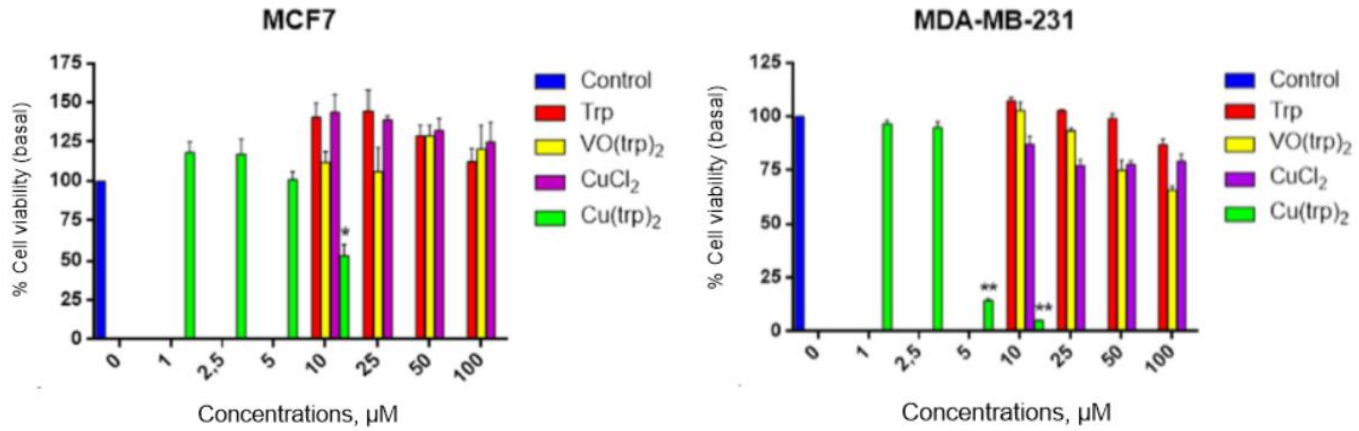



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**Figure 3.1. The influence of Tropolone, Vanadium-tropolone and Copper-tropolone complexes, Copper chloride on the MCF7 (above) and MDA-MB-231(below) cell lines after 48h treatment. The results are expressed as the percentage of the basal and represent the values of mean ± SD *(p = 0.0003); **(p <0.0001) significant with respect to Basal.**

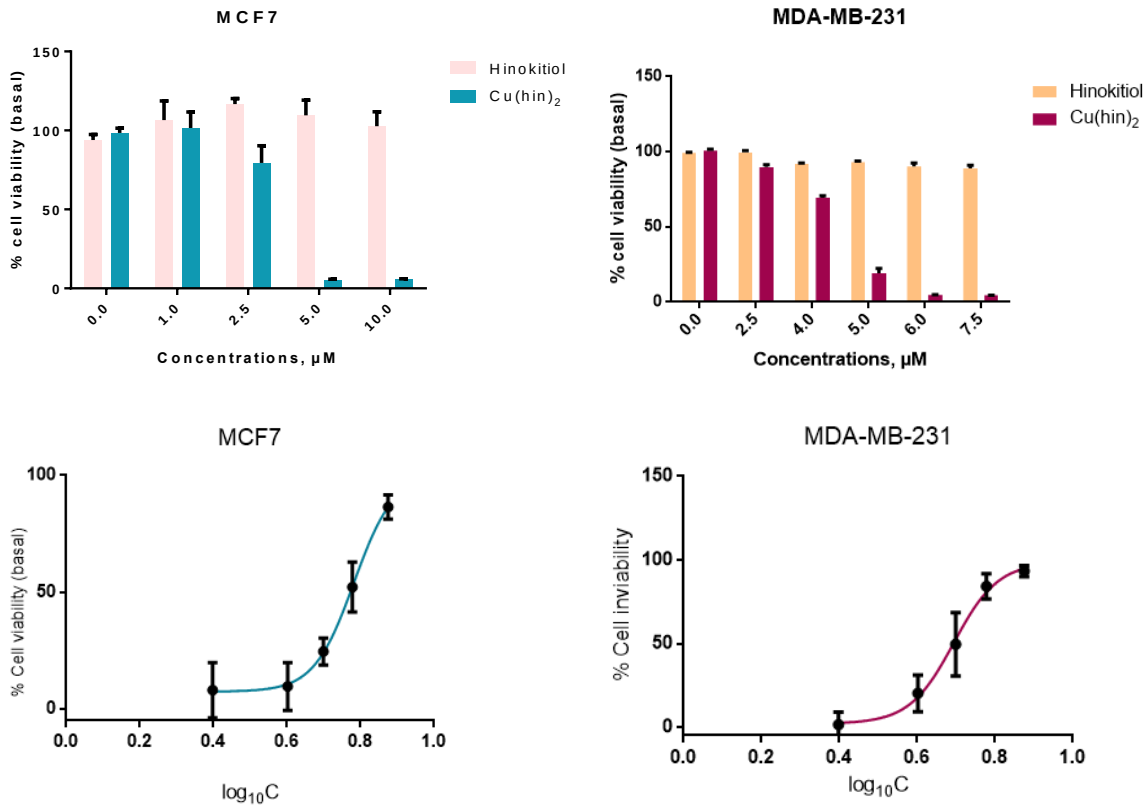



**Figure 3.2. The effect of Hinokitiol and Copper-hinokitiol complexes on the MCF7 (left) and MDA-MB-231(right) cell lines after 48h treatment. The results are expressed as the percentage of the basal and represent the values of mean ± SD. *(p = 0.0003); **(p <0.0001) significant with respect to Basal.**

The obtained results indicate the significant deleterious effect on both of the cell lines, compared to the data from the interaction of other compounds on the same cell line. The concentrations of approximate 6.05±0.48 µM for MCF7 and 4.94±0.26 µM of $Cu(hin)_2$ for MDA-MB-231 in both breast cancer lines have shown a shift in values of absorption and caused 50% of cell death, which could lead to the response of cells in the presence of the compounds. Consequently, MTT assay brought the attention to the $Cu(trp)_2$ and $Cu(hin)_2$ complexes with their potentially damaging effects on the breast cancer cell lines and the following assays were performed exclusively in the presence of complexes. Therefore, it was possible to obtain the approximate value of IC50, which is presented in Table 3.1.

As can be seen from Figure 3.1 and Table 3.1, the copper complexes $Cu(trp)_2$ and $Cu(hin)_2$ show higher effect on the viability of cells, compared to its ligands and metal cytotoxicity. Additionally, the dose-response level of the complexes is lower than their components, consequently, it could be stated that complexes have more antitumor effects than their substituents.

| Cell Lines | $Cu(hin)_2$ | $Cu(trp)_2$[2] | Hinokitiol | Tropolone | $Cu^{2+}$ |
|---|---|---|---|---|---|
| **MCF7** | 6.05±0.48 | 5.2±1.8 | >100 | >100 | >100 |
| **MDA-MB-231** | 4.94±0.26 | 4.0±0.2 | >100 | >100 | >100 |

**Table 3.1. The values of IC50, measured in μM and obtained after 48h of treatment in the presence of the Copper-hinokitiol complex. The results for complexes of the observation are the mean ± SD of independently performed experiments.**

For the purpose of proving the antitumor effect of observed copper complexes, it was taken studied values of commercially available drug and also metal-based compounds, Cisplatin and also common drugs, using against breast cancer tumors, Tamoxifen and Doxorubicin. Taking into consideration the viability effect and making a comparison to other anticancer drugs, Cisplatin in both MCF7[67] and MDA-MB-231[68] cell lines shows lower activity in terms of its concentration and IC50 on both cell lines. Also, coming up to Tamoxifen, the activity of observed complexes is higher and for Doxorubicin is lower, which is described in Table 3.2.[69] It can also be seen that the complexes have concentration-dependent effects for both cell lines.

| Cell Lines | $Cu(hin)_2$ | $Cu(trp)_2$[3] | Cisplatin | Doxorubicin | Tamoxifen |
|---|---|---|---|---|---|
| **MCF7** | 6.05±0,48 | 5.2±1.8 | 18.27 | 0.41±0.75 | 24.76±0.80 |
| **MDA-MB-231** | 4.94±0,26 | 4.0±0.2 | 25.28 | 0.60±0.36 | 25.84±1.60 |

**Table 3.2. The values of IC50, measured in μM and obtained after 48h of treatment of MCF7 and MDA-MB-231 cell lines. The values represent effectiveness in a dose-response assessment for the Copper-hinokitiol and Copper-tropolone complexes versus commercial**

[2] This IC50 value was obtained by Lucia Santa Maria de la Parra and kindly provided by Prof. Ignacio E. León
[3] This IC50 value was obtained by Lucia Santa Maria de la Parra and kindly provided by Prof. Ignacio E. León

**Cisplatin, Doxorubicin and Tamoxifen drugs. The results for complexes of the observation are the mean ± SD of independently performed experiments.**

Later on from here, for the observation and into consideration for further assays would be taking $Cu(trp)_2$ and $Cu(hin)_2$ exclusively, since only they showed anticancer activity against MCF7 and MDA-MB-231 cell lines.

#### 3.1.2.1.2. Cell Migration

As far as some of the breast cancer cell lines demonstrated migration properties to occur the metastasis in other tissues and organs in the body depending on its PR factor, the migration ability in the presence of the drug should be also considered.[70] To examine the anti-migration properties of complexes, a wound-healing assay was performed on the cell lines MDA-MB-231 and MCF7, even if the first is highly metastatic and more migrational, compared to MCF7.[71] In order to evaluate the $Cu(trp)_2$ and $Cu(hin)_2$ influence on cell migration, the Wound Healing assay was applied. The healing was performed by using the pipette tip to scratch the confluent monolayer of cells, in order to create a gap. According to the obtained results and as it is shown in Figure 3.4, complexes have an inhibitory effect on a cell migration, in comparison to the control, in MCF7 cells in a concentration of 5 μM, and in MDA-MB-231 cells already in a concentration of 5 μM the same as for $Cu(hin)_2$. Both of the values are close respectively to the estimated IC50 value, estimated by the MTT study. At the same time, increasing the concentration of the compounds will lead to a more significant effect on the migration activity of the cell lines. Figure 3.3 shows the comparative analysis in cell migration before and after 48h treatment with $Cu(hin)_2$.

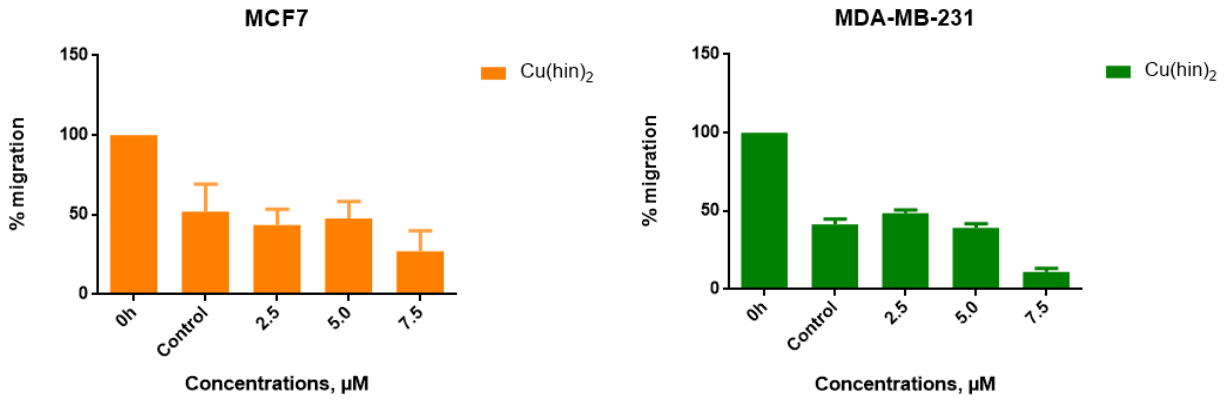


**Figure 3.3. The effect of Cu(hin)$_2$ on migrating properties of MCF7 and MDA-MB-231 cells. Results were obtained are measured in pixels an of healing before (0h) and after 48 h treatment and expressed in percentage of area, in a respect to control (0h) ± SD. *(p = 0.0003); **(p <0.0001) significant with respect to Basal.**

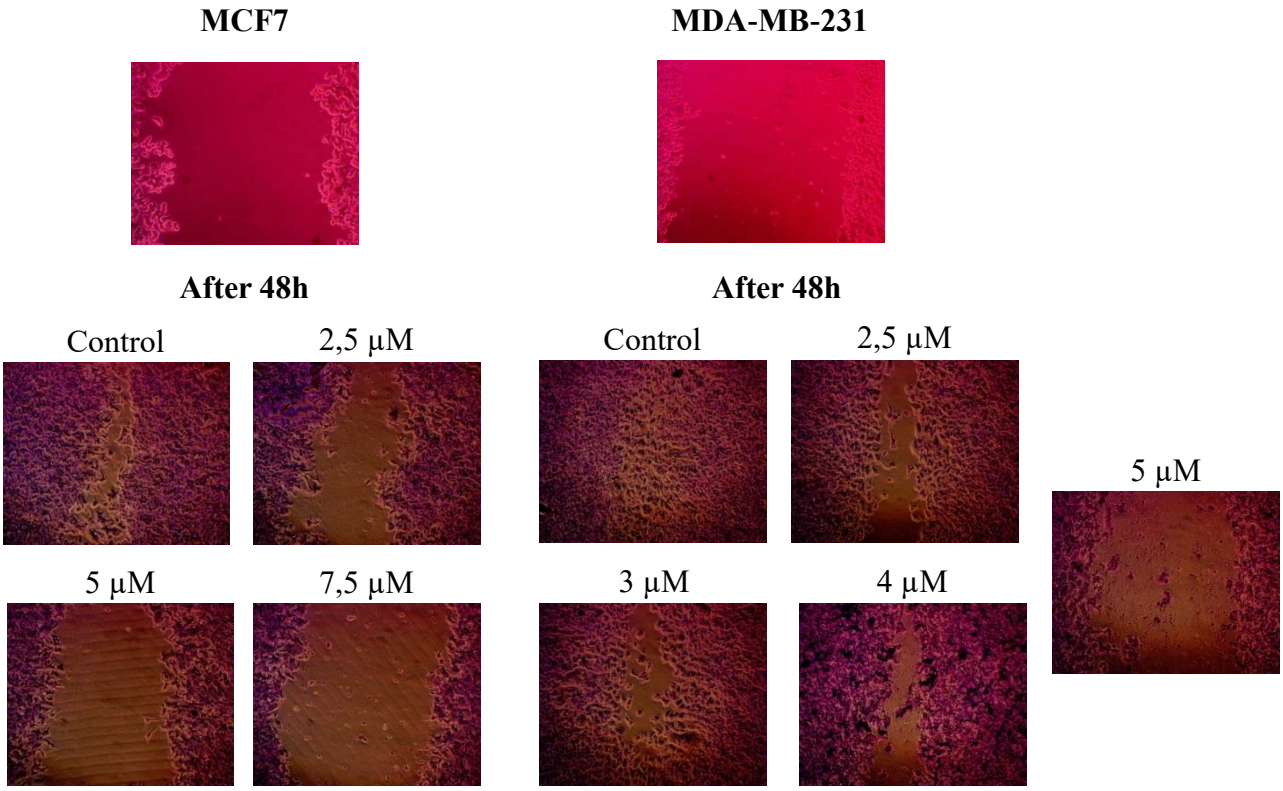



**Figure 3.4. Microscope photos of the cell monolayers and scratches, done before introducing the drug (above) and the same areas after 48h of treatment (below) with different concentrations of Cu(hin)$_2$ and a control.**

#### 3.1.2.1.3. Mechanism of Action

The above-mentioned obtained results represent the influence on both of the breast cancer cell lines. Anticancer drugs are usually targeted to suppress the multiplication and vital ability of cancer cells. Together with this, recognition and differentiation by the drug between abnormal cancer cells and normal cells is also an ongoing problem. For the purpose of determining the exact mechanism of influence of a drug on the MCF7 and MDA-MB-231 cell lines, the anticancer effect of the Cu(hin)$_2$ compound was observed. The following assays were carried out:

the detection and measurement of oxidative stress through reactive oxygen species (ROS), the interaction with calf thymus DNA (ctDNA), and the apoptosis study.

#### 3.1.2.1.3.1. Oxidative Stress. Determination of Reactive Oxygen Species (ROS Production)

Among different possible types of cellular damage, oxidative stress takes one of the main roles in cell damage. ROS can interact with the surface and intracellular receptors, modulate signaling pathways and disrupt physiological mechanisms related to proliferation, apoptosis, angiogenesis, and others.[54] To determine the presence and influence of the drug on the cell, the method of using dihydrorodamine123 (DHR123) was implemented. The valuation was performed in the MCF7 cell line in the presence of $Cu(hin)_2$. The results, presented in Figure 3.5, show no significant shift of the level of absorbance in tested samples with different concentrations. Therefore, it could be stated that in a presence of the complex that there is no significant effect and contribution on a ROS production level with respect to the basal.

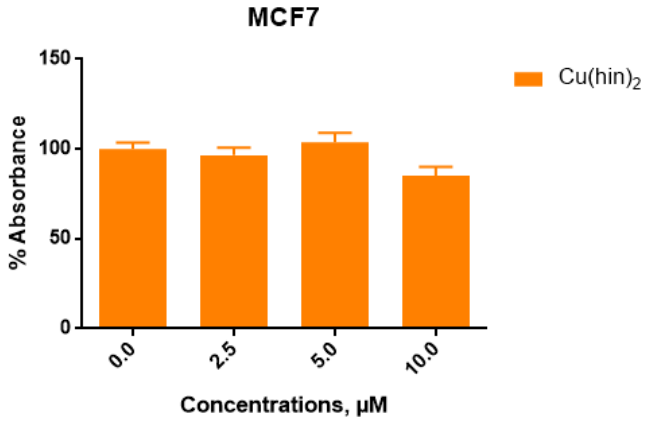



**Figure 3.5. ROS production in the presence of increasing concentrations of $Cu(hin)_2$ and Cu(trp)2 of the MCF7 and MDA-MB-231 cell lines. The generation of ROS of the cells was measured by the absorption of oxidized DHR123 (dihydrorhodamine123) into rhodamine123. The conversion of DHR123 and the changes of the absorption indicate the presence of ROS in the cells. The values represent the mean ± SD and expressed as percentages with respect to the Basal level.**

#### 3.1.2.1.3.2. Calf-Thymus DNA Interaction (ctDNA)

DNA (deoxyribonucleic acid) is the main target for many classes of drugs and for this reason the interaction between drugs and DNA is the most important and can provide fruitful information for effectively targeting therapeutic drugs for controlling gene expression.[72] In current drug–DNA binding research, few models of DNA are accepted as model DNA, from this calf thymus DNA is one of the model DNA mostly studied for the drug binding mechanism. In this work, different interaction mechanisms were studied and among them is interaction with calf-thymus DNA by using different spectroscopic techniques together with computational techniques.

##### 3.1.2.1.3.2.1. Fluorescence studies

In general, the DNA molecule has less fluorescence intensity to improve the fluorescence nature of ctDNA, thus the ethidium bromide (EB) probe is used here, as it is widely used as a fluorescent marker in DNA intercalation studies. In case the compound intercalates into ctDNA then it has to compete with EB dye for the intercalation region of ctDNA, and then ctDNA–EB system fluorescence intensity will be reduced. Figure 3.7 shows the EB–ctDNA and various concentrations of the complexes with EB–ctDNA complex. Fluorescence spectroscopy was performed twice, firstly using the range of concentrations from 1 μM to 50 μM and secondly, from 0.25 μM to 10 μM. After obtaining the results of the first measurement, which shows the response of ctDNA in a presence of complexes, it was decided to investigate the possible response of ctDNA in lower concentrations for each of the complexes. In the second measurement, the spectrometry of different concentrations with ctDNA compared to the control, was not accurate and did not demonstrate the correspondence between the shift of the graph and used concentration. Moreover, using such low concentrations is hard to achieve and operate with. After the addition of various concentrations of the $Cu(trp)_2$ and $Cu(hin)_2$ drugs, the fluorescence intensities of EB–ctDNA systems were gradually decreased. This quenching result shows the shift and change in the fluorescence intensity during the measurement of the wavelength. It characterizes the shift of the graphs for the corresponding concentrations. However, it was not observable the evidence of relation between the changes and shift to the down of the graphs and concentrations of the drug was not observed. But even with that, the shift to the baseline is

characteristic and shows the concrete interaction mechanism with ctDNA and both of the complexes.

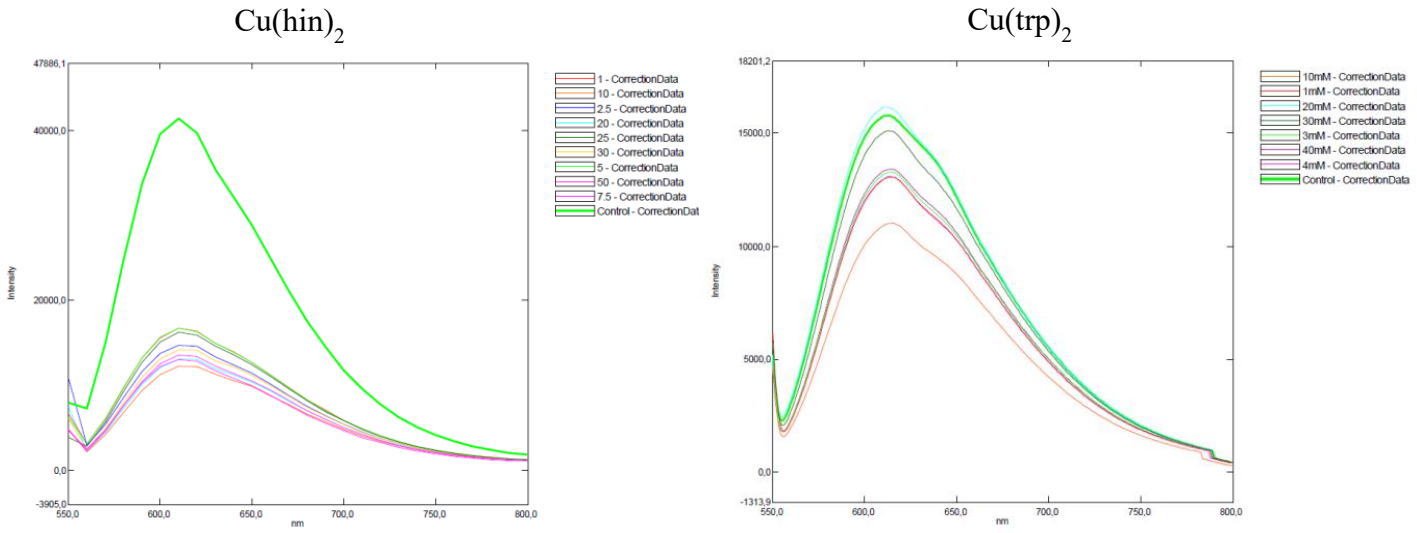



**Figure 3.6. The fluorescence, measured in a presence ctDNA, sodium chloride in water as a solvent and ethidium bromide (EB) with the various concentrations of the complexes. The data is presented corresponding with intensity to nm of the measured wavelength in the presence of Cu(hin)2 (left) and Cu(trp)2 (right). The control sample is measured as a blank sample without the presence of drugs. The shift of wavelength down and closer to the baseline characterizes the presence of the interaction into ctDNA in the presence of both of the complexes.**

#### 3.1.2.1.3.2.2. UV-vis studies

UV–vis spectroscopy is an effective method to observe the binding mode of DNA with metal complexes.[73] Thus, in order to provide information for the possibility of binding of complex to ctDNA, the absorption spectra of each of the complexes were measured in the range of 190–600 nm about 10 min after the addition of DNA solution in room temperature. UV–vis absorbance spectrums were recorded in two ways in order to observe the influence on the DNA by using the constant concentration of ctDNA. In the first case, using the constant concentration of the $Cu(trp)_2$, and $Cu(hin)_2$ in the second case. For the first case, as a control baseline, it was taken an aqueous solution of sodium chloride in 0.9% (w/w) concentration and, separately it was taken the measurements of the control as a mixture of ctDNA solution in a concentration of

104.24 μM and aqueous solution of sodium chloride in 0.9% (w/w) concentration. For the second case, as a control baseline, it was taken an aqueous solution of sodium chloride in 0.9% (w/w) concentration and, separately it was taken the measurements of the control as a mixture of each of complexes in a concentration of 10 μM together with aqueous solution of sodium chloride in 0.9% (w/w) concentration. The possible influence of the solvent on the ctDNA was not taken into consideration.

In both cases, different types of shifts of graphs were detected, compared to the control baseline. The graphs represent the comparison of the action of both of the complexes on ctDNA for the ctDNA solution in a constant concentration as shown in the Figure 3.7 and Figure 3.8 the graphs of comparison for the interaction with ctDNA with constant concentrations of $Cu(trp)_2$ and $Cu(hin)_2$ are shown in Figure 3.9 and Figure 3.10.

As can be seen from Figure 3.7, the shifts of the graph, comparing to the control line, occurred. In the minimum observed concentration of 2.5 μM, the higher influence on this level of concentration was made by $Cu(hin)_2$, compared to the same concentration interaction of $Cu(trp)_2$. The spectrum for free ctDNA shows a characteristic DNA absorption peak at the wavelength of 260 nm and a different absorbance in the presence of $Cu(trp)_2$ and $Cu(hin)_2$ with absorption peaks at the same wavelength are explained by the changes in a ctDNA structure, which could be characterized as an interaction. Shifts of the absorbance lines specify the possible interaction in a ctDNA-complex system and are presented in Annex 1.

The results demonstrated that spectra of measurement in the presence of a constant concentration of ctDNA and sodium chloride as a control solution were more intensive, compared with the absorbance of the free ctDNA. Moreover, there are blue hyperchromic shifts in the wavelength of the ctDNA of the DNA-complex mixture, proposed as a strong interaction of the complexes with ctDNA that takes place by stacking binding with DNA.[73]

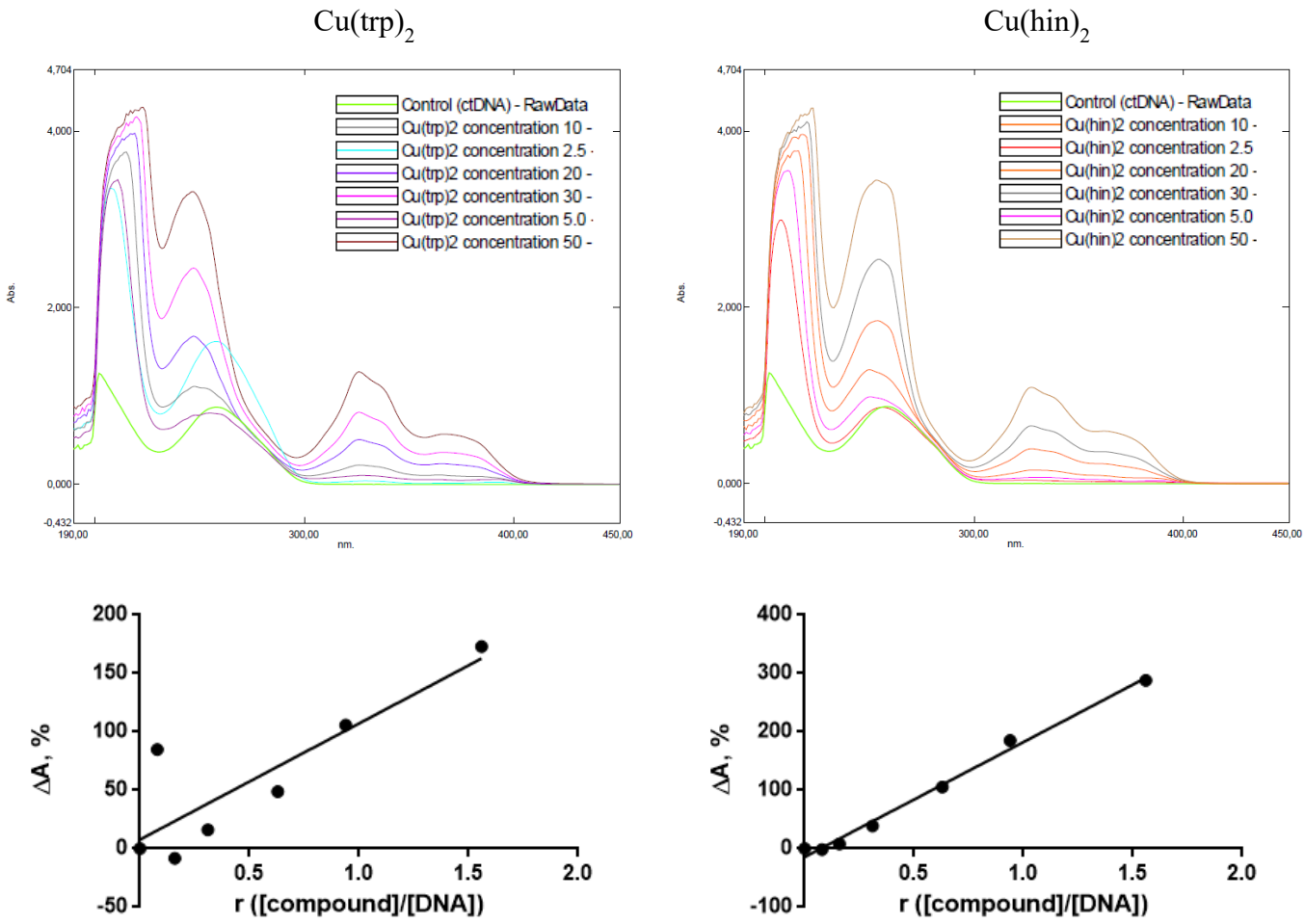



**Figure 3.7. The UV-vis absorption graphs of ctDNA interaction with drugs of interest. As a control baseline, marked as the green line, was a sodium chloride aqueous solution (0,9% w/w). The absorbance spectra is shown in a and b for $Cu(trp)_2$ and $Cu(hin)_2$ respectively. The measurement was taken in a constant concentration of ctDNA.**

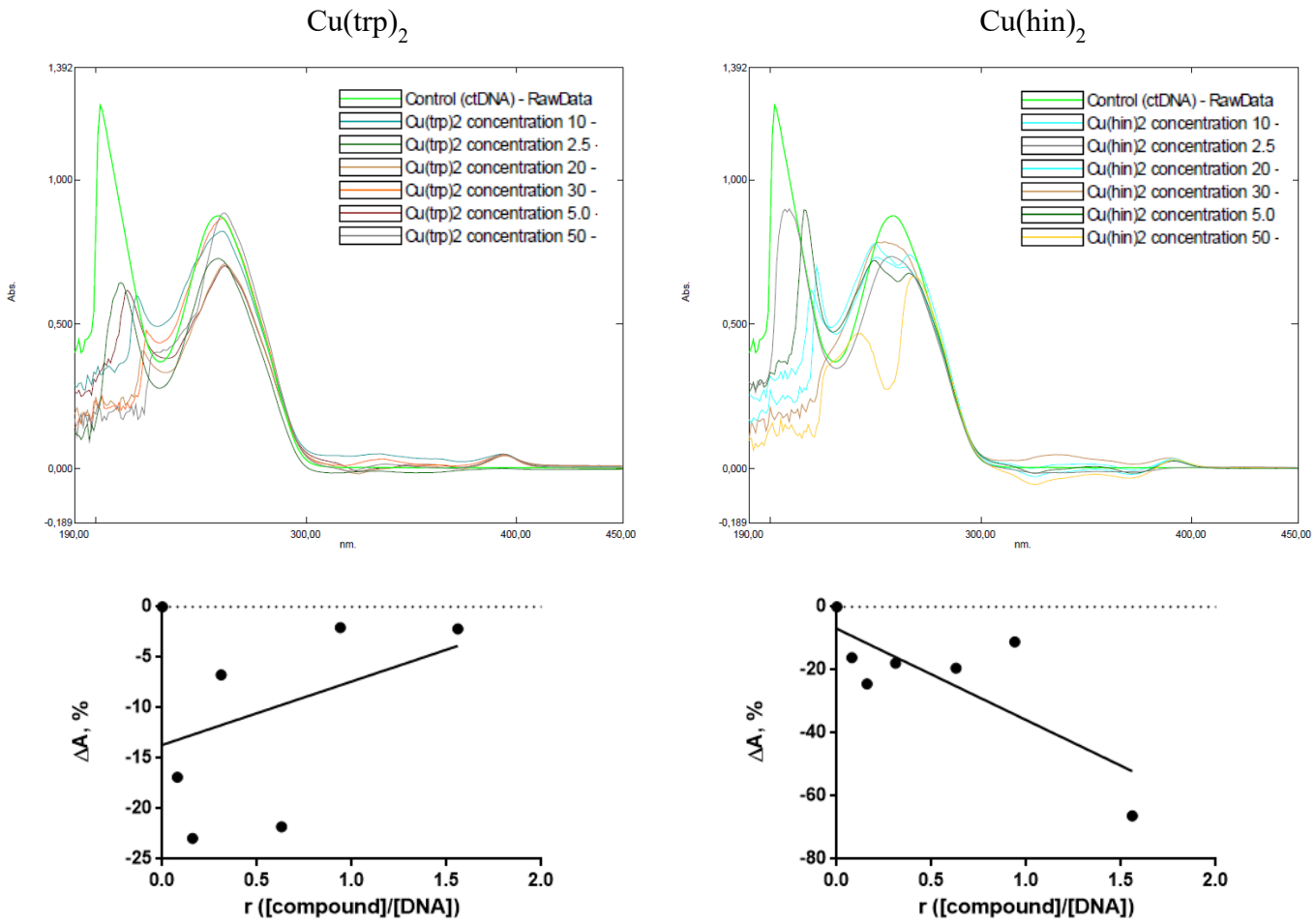
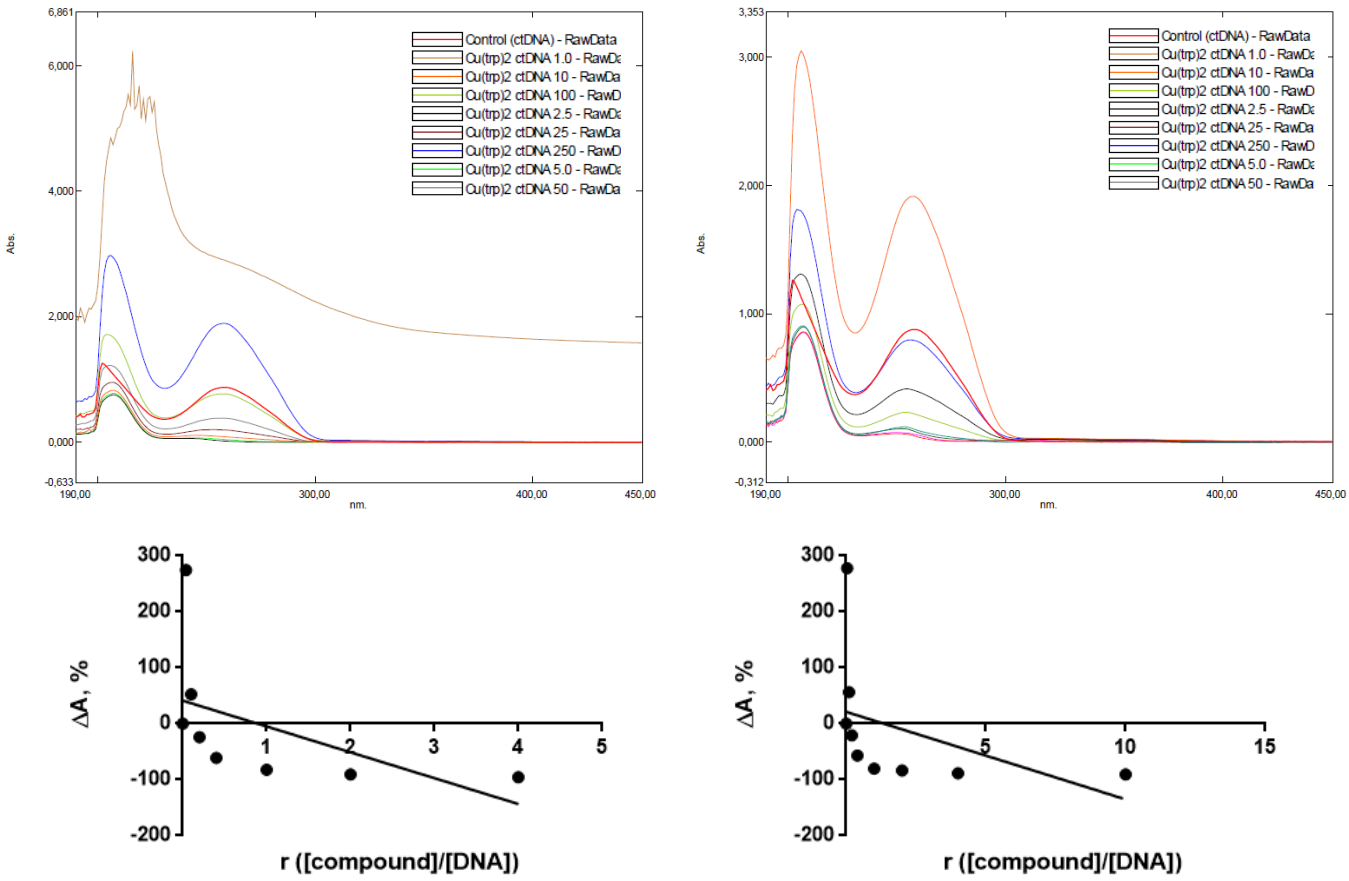



**Figure 3.8. The UV-vis absorption graphs of ctDNA interaction with drugs of interest. As a control baseline, marked as the green line, was sodium chloride aqueous solution (0,9% w/w) together with ctDNA solution. The absorbance spectra is shown in c and d for $Cu(trp)_2$ and $Cu(hin)_2$ respectively. The measurement was taken in a constant concentration of ctDNA.**

$Cu(trp)_2$ $Cu(hin)_2$



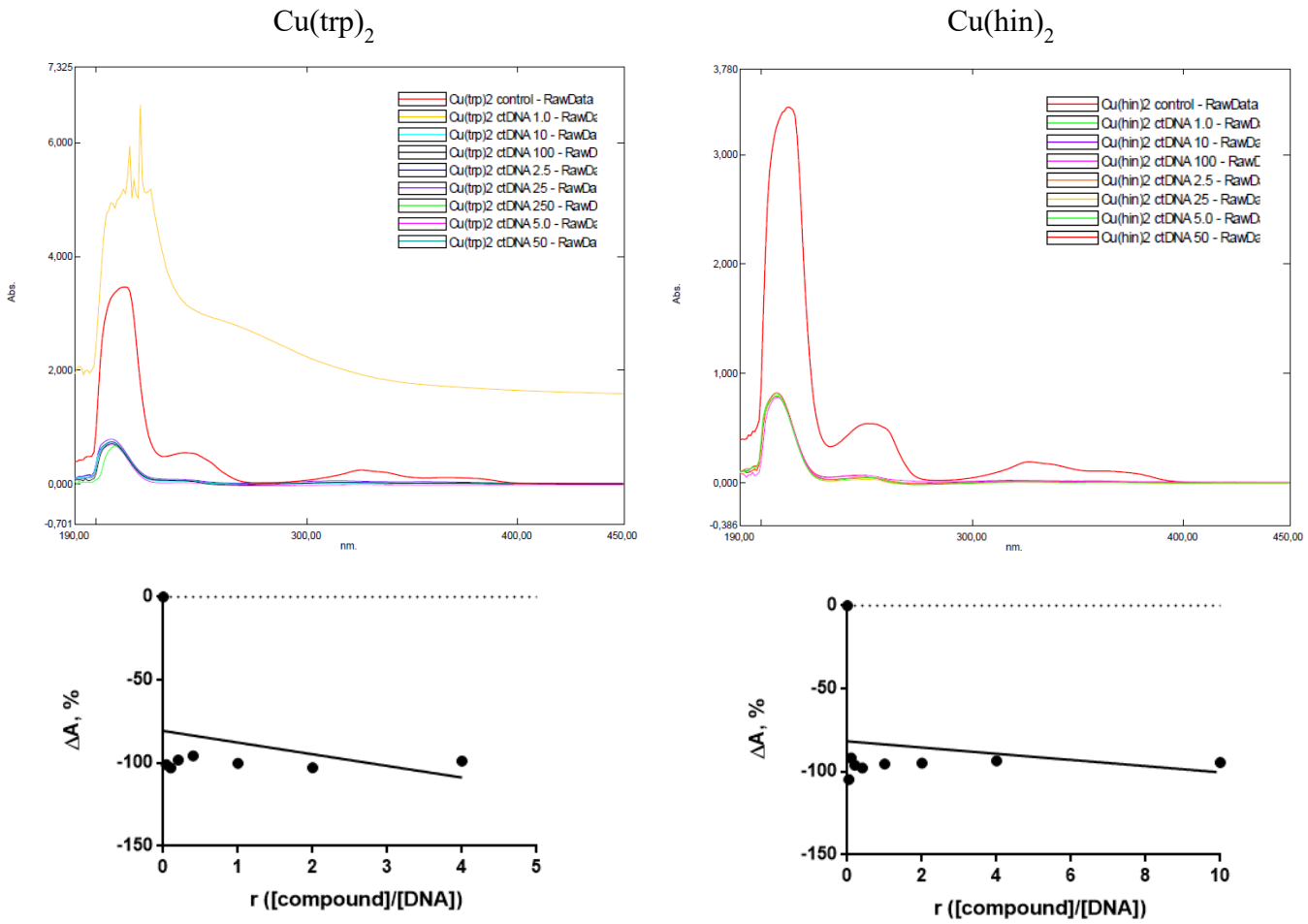

$y = (-46{,}22) \times x + 41{,}48$

**e**

$y = (-15{,}64) \times x + 21{,}33$

**f**

**Figure 3.9. The UV-vis absorption graphs of ctDNA interaction with drugs of interest. As a control baseline, marked as the red line, was a sodium chloride aqueous solution (0,9% w/w). The absorbance spectra is shown in e and f for $Cu(trp)_2$ and $Cu(hin)_2$ respectively. The measurement was taken in a constant concentration of complexes.**




$y = (-7{,}022) \times x$

**g**

$y = (-1{,}867) \times x - 81{,}67$

**h**

**Figure 3.10. The UV-vis absorption graphs of ctDNA interaction with drugs of interest. As a control baseline, marked as the red line, was sodium chloride aqueous solution (0,9% w/w) together with each of the complexes. The absorbance spectra is shown in g and h for $Cu(trp)_2$ and $Cu(hin)_2$ respectively. The measurement was taken in a constant concentration of complexes.**

#### 3.1.2.1.3.3. Apoptosis Study

The analysis of the ability to induce and promote apoptosis in the presence of $Cu(hin)_2$ compound on breast cancer cell line, MCF7, was evaluated through the detection of the binding of phosphatidylserine (PS) with Annexin V by flow cytometer.

Annexin V is a recombinant protein, which strongly interacts and binds with high affinity and specifically to phosphatidylserine residues, but not to other phospholipids, and can be used for the detection of apoptosis. In the presence of calcium, Annexin V is able to bind specifically to PS, which when exposed to the outer layer of the membrane surface, causes the early apoptosis event.[74,75] In cells the distribution of phospholipids in the plasma membrane is asymmetric, the inner membrane contains anionic phospholipids as PS and the outer one mainly neutral phospholipids. In an apoptotic cell, the rapid translocation of PS towards the outer face of the membrane occurs early, which causes the loss of asymmetry that exists under normal conditions. This process can be detected using the binding properties of Annexin V to the PS.[76]

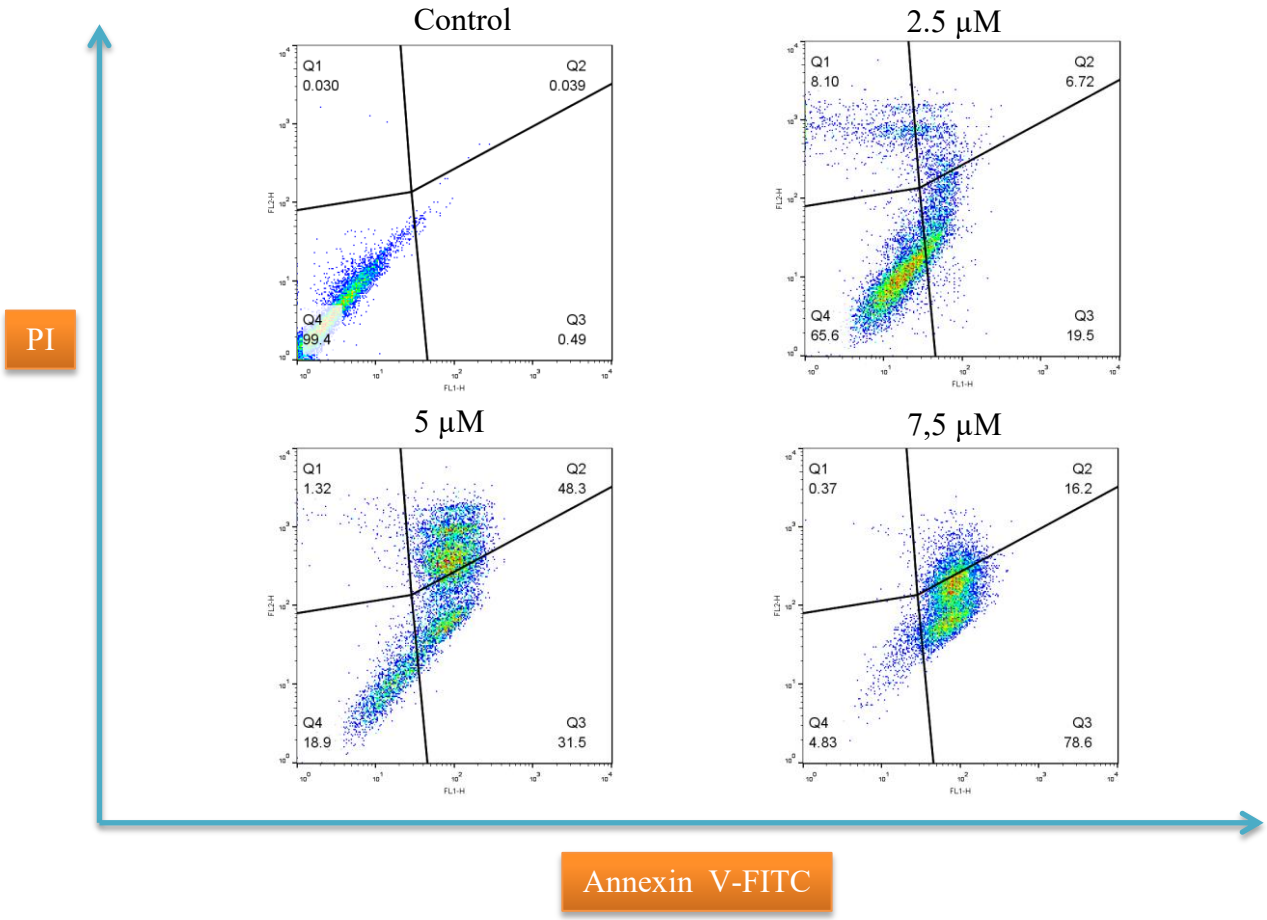



**Figure 3.11. Effect of Cu(hin)$_2$ on cell apoptosis in MCF7 cell line, determined by flow cytometer with Annexin V-FITC/Propidium Iodide (PI) staining. The 48 h incubation after treatment with different concentrations of Cu(hin)$_2$ was performed and analyzed by flow cytometer afterward.**

The cell population was analyzed by flow cytometer based on four following groups:

- viable cells (Q4);
- early apoptotic cells (Q3);
- late apoptotic cells (Q2);
- necrotic cells (Q1).

The number of cells, which have not received treatment at fills the surface of the Q4 quadrant are considered alive or viable cells. The cells that stain only for Annexin V and fill the Q3 quadrant are considered early apoptotic. The cells, which present double labeling for Annexin

V and PI and fill the quadrant of Q2 detected as late apoptotic cells. In the end, the cells that marked only with PI and are situated in a Q1 quadrant are considered as necrotic.

In the presence of increasing concentrations of $Cu(hin)_2$ the behavior of cells could be determined as early apoptotic in the concentration of 2.5 µM of complex to late apoptotic when the concentration of drug increased to 7.5 µM.

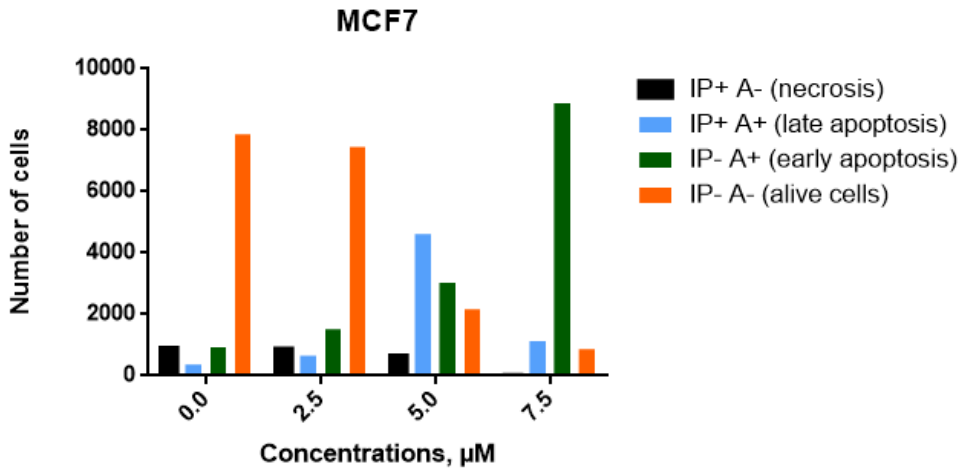



**Figure 3.12. The representation of the amount of early, late apoptotic and necrotic MCF7 cells, respectively in the presence of $Cu(hin)_2$ compared to control. The measurement was performed after 48h treatment with various concentrations of 2.5 µM, 5 µM and 7.5 µM of complex. The values represent the mean ± SD and are expressed as a number with respect to the Basal level.**

As it can be seen in Figure 3.11 and Figure 3.12, there is significant evidence in increased amounts of early apoptosis, based on the influence of the compound in cells, incubated with 7.5 µM concentration of $Cu(hin)_2$. On the other hand, a significant difference was observed in the treatment with 5 µM, where the population of early and late apoptotic cells increased.

In summary, the results obtained in this assay for the MCF7 cell line would indicate that the cytotoxic effect of the $Cu(hin)_2$ complex involves the induction of apoptosis in the line studied.

#### 3.1.2.2. 3D Cell Systems

For the drug development process, the test of response mammalian cancer cells to anticancer drugs must be performed, the *in vitro* and *in vivo* studies in its accordance. While the first one is a cost-effective and easy model for trials, the second one gives rise to difficulties in usage. Among the limitations of using animal testing, it also raises also ethical problems, which have recently gained more attention.

The IC50 values for such anticancer drugs, as Tamoxifen and Doxorubicin, significantly differ from the concentration of IC50, used in monolayer studies and concentration, used for the 3D MTS model. The fold change in IC50 concentrations between 2D-monolayer and 3D-model for Tamoxifen has been estimated to be in 22.9 times higher and for Doxorubicin in 21.2 times higher.[77] These numbers represent that fact that drug efficacy in its development is lower in using a 3D-dimensional multicellular model, compared to monolayer cell spreading, suggesting it occurs by drug distribution into cells and efficiency. Furthermore, the architecture of the 3D models is more complex, which complicates drug delivery. Together with this, different types of cell cultures have different properties, various shape structure and response on a chemical.[78]

As soon as promising *in vitro* results for a monolayer of MCF7 and MDA-MB-231 cell lines were obtained for $Cu(hin)_2$ and $Cu(trp)_2$, the formation and its viability of tumor-like 3D cellular model was taken under investigation. Formation of 3D structure of conglomerated cells into a spheroid-like form are clearly more relevant and could be considered as a practical alternative or reliable predictor to *in vivo* studies for drug efficacy and toxicity. This method requires the ability of cells to conjugate and make a conglomerate into a tumor spheroid, which more fully mimics the environment and the microenvironment tumor, comparing to the monolayer.[79] Thus, the MCF7 cell line was used for the production of cellular spheroid formations by the hanging-drop method and was incubated for 10-12 days, until the desirable size of spheroid was reached, using the liquid overlay technique for its growth and formation. In order to allow cells to grow and form the spheroid of the applicable size, the incubation of spheroids was carried out until they reach a diameter of approximately 500 μm. The Figure 3.13 shows the process of combined methods in order to reach cell spheroids reach a desirable size.

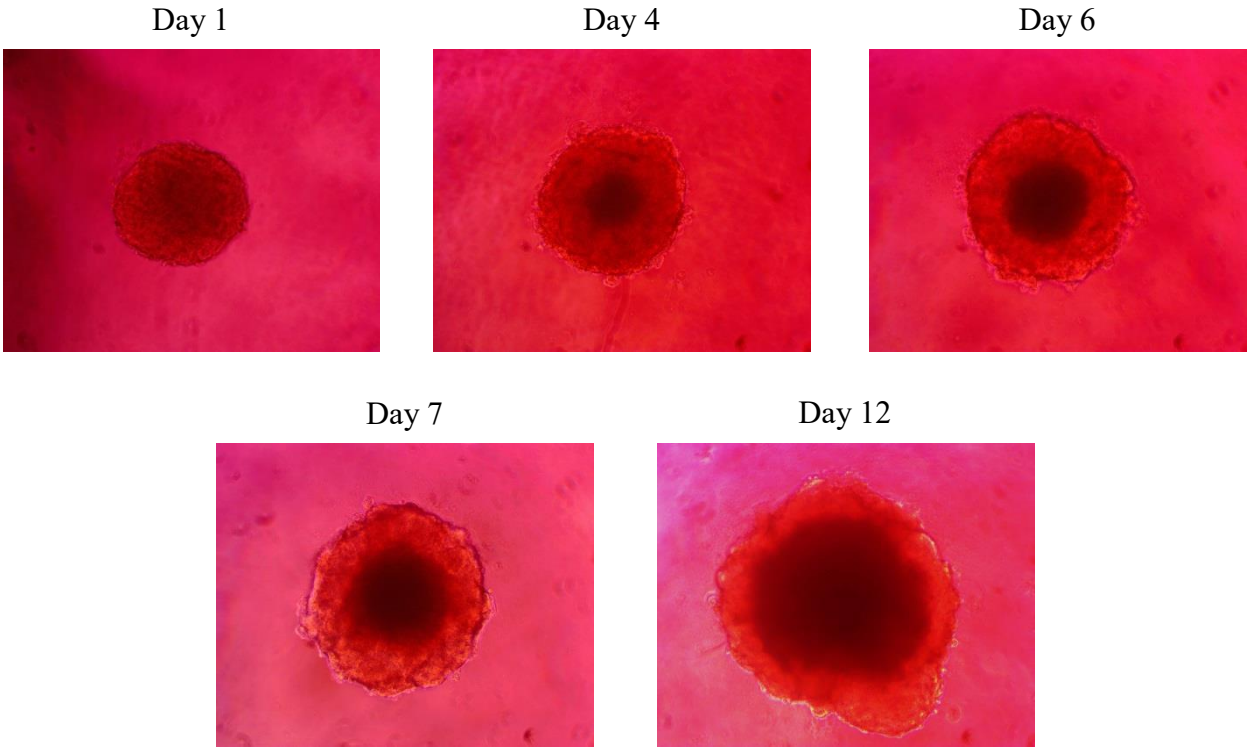



**Figure 3.13. Microscope photos of MCF7 cellular spheroids growth in DMEM medium with FBS over 12 days.**

#### 3.1.2.2.1. Cell viability of spheroids

To estimate of the effect of $Cu(trp)_2$ and Cu(hin)2 on viability in the MCF7 spheroids, it was applied the method of using resazurin, which is based on the irreversible reduction of blue and weakly fluorescent resazurin into pink(red) and strongly fluorescent resorufin. This cytotoxic method is functionally similar to the MTT assay and, at the same time, includes fluorescence detection, which makes it more sensitive. Together with this, resazurin is used in monitoring cell proliferation, since this compound is not toxic to cells.[60] The conversion of resazurin to resorufin is taking place out in mitochondria of alive cells and through the measurement of fluorescence, the detection and proportion of viable cells could be determined.

Figure 3.14 shows the results, obtained on MCF7 spheroids viability, incubated and grown for 12 days, after 48h treatment with $Cu(trp)_2$ and $Cu(hin)_2$. The cell integrity is visibly

damaged already in the presence of 2.5 μM of $Cu(trp)_2$ and 5μM of $Cu(hin)_2$, at the same time the necrotic centers of spheroids occurred with the treatment of 10μM for both of the compounds.

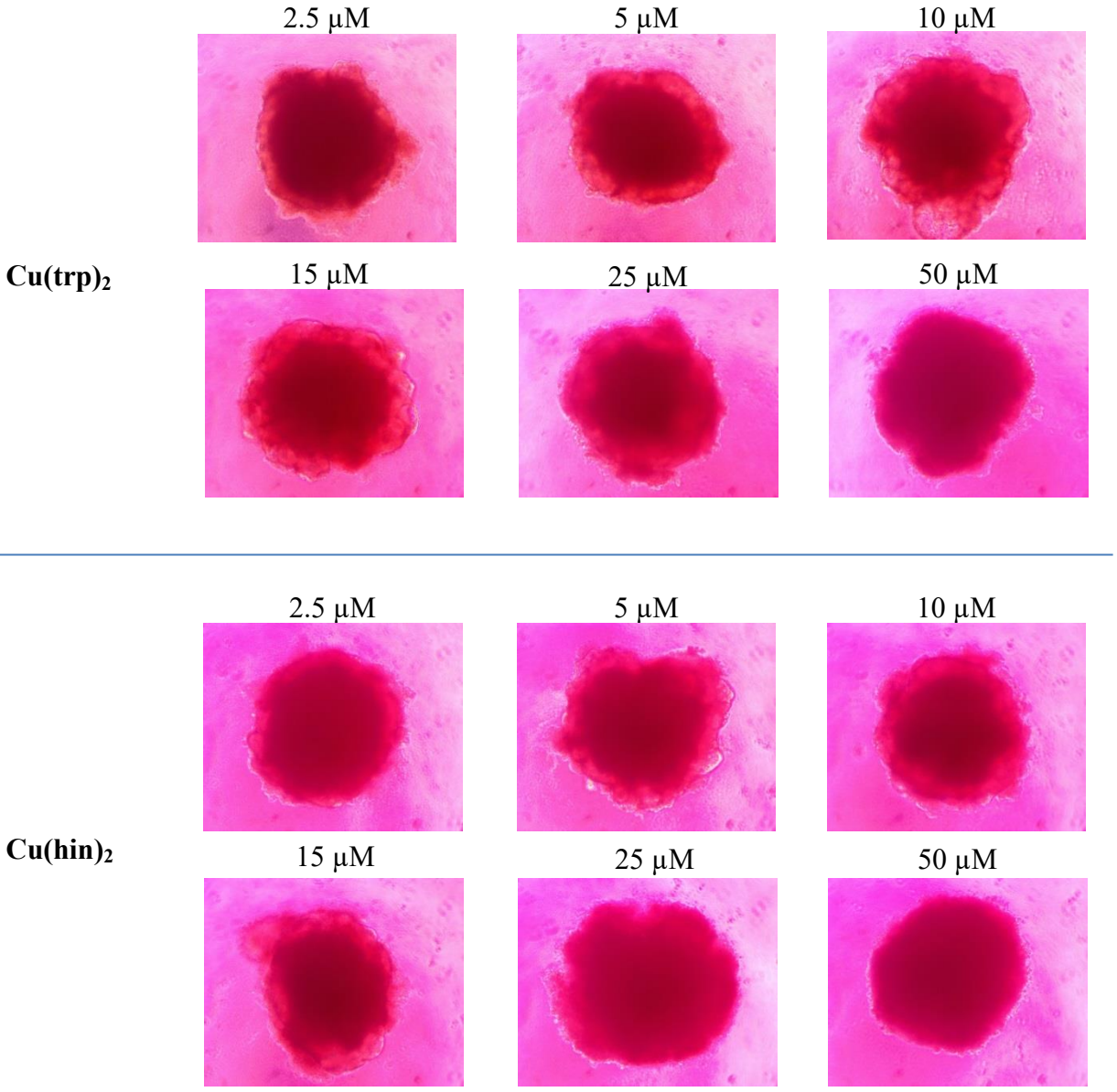



**Figure 3.14. The microscope photos of MCF7 cellular spheroids in DMEM medium after 48h treatment of $Cu(trp)_2$ (higher) and $Cu(hin)_2$ (lower).**

The obtained photos indicate the significant effect on cell spheroids after 48h of treatment for both complexes. Figure 3.15 represents the measurement of absorbance and the influence of different concentrations of both $Cu(trp)_2$ and $Cu(hin)_2$ on MCF7 spheroids.

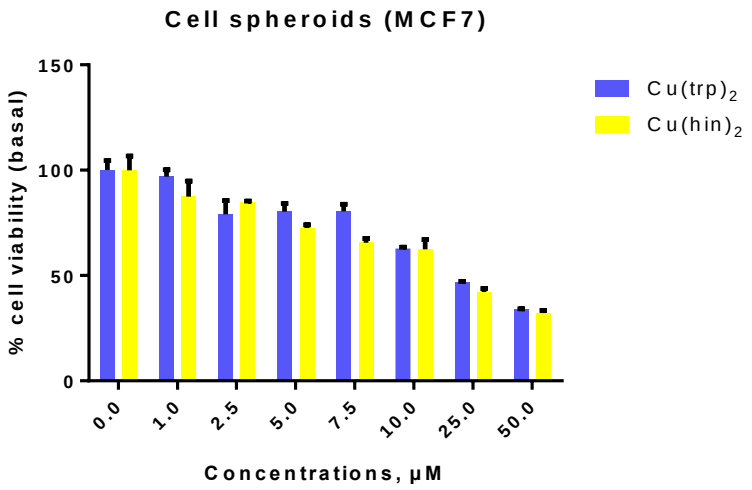



**Figure 3.15. The effect of $Cu(trp)_2$ and $Cu(hin)_2$ complexes on the MCF7 cell spheroids after 48h treatment. The results are expressed as the percentage of the basal and represent the values of mean ± SD. *(p = 0.0003); **(p <0.0001) significant with respect to Basal.**

## 4. Conclusions and Discussions

Over the last decades and nowadays cancer has become an increasingly significant and ongoing problem, which continually gains the attention of every generation, which is related to lifestyle, genetics, and environmental pollution. Existing drugs for chemotherapy, which are meant to be used, have been losing efficacy due to the acquisition of drug resistance by several types of cancer. Along with that, side effects, diseases, and mortality, caused by chemotherapy drugs are also a matter of concern of patients. For the last several decades, research and development of chemotherapy has focused on stopping or slowing the growth of cancer cells, leading to a significant increase in the survival rate of breast cancer patients, but has also lead to a significant increase in the variety and intensity of side effects for those patients.

The statistics data on breast cancer patients, according to WHO, shows that annually 2.08 million people are affected by this disease and most of them are women. Early diagnosis, right and targeted treatment become key points in surmounting in any stages of breast cancer. However, despite the successful treatment, the appearance of metastasis and remission are experienced by patients. Treatment strategy is a complex solution for cancer patients, which includes not only patient data and prognosis but also information about cancer itself: type, spreading, cell line, etc. Together with that, chemotherapy assumed as a mainly targeted type of therapy, which among others focused not only on suppressing and killing existing cancer cells but also on prevention of cancer migration and occurring metastases in other parts of the body. Nevertheless, by years the number of cancer cases, new breast cancer subtypes findings, and resistance to existing therapeutic drugs demands new diagnostic and treatment drugs.

The studied cell lines were taken under observation, due to their being an established model for breast adenocarcinoma in patients. The tests were done under 48 hours of a treatment since it was shown as the most effective time for drug response. The viability test on monolayers, carried out on both breast cancer lines, with the two compounds under study, demonstrated that only the $Cu(trp)_2$ and $Cu(hin)_2$ complexes have a markedly deleterious effect on the cellular viability of both cell lines within 48 hours of treatment. Compared to its primary compounds and to the commercially available anticancer drugs, such as Cisplatin and Tamoxifen and, these compounds have shown a response in smaller concentrations than their IC50 estimations. $Cu(trp)_2$ and $Cu(hin)_2$ have an inhibitory effect on the migratory capacity in both cell lines,

around the respective IC50, which agrees and correlates with the results observed on cell viability.

After obtaining promising results in cell viability and migratory properties, it was taken into observation the possible mechanisms of action of compounds. The study in ROS production in cell lines under $Cu(trp)_2$ and $Cu(hin)_2$ treatment have not shown any significant influence in value, estimated as IC50. Though, in both cell lines, the induction of apoptosis is used as a mechanism of action. The results correlate with the effect on cytotoxicity and the inhibition of cell migration, observing the induction of apoptosis, late in both lines and early in MDA-MB-231. Moreover, it was estimated the effect on ctDNA, which was confirmed with both fluorescence and UV-vis methods. However, the difference in absorbance, which is the main methods of investigating the influence of the drug on DNA, did not show a correlation between concentrations. At this point, the mechanism of action of these drugs should be studied further.

The MCF7 spheroids of more than 500 μm in diameter were obtained, which were expected to have an apoptotic/necrotic center, an intermediate layer of quiescent cells, and an outer layer of active cells, and thus better represented the microenvironment of a tumor. The spheroids were used as a model for further *in vivo* testing and considered as a model of solid tumor. Finally, the inhibitory effect of the invasive capacity of the cells in the spheroids possessed by $Cu(trp)_2$ and $Cu(hin)_2$ was determined, finding that it has an inhibitory effect at a concentration near 10 μM.

The biological significance of this work may be evident since it proved the cytotoxic effect on both MCF7 and MDA-MB-231 breast cancer cell lines and interaction with the calf thymus DNA, where intercalation and electrostatic effect play a major role in the binding of complex to DNA.

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**Annexes**

**Annex 1. The measurement data of UV-vis absorption in the presence of $Cu(trp)_2$ and $Cu(hin)_2$ on the changes in the interaction to ctDNA**

| Complex | | Control | r=[compound]/[DNA] | Shift of absorption (ΔA), % | | |
|---|---|---|---|---|---|---|
| Cu(hin)2 | [ctDNA] = constant | NaCl aqueous (0,9% w/w) solution | 0,08 | -1,48 | ↓ | Hypochromism |
| | | | 0,16 | 7,99 | ↑ | Hyperchromism |
| | | | 0,31 | 38,93 | ↑ | Hyperchromism |
| | | | 0,63 | 105,71 | ↑ | Hyperchromism |
| | | | 0,94 | 185,05 | ↑ | Hyperchromism |
| | | | 1,56 | 288,24 | ↑ | Hyperchromism |
| | | NaCl aqueous (0,9% w/w) solution; ctDNA | 0,08 | -16,10 | ↓ | Hypochromism |
| | | | 0,16 | -24,43 | ↓ | Hypochromism |
| | | | 0,31 | -17,81 | ↓ | Hypochromism |
| | | | 0,63 | -19,41 | ↓ | Hypochromism |
| | | | 0,94 | -11,07 | ↓ | Hypochromism |
| | | | 1,56 | -66,32 | ↓ | Hypochromism |
| | [complex] = constant | NaCl aqueous (0,9% w/w) solution | 10,00 | -90,71 | ↓ | Hypochromism |
| | | | 4,00 | -88,34 | ↓ | Hypochromism |
| | | | 2,00 | -83,60 | ↓ | Hypochromism |
| | | | 1,00 | -80,24 | ↓ | Hypochromism |
| | | | 0,40 | -56,72 | ↓ | Hypochromism |
| | | | 0,20 | -20,55 | ↓ | Hypochromism |
| | | | 0,10 | 56,32 | ↑ | Hyperchromism |
| | | | 0,04 | 278,26 | ↑ | Hyperchromism |
| | | NaCl aqueous (0,9% w/w) solution; Cu(hin)2 | 10,00 | -94,47 | ↓ | Hypochromism |
| | | | 4,00 | -93,48 | ↓ | Hypochromism |
| | | | 2,00 | -94,86 | ↓ | Hypochromism |
| | | | 1,00 | -95,26 | ↓ | Hypochromism |
| | | | 0,40 | -97,63 | ↓ | Hypochromism |
| | | | 0,20 | -96,05 | ↓ | Hypochromism |
| | | | 0,10 | -91,70 | ↓ | Hypochromism |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | 0,04 | -104,74 | ↓ | Hypochromism |
| | [ctDNA] | NaCl | 0,08 | -1,48 | ↓ | Hypochromism |
| | = constant | aqueous | 0,16 | 7,99 | ↑ | Hyperchromism |
| | | (0,9% | 0,31 | 38,93 | ↑ | Hyperchromism |
| | | w/w) | 0,63 | 105,71 | ↑ | Hyperchromism |
| | | solution | 0,94 | 185,05 | ↑ | Hyperchromism |
| | | | 1,56 | 288,24 | ↑ | Hyperchromism |
| | | NaCl | 0,08 | -16,10 | ↓ | Hypochromism |
| | | aqueous | 0,16 | -24,43 | ↓ | Hypochromism |
| | | (0,9% | 0,31 | -17,81 | ↓ | Hypochromism |
| | | w/w) | 0,63 | -19,41 | ↓ | Hypochromism |
| | | solution; | 0,94 | -11,07 | ↓ | Hypochromism |
| | | ctDNA | 1,56 | -66,32 | ↓ | Hypochromism |
| | [complex] | NaCl | 10,00 | -90,71 | ↓ | Hypochromism |
| **Cu(trp)2** | = constant | aqueous | 4,00 | -88,34 | ↓ | Hypochromism |
| | | (0,9% | 2,00 | -83,60 | ↓ | Hypochromism |
| | | w/w) | 1,00 | -80,24 | ↓ | Hypochromism |
| | | solution | 0,40 | -56,72 | ↓ | Hypochromism |
| | | | 0,20 | -20,55 | ↓ | Hypochromism |
| | | | 0,10 | 56,32 | ↑ | Hyperchromism |
| | | | 0,04 | 278,26 | ↑ | Hyperchromism |
| | | NaCl | 10,00 | -94,47 | ↓ | Hypochromism |
| | | aqueous | 4,00 | -93,48 | ↓ | Hypochromism |
| | | (0,9% | 2,00 | -94,86 | ↓ | Hypochromism |
| | | w/w) | 1,00 | -95,26 | ↓ | Hypochromism |
| | | solution; | 0,40 | -97,63 | ↓ | Hypochromism |
| | | Cu(trp)2 | 0,20 | -96,05 | ↓ | Hypochromism |
| | | | 0,10 | -91,70 | ↓ | Hypochromism |
| | | | 0,04 | -104,74 | ↓ | Hypochromism |

**Annex 2. Molecular profiles of breast carcinomas subtypes**

| Subtype | ER/PR/ HER2/ Ki67 | Basal markers, EGFR | Proliferation cluster | Characteristic genes | Corresponding Cell Lines | Benefit from chemotherapy | Other |
|---|---|---|---|---|---|---|---|
| **Luminal A** | ER+, PR+/-, HER2-, Ki67- | - | Low | ESR1, KRT8, KRT18, GATA3, XBP1, FOXA1, TFF3, CCND1, LIV1 | MCF7, T47D, SUM185 | Low (0 – 5% pCR) | Endocrine responsive |
| **Luminal B** | ER+, PR+/-, HER+/-, Ki67+ | - | High | ESR1, KRT8, KRT18, GATA3, XBP1, FOXA1, TFF3, SQLE, LAPTM4B | BT474, ZR-75 | Intermediate (10 – 20% pCR) | Usually endocrine responsive |
| **HER2** | ER-, PR-, HER+, Ki67+ | +/- | High | ERBB2, GRB7 | SKBR3, MDA-MB-453 | Intermediate (25 – 40% pCR) | |
| **Basal-like** | ER-, PR-, HER2-, Ki67+ | + | High | KRT5, KRT17, CDH3, FABP7, TRIM29, LAMC2, ID4 | MDA-MB-468, SUM190, MDA-MB-231 | High (≥40% pCR) | Endocrine nonresponsive |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Claudin-low** | ER-, PR-, +/- HER2-, Ki67+/- | High | CD44, SNAI3 | BT549, Hs578T, SUM1315 | Intermediate (25 – 40% pCR) | Claudin-3, claudinin-4 and claudinin-7 low |
| **Normal breast-like** | ER+, PR+, -/+ HER2+, Ki67+ | Low/Inter-mediate | PTN, CD36, FABP4, AQP7, ITGA7 | Unspecified | Low (0 – 5% pCR) | |
| **Molecular apocrine** | ER-, PR-, -/+ HER2+/-, Ki67+ | High | AR, FAS, ERBB2, XBP1 | Unspecified | Not examined | Androgen receptor + |

ER – Estrogen receptor, PR – Progesterone receptor, HER2 – Human epidermal receptor 2, EGFR – epidermal growth factor receptor, pCR – pathological complete response after neoadjuvant chemotherapy, + = positive, - = negative