

# **Original Research Article**

## **Effect of Carboplatin and Methotrexate on Lipid Levels in the Plasma**

### **Membrane of MCF-7 Cells and their Association with Cell Motility**

#### **Abstract:**

**Aim:** We examine in this study the effect of two antitumor drugs methotrexate (MTX) and carboplatin (CPT) on the phospholipid content of plasma membrane of MCF-7 breast cancer cells. MTX is a folate analog that inhibit dihydrofolate reductase. CPT is a platinum-containing antineoplastic drug that inter-chelates DNA and inhibits replication.

**Methods:** MCF-7 breast cancer cells were treated with different concentrations of CPT or MTX. Plasma membranes were purified and their protein contents were measured with and without drug treatment. We extracted the lipids from plasma membranes of drug-treated and control MCF-7 cells, quantitated, and separated by HPLC using a C18 reverse-phase column.

The ability of both drugs to affect cell movement was studied using a motility assay.

#### **Results:**

MTX induced 50% cell death at concentrations ranging from 50 to 100  $\mu$ M. No further decrease in viability was seen above this concentration even at 1 mM. CPT induced 50% cell death at 5  $\mu$ M. It also showed a range concentration that gives a 50% cell death after 24 hrs of incubation. Protein levels in plasma membranes of treated cells doubled compared to control. Lipid levels in plasma membranes decrease insignificantly after drug treatment. No changes in separation pattern of lipid extracted from membranes were seen after CPT treatment compared to control; however, MTX treatment showed to a single change in elution pattern in peak at 4.36 min.

Both drugs had little effect on cell motility causing a decrease of 13.3% and 17.4% with CPT and MTX respectively compared to control.

**Conclusions:** Our results show that both drugs did not affect the amount of lipids but lead to doubling in the protein concentration in the plasma membranes of MCF-7 breast cancer cells. These drugs did not lead to a significant decrease in cell motility compared to control.

**Keywords:** Antitumor drugs, plasma membrane, lipids, cell motility.

**Abbreviations:** CPT= carboplatin, MTX= methotrexate, RP-HPLC: reverse phase high pressure liquid chromatography.

#### **Introduction:**

Cancer is the most common malignant disease and a leading cause of death in the world. In the United States and a number of European countries, cancer is the

42 second leading killer after heart disease and strokes (1). In recent years, the  
43 occurrence of cancer has increased rapidly and cancer research became one of the  
44 most intense areas of research.

45 Breast cancer is the leading type of cancer death in women worldwide with  
46 more than one million new cases occurring annually (2). Breast cancer is treated by  
47 surgery and/or combined with other treatments such as radiation therapy, hormone  
48 therapy and chemotherapy (3). Different classes of chemotherapeutic drugs are used  
49 to treat breast cancer. The most common ones are taxanes, anthracyclines,  
50 antimetabolites and alkylating.

51 Antimetabolites drugs (i.e. MTX, Fluorouracil) interfere with the normal metabolic  
52 processes within cells. They interfere with one or more enzymes that are necessary for  
53 DNA synthesis (4). While alkylating drugs (i.e. cisplatin, CPT) bind covalently to  
54 nucleophilic moieties in the cellular DNA forming DNA cross-links that cause  
55 changes in DNA conformation, resulting in disruption of DNA function and cell death  
56 (5).

57 Carboplatin, cis-diamine (1,1-cyclobutanedicarboxylato)platinum(II) (CPT),  
58 commercially available under the name of Paraplatin or Platinol, is a platinum analog  
59 of cisplatin. It was introduced into clinical trials in 1981 to overcome the toxic side  
60 effects of cisplatin such as nausea, vomiting, neuro-toxicity, nephrotoxicity, and  
61 ototoxicity. Currently, the use of CPT based treatments is considered standard  
62 treatment for many cancers (6).

63 CPT uptake across the plasma membrane occurs via a specific mediator,  
64 copper transporter 1 (CTR1), which is the major copper influx transporter expressed  
65 on the cell membranes (7).

NC1=NC=NC2=C(N1)N=CN=C2CN(C)c3ccc(cc3)C(=O)N[C@@H](CCC(=O)O)C(=O)O

**Figure 2.** Chemical structure of MTX

3

86 decreased affinity of MTX to mutated DHFR or a combination of these mechanisms  
87 (9).

88 The ability of cancer cells to invade and metastasize depends on the  
89 composition of the plasma membrane. The plasma membrane is an effective barrier  
90 that separates the inside of cells from environment and regulates molecular  
91 trafficking. Like other biological membranes, the general structure of the plasma  
92 membrane consists mainly of protein and lipids linked together by non-covalent  
93 interactions with a mass ratio ranging from 1:4 to 4:1 (10,11).

94 Lipids can move either laterally in the plane of the membrane or transversally  
95 from one leaflet to the other by flippases, floppases and scramblases (12). Lipids  
96 perform multiple important and vital biological roles at cellular and organismal levels.  
97 They act as fuel molecules, are crucial structural components of membranes, and  
98 considered important signaling molecules (10, 13).

99 Phospholipids are the most abundant lipids in all biological membrane.  
100 Phospholipids play essential roles for cell survival. They are involved in the equilibria  
101 of the electric charge between the membrane and the surrounding solutions  
102 components (14).

103 Malignant neoplastic cells are distinguished from their normal counterparts  
104 by alteration in the cellular behavior and disorganization of the plasma membrane  
105 (15). These perturbations are reflected by modification in the content of membrane  
106 phospholipids (15), as well as dysregulation in their metabolism (16).

107 The importance of lipids in cancer biology has started to reveal in the past  
108 three decades of research. Extensive studies have elucidated the abnormalities at the  
109 surface of neoplastic cells. Such changes are elucidated by alteration in membrane

110 lipids that are considered major cellular indicators of tumor evolution and  
111 progression.

112 Major hallmarks of cancer are the abnormalities in phospholipids metabolism  
113 (17, 33). Larger amounts of phospholipids were shown to be present in various types  
114 of tumors compared to normal cells (17). Furthermore, malignant tumors contain  
115 larger amounts of both cholesterol and phospholipids than benign tumors. Both  
116 programmed cell death and proliferative growth are affected by plasma membrane  
117 phosphatidylcholine and choline metabolites (17). Szachowicz-Petelska *et al.* (14)  
118 quantitated the phospholipids extracted from healthy and cancerous renal tissue from  
119 patients and from plasma membrane of non-metastatic colorectal cancer cells. This  
120 study concluded that cancerous cells showed an increase in the amount of  
121 phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE)  
122 and phosphatidylserine (PS) in cell membranes. Hence, alteration of membrane  
123 phospholipid in malignant transformation and progression represents a major hallmark  
124 of cancer cells. Cancer research has focused on the effect of chemotherapeutic drugs  
125 intracellularly with less emphasis on their effect on the cell's plasma membrane. Our  
126 study aims at investigating the effect of the two anti-neoplastic drug CPT and MTX  
127 on the lipid levels in the plasma membrane of breast cancer cells (MCF-7) and to  
128 evaluate the effect of these drugs on the invasiveness of MCF-7 cells in an *in vitro*  
129 system using time lapse assay.

130

## 131 **Materials and Methods:**

### 132 **1. Cell strain and culture**

133 MCF-7 (human breast cancer cell line) (cat# HTB-22) used in this study was  
134 purchased from the American Type Culture Collection (ATCC), Manassas, VA, US.

135 Cells were grown in RPMI medium supplemented with 10% fetal bovine serum (FBS)  
136 and 2% PEN-Strep at 37°C in a humidified incubator containing 5% CO<sub>2</sub>.

## 137 **2. *In vitro* cytotoxicity assay (MTT Assay)**

138 Cell Proliferation Kit I (MTT) (cat# 11699709001) purchased from Roche was  
139 used to determine cell viability. MCF-7 (passage number between 15 to 20) cells were  
140 seeded in a flat bottomed 96-well plate at a density of 5000 cells/well in a final  
141 volume of 100µl medium in a humidified incubator with 5% CO<sub>2</sub> for 24 h. The cells  
142 were treated with 100 µl of different concentrations of MTX (0.1µM to 50 µM) or  
143 CPT (1 µM to 1 mM) suspended in complete media or media lacking FBS and  
144 incubated for 24 h. Control cells were treated similarly with both types of media  
145 lacking the drugs. 10µl of 3-(4-5 dimethyl thiazol-2yl)-2,5 diphenyl tetrazolium  
146 bromide (MTT reagent) were then added and the plate was incubated for 4 h, followed  
147 by the addition of 100µl of solubilizing reagent and overnight incubation. Cell  
148 viability was determined by the formation of purple formazan product. Absorbance of  
149 the color obtained was read at a wavelength of 595 nm using an ELISA plate reader.  
150 The percent survival was measured according to the following equation:

$$151 \quad \% \text{ survival} = (\text{abs of treated cells} / \text{abs of control cells}) * 100.$$

## 152 **3. Membrane Isolation**

153 Plasma membrane isolation was carried out as described by Lin *et al.* (18)  
154 with a minor modification. MCF-7 cells were seeded at a density of  $2 \times 10^6$  cells/10ml  
155 and then treated with MTX and CPT at their determined IC<sub>50</sub> for 24 h. Both control  
156 and treated cells were harvested, washed twice with saline, and pelleted by  
157 centrifugation at 500 x g for 5 min. Cell pellets were homogenized in 500µl HEPES-  
158 Mannitol solution for 5 min using a 27 gauge needle. The volume was made up to 3

159 ml followed by addition of 1M CaCl<sub>2</sub> to a final concentration of 10 mM. Homogenate  
160 was stirred for 10 min to evenly distribute the Ca<sup>2+</sup> cation and then centrifuged at  
161 3000xg for 15 min. The obtained supernatant was collected and ultracentrifuged at  
162 100000xg for 24 h using a Sorvall WX Ultra Centrifuge in AH-627 rotor to pellet the  
163 plasma membrane. All steps were performed at 4°C.

#### 164 **4. Enzymatic characterization of isolated plasma membrane from MCF-7**

165 The purity of the extracted plasma membrane was assessed by measuring the  
166 activities of a membrane specific enzyme as well as marker enzymes for endoplasmic  
167 reticulum (ER) and mitochondria. The presence of 5'-nucleotidase activity and  
168 absence of glucose-6-phosphatase and succinate dehydrogenase from the plasma  
169 membrane preparation confirmed its purity from both ER and mitochondria,  
170 respectively. Purity of the plasma membrane was assessed by measuring the activity  
171 of 5'-Nucleotidase as described by Aronson and Touster (19). Absence of ER and  
172 mitochondrial contaminants was confirmed by assessing the activity of glucose-6-  
173 phosphatase, and succinate dehydrogenase as described by Aronson and Touster (19)  
174 and Bonner (20) respectively.

175

#### 176 **5. Protein quantification**

177 Proteins concentrations of the cell homogenates and the different fractions  
178 were estimated using Bradford assay. Concentrations were determined from a  
179 standard curve obtained using different amount of 0.01% of bovine serum albumin  
180 (BSA).

181

#### 182 **6. Extraction of the plasma membrane phospholipids**

183           The Bligh and Dyer (21) method was used to extract the total lipids from the  
184 isolated plasma membrane. Membranes were suspended mixed with  
185 chloroform/methanol (1:2), vortexed very well. Double distilled water was added to  
186 the mixture with vigorous vortexing. Samples were then centrifuged at 200x g for 5  
187 min at room temperature to separate the aqueous and organic layers. The chloroform  
188 layer containing the extracted phospholipids was collected for analysis. The extraction  
189 procedure was repeated 3 times on the aqueous layer to remove any residual lipids.

#### 190 **7. Total inorganic phosphate determination**

191           The total inorganic phosphate was determined as described by Lowry and  
192 Lopez with slight modifications (22). Different amount of inorganic phosphate  
193 standard were added to a solution containing 10 N H<sub>2</sub>SO<sub>4</sub>: 70% HClO<sub>4</sub>: H<sub>2</sub>O with a  
194 ratio of 9:1:40. Samples were vortexed and incubated overnight at 160°C. Tubes were  
195 washed with double distilled water followed by addition of 0.9 % ammonium  
196 molybdate reagent. After vortexing, ascorbic acid solution (9%) was added. The tubes  
197 were then incubated at 45°C for 30 min for the blue color to develop. The absorbance  
198 of the developed blue color was read at 820 nm.

199

#### 200 **8. Separation of lipids by HPLC**

201           The isolated phospholipids were separated by isocratic RP-HPLC. The separation  
202 was performed using an Agilent 1100 series HPLC with an HS C18 Discovery  
203 chromatographic column (25 cm, 4.6 mm, 5 µm) from Sigma. Elution was performed  
204 isocratically with a solvent consisting of a mixture of Acetonitrile/Methanol/Water  
205 (70:22:8). The flow rate was 0.5 ml/min. The eluted samples were detected at 210 nm.  
206 Every run was completed till absorbance returned to baseline.

## 207 **9. Motility Analysis by Time Lapse Assay**

208 Cells were plated on a 35mm petri dish with a density of  $2 \times 10^4$  cells/well and  
209 incubated under optimal conditions in a humidified incubator with 5% CO<sub>2</sub> at 37°C  
210 for 24 h. CPT at IC<sub>50</sub> (100 µM) or MTX (5 µM) were then added for a period of 24 h.  
211 For motility analysis, cells were imaged in RPMI media, buffered using HEPES and  
212 overlaid with mineral oil on a 37°C stage. Images of moving cells were collected  
213 every 60 seconds for duration of 2 hours using a 20X objective lens on Zeiss  
214 Observer Z1 microscope. The speed of cell movement was quantified using the ROI  
215 tracker plugin in ImageJ software where every cell was tracked over 120 frames.  
216 Eight cells were tracked for each time-lapse movie, and average of distances moved  
217 were measured.

## 218 **10. Statistical Analysis**

219 All statistical analyses were performed with the standard statistical program  
220 SPSS 13.0 PL. The data obtained in this study were expressed as the mean ± standard  
221 deviation (SD). Statistical significance was tested using ANOVA test. P-values less  
222 than 0.05 were considered significant.

223

## 224 **Results:**

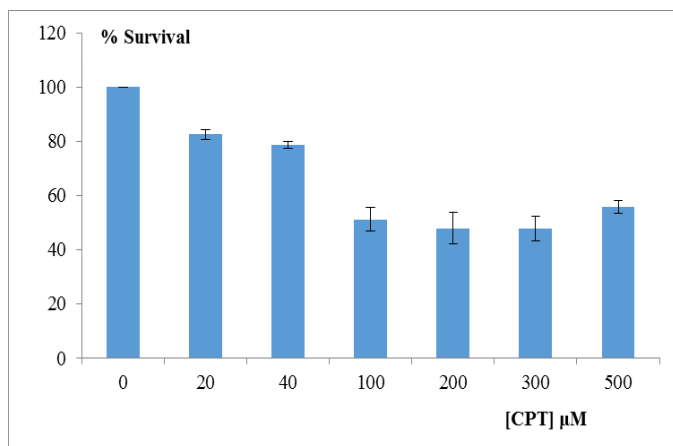
### 225 **1. Effect of CPT and MTX on MCF-7 viability**

226 The cytotoxicity of CPT and MTX on MCF-7 cells was evaluated in an *in*  
227 *vitro* assay using MTT assay. Cells grown in RPMI media with or without FBS were  
228 treated with CPT or MTX at concentration varying from 1 µM to 1 mM and from  
229 0.1µM to 50 µM respectively for 24 hours. No cytotoxic effect was observed with  
230 both drugs when mixed in FBS containing media. Cytotoxicity of both drugs was seen

231 only when FBS was eliminated from media. IC<sub>50</sub> was reached at a concentration of  
232 100 µM for CPT and 5 µM for MTX (Figure 3 and 4). Additional concentration of  
233 CPT up to 1 mM did not show any enhanced cytotoxicity.

234 On the other hand, MTX gave a 50 percent decrease in viability at 5 µM  
235 Lower doses did not show dose dependent decrease. Higher doses up to 50 µM did  
236 not have enhanced killing effect. It did not yield enhanced cell death at concentrations  
237 above 50 µM.

238



239

240 **Figure 3.** Effect of CPT on MCF-7 viability using MTT assay. Cells were cultured with CPT at  
241 concentrations ranging from 0 to 500 µM in the absence of FBS for 24 hours. Results represent mean of  
242 3 determinations ± SD.

243

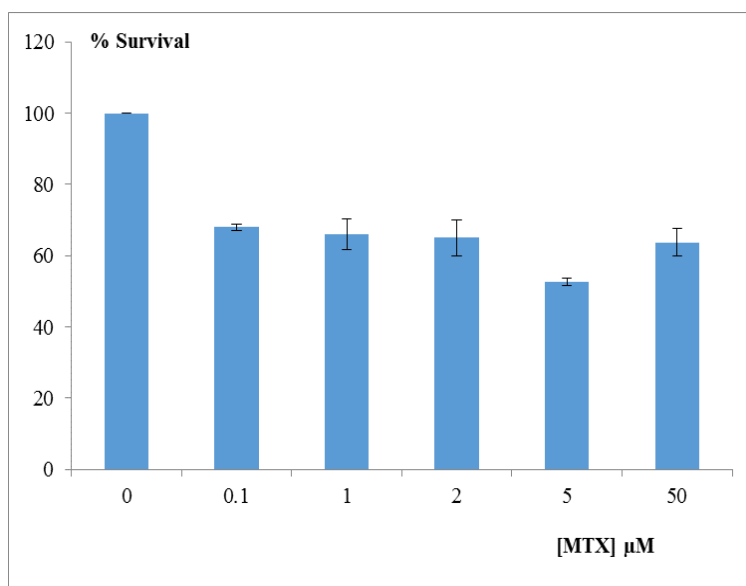


**Figure 4.** Effect of MTX on MCF-7 viability using MTT assay. Cells were cultured with MTX at concentrations ranging from 0 to 50 $\mu\text{M}$  for 24h in the absence of FBS. Results represent mean of 3 determinations  $\pm$  SD.

## 2. Protein quantification

Protein concentrations were determined using Bradford Assay. Table 1 summarizes the results. The concentration of proteins in total homogenate did not significantly change upon CPT or MTX treatment. However, the concentration of proteins in plasma membrane increased twice after treatment with both drugs.

**Table 1.** Protein concentrations of cell homogenate and isolated plasma membrane from control and CPT or MTX treated cells. Results represent  $\pm$  SD of 3 different determinations of three different experiments. P-value in CPT treatment were 0.218 and 0.039 for the cell homogenate and the plasma membrane respectively. For MTX treatment, the p-values were 0.263 and 0.023 for the homogenate and the plasma membrane respectively.

| Sample | Total protein in control ( $\mu\text{g}$ ) | Total protein after CPT treatment ( $\mu\text{g}$ ) | Total protein after MTX treatment ( $\mu\text{g}$ ) |
|--------|--------------------------------------------|-----------------------------------------------------|-----------------------------------------------------|
|--------|--------------------------------------------|-----------------------------------------------------|-----------------------------------------------------|

|                       |           |            |            |
|-----------------------|-----------|------------|------------|
| Total cell homogenate | 640.7 ± 8 | 727.2 ± 61 | 591.6 ± 39 |
| Plasma membrane       | 25.12 ± 4 | 51.1 ± 4   | 49.84 ± 5  |

### 3. Total inorganic phosphate determination

The amount of inorganic phosphate was determined as described under material and methods. Table 2 summarizes the results obtained. Upon comparing control with CPT treated cells, no significant changes in inorganic phosphate were detected in purified plasma membranes and in total lipids extracted from plasma membranes in CPT treatment. The same is true for MTX treatment except for total cell homogenate.

Table 2. Amount of inorganic phosphate in control and CPT or MTX treated cells obtained from total cell homogenate, plasma membranes, and their extracted lipids. Results represent ± SD of 3 different determinations of three different experiments. P-values for CPT treatment for the cell homogenate, plasma membrane and the extracted lipids were 0.141, 0.845 and 0.156 respectively. As for MTX treatment, the p-values for cell homogenate, plasma membrane and the extracted lipids were 0.029, 0.1, and 0.386 respectively.

| Sample                                | Total inorganic phosphate in control cells | Total inorganic phosphate in CPT treated cells | Total inorganic phosphate in MTX treated cells |
|---------------------------------------|--------------------------------------------|------------------------------------------------|------------------------------------------------|
| Total cell homogenate                 | 960 ± 6                                    | 805.8 ± 65                                     | 664.33 ± 82                                    |
| Plasma membrane                       | 199 ± 29                                   | 190 ± 30                                       | 116 ± 6                                        |
| Lipids extracted from plasma membrane | 118 ± 12                                   | 106 ± 4                                        | 105 ± 3                                        |

273           However, when comparing the amount of lipids per microgram of proteins, we  
274 noticed that there is a decrease in the total amounts of lipids (as measured by free inorganic  
275 phosphate). Total inorganic phosphate (Pi) in plasma membrane decreased 2 to 3 times in  
276 CPT and MTX treatment respectively (from 7.96 nmol/ml/μg of protein to 2.32 nmol/ml/μg  
277 in MTX treatment and to 3.72 nmol/ml/μg in CPT treatment). In the lipids extracted from the  
278 plasma membrane, it decreased about 2.4 times (from 4.72 nmol/ml/μg to 2.0 nmol/ml/ μg in  
279 both treatment). No significant changes were seen in the concentrations of inorganic  
280 phosphate per microgram of protein in total homogenate versus drug treatment (1.5  
281 nmol/ml/ μg of protein versus 1.1 for both treatment).

#### 282   **4. Separation of lipids by HPLC**

283           Figure 5, 6, and 7 show representative chromatograms of extracted lipids from  
284 plasma membranes of untreated, CPT, and MTX treated cells respectively. No  
285 changes in the elution pattern were noticeable between control and CPT treatment  
286 while MTX treatment showed an additional peak eluting at 4.36 min.  
287 Measurement of inorganic phosphate in the eluted peaks at 4, 6.7, and 7.2 min showed  
288 that the changes in the ratio of inorganic phosphate between MTX treatment and  
289 control were 1.3, 1.9, and 1.22 respectively.

290           In CPT treatment, changes in inorganic phosphate was seen mainly at 13.2  
291 min where the ratio between Pi in CPT treatment to control was 0.6.

292

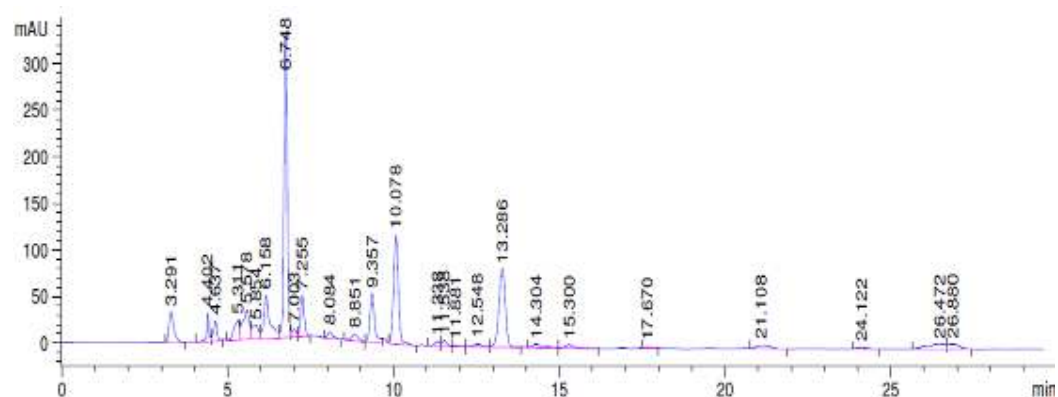


**Figure 5.** Chromatogram showing the separation pattern of lipids extracted from plasma membrane of control MCF-7 cells.

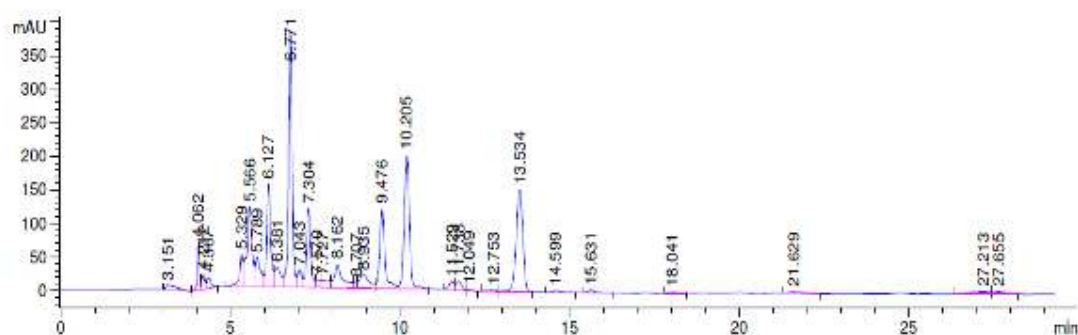
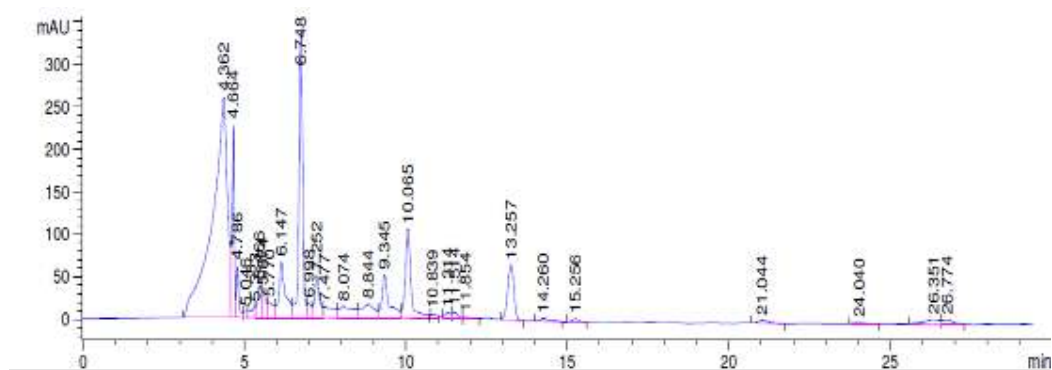


**Figure 6.** Chromatogram showing the separation pattern of lipids extracted from plasma membrane of CPT treated MCF-7 cells.

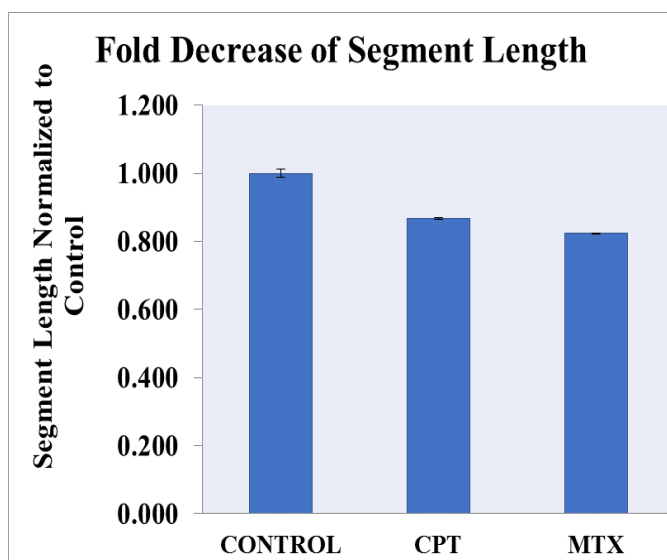


303 **Figure 7.** Chromatogram showing the separation pattern of lipids extracted from plasma membrane of  
304 MTX treated cells.

### 305 **5. Effect of CPT and MTX on MCF-7 motility**

306 Motility of MCF-7 breast cancer cells treated with 5 $\mu$ M MTX or 100  $\mu$ M of  
307 CPT resulted in a non-significant decrease by 13.3 % and 17.4% respectively  
308 compared to the untreated cells (Figure 8) as observed by time-lapse assay.

309



310

311

312 **Figure 8.** Effect of CPT and MTX on MCF-7 cells' motility. Results represent mean of 3  
313 determinations  $\pm$  SD. (P-value 0.492 for CPT and 0.341 for MTX).

### 314 **Discussion:**

315 In this study, we aimed at examining the effect of the anti-cancer drugs CPT  
316 and MTX on the lipid levels of the plasma membrane of human breast cancer cell line  
317 (MCF-7). In addition, we investigated their effects on the invasive properties of these  
318 cells.

319 A 50% decrease in MCF-7 viability was obtained at a CPT concentration of  
320 100  $\mu$ M. Higher concentrations of the drug up to 1mM were not accompanied by any  
321 enhanced cytotoxicity on MCF-7 cells. CPT was shown to be cytotoxic on several  
322 cancer cell lines with different reported IC<sub>50</sub>. For example, IC<sub>50</sub> was 289  $\mu$ M in  
323 human urinary bladder cancer cell line (TCC) (23), 0.016  $\mu$ M in four leukemic cell  
324 lines (CEM, HL60, U937 and K562) (3), and 1.32  $\mu$ M in human ovarian cancer cell  
325 lines (A721, A90, A286, AI, and A121A) (24). Other study performed on epithelial  
326 ovarian cancer cell lines (SKOV-3 and OVCAR-3) showed that OVCAR-3 was  
327 sensitive to CPT, with an IC<sub>50</sub> of 57.3  $\mu$ M, while SKOV-3 cells were resistant to CPT  
328 with an IC<sub>50</sub> of 211  $\mu$ M. In addition, CPT was shown to inhibit the viability of the  
329 ovarian cancer cells BG-1 with an IC<sub>50</sub> of 8  $\mu$ M (25). The IC<sub>50</sub> of CPT on MCF-7  
330 cells determined by Kwon *et.al.* was 62.19  $\mu$ M after 72 hrs incubation (34) while  
331 Zhou *et.al.* obtained IC<sub>50</sub> of 120  $\mu$ M with similar incubation time (35).

332 As for the effect of MTX on the viability of MCF-7, cell viability was almost  
333 the same (~50% cell death) at MTX concentrations ranging between 0.5 $\mu$ M and 5 $\mu$ M.  
334 However, at 10 times higher drug concentrations, the maximum decrease in viability  
335 obtained was only 35%. MTX has been actively used in the treatment in several  
336 malignancies, including head and neck, breast, and bone tumors, leukemias and  
337 lymphomas (8). The IC<sub>50</sub>s of MTX in different cells lines (MCF-7, AGS, A549,  
338 NCI-H23, HCT-116 and Saos-2) were in an extensively broad range from 6.05 $\pm$ 0.81  
339 nM to more than 1000 nM. For MCF-7 cells, IC<sub>50</sub> determined by Yoon *et.al.* was  
340 114.31 $\pm$ 5.34 nM (9) while Zhou *et.al.* obtained a value of 35 nM (35) after 72 hrs of  
341 incubation.

342 The IC<sub>50</sub> values obtained in our study using both MTX and CPT were higher  
343 than those obtained by others after 24 hrs incubation. It is generally established that

the viability of cancer cells is inversely proportional to the increase in the concentration of drug used. In this study, cell viability was almost the same (~50% cell death) at MTX concentrations ranging between 0.5 $\mu$ M and 5 $\mu$ M. However, at 10 times higher drug concentrations, the maximum decrease in viability obtained was only 35%.

Generally, the cytotoxicity of anti-cancer drugs is directly correlated with its uptake and cellular accumulation. The obtained effects of both drugs on the viability of MCF-7 cells in our study might be attributed to several factors. First, the drugs could have been chelated by the proteins found in the FBS (we can exclude this possibility as we omitted the FBS from the media used in our experiments); second, the unavailability of sufficient transporters on the cell surface to allow their entry; third, the likelihood that the parental MCF-7 cells cell passage have developed drug resistance due to the propagation of an inherently resistant subclone. Additionally, the cell passage might play a role in the cytotoxicity assay.

CPT enters the cell through active transport via the major copper influx transporter, the copper transporter 1 (Ctr1), considered as the main path of platinum drugs import into the cell (26). Ctr1 controls the cytotoxicity of platinum drugs by affecting drug uptake. Absence of Ctr1 expression significantly reduced the cellular uptake of platinum drugs making mammalian cells *in vitro* and *in vivo* resistant to these drugs (27). Quantitation of the cellular CTR1 mRNA and protein in plasma membrane should be performed to confirm this notion.

On the other hand, MTX primarily enters the cell by an active transport mechanism through the action of the reduced folate carrier (RFC). At a very high concentration (> 20 $\mu$ M), the drug may enter through passive diffusion. Some studies proved that acidic pH may facilitate drug entrance through passive diffusion and may

369 hinder entrance through active transport. Folic acid may also hinder MTX uptake by  
370 the cells since it binds to the RFC transporter thus competing with the drug (28). The  
371 concentration of folic acid in RPMI medium is 2.26  $\mu$ M almost half the concentration  
372 of MTX used in this study. Hence, we speculate that the effect seen may be due to the  
373 competition between the two chemicals for RFC binding thus, leading to uptake  
374 defects, target amplifications or alterations in polyglutamation. Furthermore,  
375 reduction in the RFC expression on the surface of the cancer cells may contribute to  
376 reduced potency.

377 Cancer cells perform their activities through their plasma membranes which  
378 are considered as a potential target for chemotherapeutic drugs. These activities are  
379 reflected by alterations in the membrane lipids composition. Todor *et.al.* studied the  
380 total lipid content in cisplatin and doxorubicin-sensitive and resistant human breast  
381 cancer cells (MCF-7) (29). Marked differences in the total lipid composition between  
382 sensitive and resistant MCF-7 cell strains were found. Cholesterol as well as  
383 cholesterol esters content were significantly higher but diacylglycerols content were  
384 significantly lower in resistant cell strains than in parental cells. In addition, the  
385 analysis of individual phospholipids showed increased levels of sphingomyelin,  
386 phosphatidylserine, cardiolipin, phosphatidic acid and decreased levels of  
387 phosphatidylethanolamine, phosphatidylcholine in cisplatin and doxorubicin resistant  
388 MCF-7 cells compared to sensitive MCF-7 cells (29). The same study found that  
389 alterations and changes in the lipid composition of MCF-7 cancer cells were  
390 accompanied with the formation and development of resistance to anticancer drugs.  
391 A decrease in membrane fluidity is caused by increased levels of cholesterol and  
392 sphingomyelin, and cytotoxic substances elimination by means of P-glycoprotein is  
393 activated by increased phosphatidylserine content. Additionally, characterization of

the plasma membrane lipid content of non-metastatic human colorectal cancer cells was studied (14). The obtained results showed a modification of the plasma membrane structure and function in neoplastic lesions reflected by changes in the membrane's phospholipids and free fatty acids content. Cancer transformation was accompanied by an increase in total phospholipids as well as a decrease in free fatty acids levels. Our results did not show an overall change in the plasma lipids content as measured by inorganic phosphate content; however, this does not reflect changes at the level of the different types of phospholipids. The changes in Pi in the different collected peaks confirm our suggestion (data not shown for both drugs).

Cancer research has used proteomics as a tool for the identification of biomarkers and chemotherapeutic targets. Recently, lipidomics have gained a great attention as a way to detect biomarkers and eventually a potential therapeutic target. A change in the lipidomics of cancer cells may affect the way this cancer is approached. Alteration in the levels of different plasma membrane lipids might open new perspectives in identifying efficient drugs that can overcome classical resistance mechanisms exhibited by cancer cells.

As for our second aim that targets the effect of CPT and MTX on MCF-7 invasiveness, our results showed that CPT at 100 $\mu$ M caused a 13.3% decrease in the motility of MCF-7 compared to the control cells while MTX gave a 17.4 % at 5  $\mu$ M. However, these decreases were not significant. Invasiveness and metastasis are the main cause of death of breast cancer patients (30). Chemotherapy drugs play a crucial and important role in regulating and controlling cell invasion and metastasis. Menhofer *et.al.* showed that chondramide exerts its effect by disrupting the actin cytoskeleton and hence decrease the ability of cells for contraction (31). Chen *et.al* studied the effect of CPT on the level of actin polymerization and showed that actin

binds CPT and leads to decreased expression and disorganization of several cytoskeletal proteins providing a molecular and cellular basis for the known defect in endocytosis in these cisplatin resistant cells (32). Hence, CPT may play a role in inhibiting cellular contractility and thus cellular invasion.

#### **Conclusions:**

Our results show that both CPT and MTX lead to changes in protein level in the plasma membrane of MCF-7 cells. However, they exhibited an insignificant decrease in the amount lipids of these membranes. Furthermore, both drugs did not lead to significant decrease in cell motility compared to control.

#### **References:**

- 1- Cairns, R.A., Harris, I.S., & Mak, T. W. Regulation of cancer cell metabolism. 2011; *Nature Reviews Cancer*, 11, 85.
- 2- Ruddon, R. D. Characteristics of human cancer. 2007; *Cancer biology fourth edition* (pp. 3-14).
- 3- Su, W. C., Chang, S. L., Chen, T. Y., Chen, J. S., & Tsao, C. J. Comparison of in Vitro growth-inhibitory activity of Carboplatin and cisplatin on leukemic cells and hematopoietic progenitors: The myelosuppressive activity of Carboplatin may be greater than its anti-leukemic effect. 2000; *Japanese Journal of Clinical Oncology*, 30, 562-567.
- 4- Kaye, S. B. New antimetabolites in cancer chemotherapy and their clinical impact. 1998; *British Journal of Cancer*, 78, 1-7.
- 5- Damia, G., & D'Incalci, M. Mechanisms of resistance to alkylating agents. 1998; *Cytotechnology*, 27, 165–173.
- 6- Tiseo, M., & Ardizzoni, A. Cisplatin or Carboplatin in the treatment of non-small cell lung cancer: A comprehensive review. 2007; *Oncology Review*, 1, 162–169.
- 7- Holzer, A. K., Manorek, G. H., & Howell, S. B. Contribution of the major copper influx transporter CTR1 to the cellular accumulation of cisplatin, Carboplatin, and oxaliplatin. 2006; *Molecular Pharmacology*, 70, 1390–1394.

- 449 8- Jolivet, J., Cowan, K.H., Curt, G.A., Clendeninn, N.J., Chabner, B.A. The  
450 pharmacology and clinical use of Methotrexate. 1983; *N. Engl. J. Med.*, 309, 1094.
- 451 9- Yoon, S.A, B.S., Choi, J.R., Kim, J.O., Shin, J.Y., Zhang, X.H., Kang, J.H. Influence  
452 of reduced folate carrier and dihydrofolate reductase genes on Methotrexate-induced  
453 cytotoxicity. 2010; *Cancer Research Treatment*, 42, 163-171.
- 454 10- Berg, J. M., Tymoczko, J. L., & Stryer, L. Lipids and cell membranes. 2002;  
455 *Biochemistry* fifth edition pp. 319-329.
- 456 11- Nelson, D. L., Cox, M. M. Biological membranes and transport. 2006; *Lehninger*  
457 *Principles of Biochemistry* Fourth Edition (pp 369- 420).
- 458 12- Lodish, H., Berk, A., Kaiser, C. A., Krieger, M., Bretscher, A., Ploegh, H., Amon, A.,  
459 & Scott, M. P. Biomembrane structure. 2013; *Molecular cell biology seventh edition*  
460 pp 443-468.
- 461 13- Santos, C. R., & Schulze, A. Lipid metabolism in cancer. 2012; *The Febs Journal*,  
462 279, 2610–2623.
- 463 14- Szachowicz-Petelska, B., Dobrzynska, I., Sulkowski, S., & Figaszewski, Z.  
464 Characterization of the cell membrane during cancer transformation. 2010; *Journal of*  
465 *Environmental Biology*, 31, 845-850.
- 466 15- Kojima, K. Molecular aspects of the plasma membrane in tumor cells. 1993;  
467 *Nagoya Journal of Medical Science*, 56, 1–18.
- 468 16- Podo, F., Canevari, S., Canese, R., Pisanu, M. E., Ricci, A., & Iorio, E. Tumour  
469 phospholipid metabolism. 2011; *Proceedings of the International Society for*  
470 *Magnetic Resonance in Medicine*, 19.
- 471 17- Aboagye, E. O., & Bhujwalla, Z. M. Malignant transformation alters membrane  
472 choline phospholipid metabolism of human mammary epithelial cells. 1999; *Cancer*  
473 *Research*, 59, 80-84.
- 474 18- Lin, P.H.; Selinfreund, R.; Wakshull, E.; Wharton, W. Rapid and efficient  
475 purification of plasma membrane from cultured cells: characterization of epidermal  
476 growth factor binding. 1987; *Biochemistry* 26 (3), pp 731–736
- 477 19- Aronson, N. Jr., and Touster, O. Isolation of rat liver plasma membrane  
478 fragments in isotonic sucrose. 1974; *Methods Enzymol.* 31 :90-102.
- 479 20- Bonner, W.D. Enzymes of Citric Acid Cycle. 1955; In: Colowich S.P. and Kaplan  
480 N.O., eds. *Methods Enzymol*, Academic Press Inc., New York, USA, vol. 1, p. 722-  
481 729.
- 482 21- Bligh, E.G., and Dyer, W.J. A rapid method of total lipid extraction and  
483 purification. 1959; *Canadian Journal of Biochemistry and Physiology* 37 (8): 911-  
484 917

- 485 22- Lowry, O. H. & Lopez, J. A. The determination of inorganic phosphate in the  
486 presence of labile phosphate esters. 1946; *J. Biol. Chem.* 162:421-8.
- 487 23- Wang, S., Zhang, H., Cheng, L., Evans, C., Pan, C.X. Analysis of the Cytotoxic  
488 Activity of Carboplatin and Gemcitabine Combination. 2010; *Anticancer Research*  
489 30: 4573-4578.
- 490 24- Fanning, J., Biddle, WC., Goldrosen, M., Crickard, K., Piver, MS., Foon, KA.  
491 Comparison of cisplatin and Carboplatin cytotoxicity in human ovarian cancer cell  
492 lines using the MTT assay. 1990; *Gynecology Oncology* 39(2):119-22.
- 493 25- Engblom, P., Rantanen, V., Kumala, J., Grenman, S. Carboplatin–paclitaxel- and  
494 Carboplatin–docetaxel-induced cytotoxic effect in epithelial ovarian carcinoma in  
495 vitro. 1999; *Cancer*, 86(10):2066-73.
- 496 26- Howell, S. B., Safaei, R., Larson, C. A., & Sailor, M. J. Copper transporters and the  
497 cellular pharmacology of the platinum-containing cancer drugs. 2010; *Molecular*  
498 *Pharmacology*, 77, 887-894.
- 499 27- Stewart, D. J. Mechanisms of resistance to cisplatin and Carboplatin. 2007; *Critical*  
500 *Reviews in Oncology/Hematology*, 63, 12-31.
- 501 28- Cowan, D.S.M, & Tannock, I.F. Factors that influence the penetration of  
502 Methotrexate through solid tissue. 2001; *International Journal of Cancer*, 91, 120–  
503 12.
- 504 29- Todor, I. N., Lukyanova, N. Y., & Chekhun, V. F. The lipid content of cisplatin-and  
505 doxorubicin resistant MCF-7 human breast cancer cells. 2012; *Experimental*  
506 *Oncology*, 34, 97–100.
- 507 30- Weigelt, B., Peterse, J. L., & van 't Veer, L. J. Breast cancer metastasis: Markers and  
508 models. 2005; *Nature Reviews Cancer*, 5, 591-602.
- 509 31- Menhofer, M.H., Kubich, R., Schreiner, L., Foerster, F., Mueller, R., Raedler, JO.,  
510 Wagner, E., Vollmar, AM., & Zahler, S. The actin targeting compound Chondramide  
511 inhibits breast cancer metastasis via reduction of cellular contractility. *PLoS One*,  
512 2014; 9(11): e112542. Doi:10.1371.
- 513 32- Shen, D. W., Liang, X. J., Gawinowicz, M. A., & Gottesman, M. M.. Identification of  
514 cytoskeletal [14C] Carboplatin-binding proteins reveals reduce expression and  
515 disorganization of actin and filamin in cisplatin resistant cell lines. *Molecular*  
516 *Pharmacology*, 2004; 66, 789-93.
- 517 33- Dobrzynska, I., Szachowicz-Petelska, B., Darewicz, B., & Figaszewski, Z.  
518 Characterization of Human Bladder Cell Membrane During Cancer Transformation.  
519 *Membr Biol.* 2015; 248(2): 301–307.

520 34. Kwon, Y., Park, J-Y., Kim, W-K. In Vitro Histoculture Drug Response Assay and In  
521 Vivo Blood Chemsitry of a Novel Pt(IV) coumpound, K104. Anticancer Research. 2007;  
522 27: 321-326.

523 35. Zhou, J., Oliveira, P., Li, X., Chen, Z., Bepler, G. Modulation of the Ribonucleotide  
524 Reductase-Antimetabolite Drug Interaction in Cancer Cell lines. J. Nucleic Acid. 2010;  
525 2010 (ID 597098).

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