Different in vitro experimental approaches to treat breast cancer: from combination treatment of olaparib, oxaliplatin and PI3K inhibitor to targeting the mitochondria with a novel Mito-CP derivative HO-5114

PhD Thesis

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1. INTRODUCTION

In terms of incidence, breast cancer is the leading cancer type among women. It is a heterogenous and hormone-dependent disease; approximately 65–75% of cases are hormone receptor-positive (HR+; estrogen receptor-positive or progesterone receptor-positive), while 15–20% are human epidermal growth-factor receptor 2 (HER2)-positive. The triple-negative subtype (TNBC) represents 15% of all cases. For HR+ and/or HER2+ breast cancers, targeted therapies are available. In contrast, the treatment protocol for TNBC is mainly limited to chemotherapy. Besides the limited therapy options, recurrent tumor resistance and poor prognoses are emerging challenges in TNBC.

Cisplatin and carboplatin are widely used platinum-based chemotherapeutic agents in the treatment of non-small-cell lung cancer, and breast, ovarian and testicular cancer. One major limiting factor in their therapeutic use is the possibility that the cancer cells develop intrinsic or acquired resistance to the treatment. Oxaliplatin is a third-generation platinum compound. One of its benefits is decreased mutagenic activity compared to cisplatin and carboplatin, and it is often effective in cisplatin-resistant tumors. It has been approved by the FDA for treatment of colorectal cancer, and it has the potential to replace other platinum compounds in therapy for other types of cancer including TNBC.

The nuclear poly(ADP-ribose) polymerases (PARP) are a family of enzymes involved mainly in DNA repair, namely in base excision and nucleotide excision repair. PARP inhibition is cytotoxic in germline mutated BRCA1 and BRCA2 ovarian and breast cancer. Olaparib is the most widely used PARP inhibitor in clinical therapy nowadays. It has been approved for the treatment of BRCA 1/2 mutated ovarian and metastatic breast cancer.

The PI3K/Akt/mTOR pathway plays an important role in carcinogenesis and apoptosis resistance, promoting tumor growth and proliferation, and the activation of phosphatidyl-inositol 3-kinase (PI3K) has been associated with endocrine resistance, a major problem in the treatment of breast cancer. Recently, the FDA approved (in combination with fulvestrant, an estrogen receptor antagonist) the PI3K p110α-isoform-specific inhibitor, alpelisib for use in the treatment of HR+/HER2- metastatic breast cancer. However, combination of isoform-specific PI3K inhibitors with immune checkpoint-, receptor- or enzyme inhibitors, including PARP inhibitors, could extend their therapeutic use to HER2+ or triple-negative breast cancers.

Anticancer drugs are most effective when given in combination. Many DNA adduct-forming metallodrugs, in combination with other drugs of different molecular action, such as

inhibition of protein synthesis or DNA repair mechanisms, can increase their therapeutic efficacy over monotherapy and can overcome chemotherapy resistance. Several preclinical studies found a synergistic effect between platinum drugs and PARP inhibitors.

Targeting the mitochondria shows great promise to enhance the efficiency of anti-cancer drugs. Additionally, targeting the mitochondria could mitigate treatment resistance, another crucial factor of today's anti-cancer therapy. Mitochondria-targeted nanocarriers and drugs conjugated to mitochondria-targeting ligands are the most common approaches.

Lipophilic cations, such as tryphenylphosphonium (TPP), are frequently used conjugates in the design of mitochondria-targeted anti-cancer drugs, and they also have antifungal, antiparasitic, and antioxidant uses. The chemical background of mitochondrial targeting by lipophilic cations, such as TPP, is that the delocalized positive charge enables the drug to easily permeate lipid bilayers, which is an advantage compared to hydrophobic compounds that should rely on tissue-specific carriers. Lipophilic cations can achieve efficient uptake and accumulation several hundredfold within the mitochondria depending on the mitochondrial membrane potential (−150 to −180 mV). The mitochondrial membrane potential ($\Delta\Psi_m$) of cancer cells is higher compared to their cytosol and to non-cancer cells, and hence a selective targeting can be achieved.

Recently, the bioenergetics of cancer cells is receiving increased interest among researchers in the field. Breast cancer cells have profound bioenergetic, histological, and genetic differences compared to normal cells. As triple-negative breast cancer (TNBC) represents about 15–20% of all cases and is associated with a poor prognosis and limited therapeutic options, the development of novel therapeutic means is needed. Targeting metabolism and the mitochondria could be a useful therapeutic approach in TNBC cases because mitochondria play pivotal roles in early relapse and the metastatic spread of TNBC. Previous research has also demonstrated that targeting glycolysis might not be an effective strategy in TNBC therapy and has suggested that the mitochondrial aid-in-reserve must be selectively blocked.

Mito-CP, a TPP conjugated superoxide dismutase mimetic, was the first mitochondria-targeted nitroxide compound. It was used for studying the role of the mitochondrial superoxide in cancer cell proliferation. Mito-CP has shown cytotoxic properties in various cancer cells, including breast cancer cells, without markedly affecting non-cancerous ones. Recently, a novel component-derivative of Mito-CP, a pyrroline nitroxide attached dyphenylphosphine compound, HO-5114, was synthesized, which demonstrated markedly higher cytotoxicity against TNBC and hormone receptor positive human breast cancer (HR+BC) lines than Mito-CP.

2. OBJECTIVES

In this study, we investigated the response of different types of breast cancer cell lines to:

- a combination of conventional chemotherapeutic agent and synthetic lethality-based therapeutic compound supplemented with Akt inhibition to prevent undesired cytoprotective effects of the latter. We used the TNBC cell line MDA-MB-231 and estrogen and progesterone receptor-positive cell line MCF7, and treated these with the third-generation platinum compound oxaliplatin, the PARP inhibitor olaparib and the PI3K inhibitor LY294002. The triple combination of them is warranted to be examined in vitro to improve further the chemotherapeutic strategies. If in this experiment proper anti-cancer effects can be presented it will pave the way towards in vivo experiments, in order to develop new human chemotherapeutic protocols to improve oncological therapies.

- a novel component-derivative of Mito-CP, a pyrroline nitroxide attached dyphenylphosphine compound, HO-5114. Our goal was to investigate whether the mitochondrial effects of HO-5114 could participate in its cytotoxic and antiproliferative effects. In these experiments MDA-MB-231 and MCF7 cells were used. Based on the results of the study, in vivo research could be added to investigate the toxicity and anti-neoplastic effect of HO-5114 in vivo.

3. RESULTS I - Olaparib, oxaliplatin, LY294002 and their combination treatment on MDA-MB-231 and MCF7 cells

3.1 Effect of olaparib, oxaliplatin and LY294002 on cell viability

To determine the effect of olaparib, oxaliplatin and LY294002 alone and in combination on the viability of MDA-MB-231 and MCF7 cells, MTT assay was performed. The PARP inhibitor olaparib alone did not decrease viability significantly in either cell line. The viability of MDA-MB-231 reached more than 90 % and for the MCF7 cells this value was over 80 %. However, the platinum drug oxaliplatin reduced viability of the TNBC line MDA-MB-231 to approximately 60 % substantially and, more pronouncedly, the estrogen and progesterone receptor-positive non-TNBC line MCF7 where viable cell make up around 40 %. Although there are several articles implying synergy between PARP inhibitors and platinum compounds our results show that olaparib and oxaliplatin had neither a synergistic, nor an additive effect at the applied concentrations, since their combination caused about the same extent of cell death as oxaliplatin alone. More than 95 % of MDA-MB-231 cells were viable after LY294002 treatment, indicating that the TNBC cells were resistant to the Akt pathway inhibitor.

In contrast, LY294002 caused a significant amount of cell death (~50 %) in the MCF7 cell line. While the cytotoxic effect of oxaliplatin was enhanced by LY294002 in both cell lines, this effect was not observed in the olaparib treatment. Furthermore, addition of the PARP inhibitor to the co-treatment of oxaliplatin and LY294002 didn't increase cell death in MDA-MB-231 and MCF7.

3.2 Effect of olaparib, oxaliplatin and LY294002 on cell death processes

Flow cytometry was performed to examine which type of cell death olaparib, oxaliplatin and LY294002 elicited. Flow cytometric measurements were performed after double-staining the treated cells with fluorescently labelled Annexin V and propidium iodide, to demonstrate apoptosis and necrosis, respectively. We found that less than 5% of cell death caused by the compounds investigated was necrotic, and most of the dying cells were in their early apoptotic stage under the experimental conditions we used. The olaparib treatment did not generate apoptosis in the breast cancer cell lines, as live cell ratio of both cells was approximately 82 %. Oxaliplatin significantly increased early apoptosis in both cell lines, late apoptosis also increased in MDA-MB-231 and MCF7 cells, significantly in the MCF7 cells. LY294002 alone caused about the same level of apoptosis in both cell lines as olaparib did. On the other

hand, treatment of the cells with LY294002 together with olaparib, oxaliplatin or their combination did not significantly change the distribution of cells among live, early, and late apoptotic populations in either of the cell lines.

3.3 Effect of olaparib, oxaliplatin and Akt pathway inhibitor on ROS production

The ROS generation capacity of olaparib, oxaliplatin and LY294002 was measured using a carboxy-H2DCFDA assay. ROS can oxidize the non-fluorescent dye yielding its fluorescent form, which intensity can be measured. Marked differences were found between MDA-MB-231 and MCF7 cells among the treatment groups. Olaparib increased ROS production in MCF7 cells, although the effect did not reach a statistically significant level. In contrast, the drug did not induce any ROS production in MDA-MB-231 cells. Oxaliplatin caused significant ROS production in the triple-negative breast cancer cells which was enhanced by olaparib co-treatment, although olaparib's enhancing effect did not reach a statistically significant level. The Akt pathway inhibitor did not affect ROS production either alone or in any combination with olaparib and oxaliplatin. On the other hand, oxaliplatin did not affect ROS production of MCF7 cells and attenuated ROS production induced either by olaparib, LY294002, or their combination. However, these negative effects of oxaliplatin did not reach a statistically significant level. The Akt pathway inhibitor alone induced a similar level of ROS production in MCF7 cells as that caused by olaparib.

3.4 Effect of olaparib, oxaliplatin and Akt pathway inhibitor on the cell cycle

Flow cytometry was used to determine which cell cycle phase the cells had reached after treatment with different combinations of olaparib, oxaliplatin and LY294002. The distribution of control MDA-MB-231 cells among G1, S and G2/M phases was 55.57%, 22.8% and 21.63%, respectively, which was not affected by the PARP inhibitor olaparib, the Akt pathway inhibitor LY294002, or their combination. In contrast, oxaliplatin treatment arrested the cells in the S phase of their cycle which was not affected by olaparib co-treatment. However, LY294002 co-treatment attenuated oxaliplatin's arresting effect, which was further reduced when olaparib was included in the combination treatment.

The cell cycle phase distribution of control MCF7 cells was slightly different from that of MDA-MB-231 cells. Approximately 51.26% of control cells were in G1; 28.64% in S and 20.1% were in G2/M phase. As with the MDA-MB-231 line, oxaliplatin arrested MCF7 cells in the S phase of their cycle and olaparib co-treatment did not have any further effect on it.

However, the PARP inhibitor increased the number of G2/M phase cells at the expense of S phase, although this effect did not reach a statistically significant level. Additionally, LY294002 significantly enhanced the number of G1-phase cells at the expense of S and G2/M phase cells. This effect was not affected by olaparib co-treatment but was significantly counteracted by oxaliplatin.

3.5 Effect of olaparib, oxaliplatin and Akt pathway inhibitor on colony formation

Colony formation assay was performed to assess cellular proliferation capacity. Olaparib and oxaliplatin significantly decreased colony numbers of both cell lines, although the non-TNBC line MCF7 was more sensitive to the cytostatic drugs than the TNBC line MDA-MB-231. Furthermore, oxaliplatin attenuated colony formation to a much greater extent than olaparib did. A combination of olaparib and oxaliplatin caused a similar decrease in colony formation as oxaliplatin did alone, indicating a lack of synergy, even additivity, between the two substances. LY294002 alone decreased colony formation to about the same extent as olaparib did in MDA-MB-231 cells. In case of MCF7 cells LY294002 caused an even greater decrease in colony numbers compared to olaparib treatment. The PARP inhibitor and to a greater extent, oxaliplatin both augmented the Akt pathway inhibitor's effect on colony formation. Again, the combination of olaparib and oxaliplatin had about the same effect as the latter alone.

3.6 Effect of olaparib and oxaliplatin on invasive growth

Invasive growth of the cell lines was assessed by the xCelligence Real-Time Cell Analysis (RTCA) system. As with the colony formation experiments, olaparib and, to a much greater extent, oxaliplatin decreased invasive growth in both cell lines. Again, MCF7 line was more sensitive to the PARP inhibitor and to the cytostatic drugs than MDA-MB-231, and combination of the two drugs had about the same effect as oxaliplatin alone had.

3.7 Effect of olaparib, oxaliplatin and Akt pathway inhibitor on invasive growth

The Akt pathway inhibitor decreased invasive growth, and its effect on MCF7 cells was more pronounced than on MDA-MB-231 cells. Both olaparib and oxaliplatin treatment augmented LY294002′s effect, decreasing invasive growth to the detection limit.

4. DISCUSSION AND CONCLUSION I

Several studies have reported the synergistic effect of PARP inhibitors and chemotherapeutic platinum agents in various tumors. Olaparib and platinum compound carboplatin have been found to have modest activity in patients with sporadic TNBC. Combination of PARP inhibitor PJ34 and anti-neoplastic agent cisplatin has been found to have cytotoxic synergy in non-small-cell lung-cancer line A549. Furthermore, PJ34 enhanced the proliferation suppressive effect of cisplatin in liver-cancer cell line HepG2. The importance of the PI3K/Akt pathway in therapy resistance has been highlighted, demonstrating that its activation results in decreased sensitivity to chemotherapeutic agents. Furthermore, PI3K/Akt pathway inhibitors have been known to cause more favorable outcomes when co-administered with usual anti-cancer drugs. To provide experimental support for the rationale of combination therapy in TNBC, Zhao et al. investigated various combinations of olaparib, carboplatin and buparlisib, a pan-PI3K inhibitor in two human TNBC lines and a HR+ breast-cancer line. By using a calculation based on the median-effect equation, they found a synergistic cytostatic effect of the combination therapy in TNBC lines but not in the HR+ line. We approached the question of synergy from a practical point of view. Instead of determining dose-response effects, we used single, therapeutically relevant concentrations of each drug, and applied these individually and in all possible combinations in experiments on viability, type of cell death, ROS production, cell-cycle phase, colony formation and invasive growth.

We found that 72 h of olaparib treatment decreased viability of MCF7 cells to a much greater extent than that of MDA-MB-231 cells. Elevated PARP-1 expressions have been reported in a wide range of human cancers including breast cancer, and an especially high PARP-1 expression has been found in TNBC which can explain our results. Furthermore, in complete agreement with our data, other studies have found the cytotoxic effect of oxaliplatin to be higher in MCF7 than in TNBC cells. Several studies have reported the synergistic cytostatic effect of PARP inhibitors and platinum agents, and one study reported synergism in combined therapy comprising olaparib, carboplatin and the PI3K inhibitor buparlisib in TNBC lines but not in a HR+ breast-cancer line. In contrast, under our experimental conditions, olaparib did not enhance the cytotoxic properties of oxaliplatin, and we could not detect synergism, nor even an additive effect between these two drugs. The PI3K inhibitor LY294002 decreased

viability of the TNBC but not the HR+ line when combined with olaparib, oxaliplatin or both. However, these effects did not reach a statistically significant level. These data compellingly indicate that, at therapeutically relevant concentrations, cytotoxicity of the platinum compound dominated that of the PARP inhibitor and the PI3K inhibitor. At lower platinum compound and higher concentrations of PARP and PI3K a synergistic effect likely appears and a regression-based model could indicate an overall synergy that may explain the conflict between our results and the findings of others. Additionally, platinum compounds induce ROS production and PARP inhibitors are known to protect against oxidative stress, which could explain the absence of synergy between the PARP inhibitor and the platinum agent that we observed. Accordingly, blocking the PI3K/Akt pathway by the PI3K inhibitor LY294002 increased the cytotoxicity of olaparib and oxaliplatin co-treatment, although the effect did not reach a statistically significant level.

We found that olaparib and oxaliplatin killed MDA-MB-231 and MCF7 cells predominantly by apoptosis. The apoptosis resistance of the two cell lines is different. MDA-MB-231 line has high levels of mutant p53, whereas MCF7 line has wild-type p53. Additionally, TNBC cells have 10-fold greater phospholipase D (PLD) activity than MCF7 cells. Mutant p53 and elevated PLD activity play a significant role in the survival of cancer cells and can contribute to the suppression of apoptosis. Nevertheless, the effect of the various treatments on distribution among live, early and late-apoptotic populations was similar in both cell lines. In this respect it is worth noting that the washing steps before and after the staining procedure remove most non-adherent cells, and the flow cytometry method used to determine the type of cell death analyses stained cells only, regardless of the original cell number and their sensitivity to the various treatments.

Among other mechanisms, ROS-mediated processes play a prominent role in remodelling cancer phenotypes resistant to apoptosis which acquire enhanced metastatic properties. In solid tumors, hypoxia and the resulting hypoxia-inducible factor (HIF)-1α mediated metabolic plasticity play a pivotal role in malignant transformation. However, in cell culturing conditions, uniform oxygen partial pressure and practically inexhaustible extracellular fuel supply obscure these processes. Accordingly, we studied ROS production which reflects metabolic plasticity and is compatible with cell culturing conditions. Increased ROS production by the platinum compounds could induce DNA breaks that may accumulate when PARP is inhibited leading to cell death. Such a mechanism could account for the observed synergism between platinum compounds and PARP inhibitors. In complete agreement with the literature, we found that oxaliplatin- but not olaparib or the Akt pathway inhibitor

LY294002- induced ROS formation in the TNBC MDA-MB-231 line and LY294002, but olaparib did not augment oxaliplatin's effect. On the other hand, the treatments alone or in combination failed to induce significant ROS production in the non-TNBC MCF7 line. However, increased vulnerability of MCF7 cells to the treatments resulted in a death rate, compared to that of MDA-MB-231 cells, leaving fewer surviving cells to produce ROS. Furthermore, MCF7 cells could produce less ROS as they represent an earlier stage of metabolic transformation than the TNBC MDA-MB-231 cell line does. Combination of these and possibly other factors could account for the observed difference between the cell lines.

Centrosome amplification occurring in the S phase of the cell cycle, is known to be associated with malignant transformation in various tissue types. Centrosome amplification is regarded as a marker for aggressiveness, even with invasive breast and prostate cancers. Accordingly, we expected to find that the MDA-MB-231line had a higher percentage of cells in the S phase of their cycle than the MCF7 cells. However, we observed the opposite trend in the two cell lines indicates that other factors, probably synchronization of cell cycles due to passages during culturing dominated centrosome amplification in determining the distribution of cell-cycle phases in these two breast cancer cell lines. In both cell lines, although to a different extent, oxaliplatin arrested most of the cells in their S phase, and this was not affected by olaparib co-treatment. These data are consistent with the DNA crosslinking effect of the platinum compound, which prevents cells from crossing the G2 checkpoint.

The TNBC cell line MDA-MB-231 represents a more aggressive, apoptosis- and therapy-resistant phenotype than the non-TNBC MCF7 line does. As measures of this aggressiveness, we assessed colony formation and invasive growth. These data were completely consistent with the results for viability, with the literature and with the aforementioned view about aggressiveness of the two cell lines. They provide two additional experimental evidence for the lack of synergy between olaparib and oxaliplatin. Furthermore, they indicate that Akt pathway inhibition could be advantageous in combined therapy with PARP inhibitors, as it blocks their Akt-mediated cytoprotective effects.

In conclusion, we provided experimental evidence for the lack of synergy between olaparib, a PARP inhibitor widely used in cancer therapy, and oxaliplatin, a third-generation platinum compound. These results are in conflict with the findings of others probably because, at therapeutically realistic concentrations, the cytostatic effect of the platinum compound dominates that of the PARP inhibitor. We have also demonstrated the advantage of using an Akt pathway inhibitor to augment the cytostatic properties of the platinum compound and/or to prevent the cytoprotective effects of PARP inhibition. Furthermore, we have shown the

therapy resistance of the TNBC line MDA-MB-231 over the estrogen- and progesterone receptor-positive line MCF7, although we failed to advance our understanding of differences in sensitivity to chemotherapy among different types of breast cancers.

5. RESULTS II - Treatment of MDA-MB-231 and MCF7 cells with novel mitochondria-targeted pyrroline nitroxide HO-5114

5.1 Effect of HO-5114 on cell viability

To assess its anti-neoplastic potential, TNBC MDA-MB-231 and HR+BC MCF7 cells were treated with 1, 2.5, 5 or 10 μM HO-5114 for 24 and 48 h and then determined their viability using the sulforhodamine B (SRB) assay. The SRB assay measures protein content that is considered to be more proportional to the cell count than metabolic activity, which can under- or over-estimate the cell count if the studied substance inhibits or uncouples mitochondrial oxidative phosphorylation. HO-5114 decreased viability in both breast cancer lines in a concentration- and time-dependent manner and significant differences could be observed between 24 and 48 h treatment in both cell lines except in 10 μM treatment, where viability was below 10 % after 24 h. In agreement with the view that TNBC is more chemotherapy-resistant than HR+BC, the MDA-MB-231 cells were more resistant against HO-5114 treatment than the MCF7 cells.

5.2 Determination of the type of HO-5114-induced cell death

The type of HO-5114-induced cell death was determined using flow cytometry. The cells were treated exactly as for the viability measurement for 24 h, and then they were double-stained with fluorescein isothiocyanate (FITC) conjugated Annexin V and propidium iodide (PI). The latter enters the cell if the plasma membrane is disrupted, binds to the double-stranded DNA, and becomes intensely fluorescent, indicating necrosis. The former binds to phosphatidylserine, a marker of apoptosis when it is on the plasma membrane's outer layer. Double positivity indicates late apoptotic/dead cells. In MCF7 cells, HO-5114 treatment increased the ratio of early—and to a much higher extent—late apoptotic/dead cells on the expense of live cells in a concentration-dependent manner in the whole concentration range tested. In contrast, <5 μM concentrations of HO-5114 did not have a significant effect on the MDA-MB-231 cells; however, 10 μM of HO-5114 had a pronounced effect. It lowered the live cell ratio to 25% while increasing the ratio of early and late apoptotic/dead cells to 10% and 65%, respectively.

5.3 Effect of HO-5114 on reactive oxygen species generation

In many cases, anti-neoplastic agents induce ROS production in cancer cells. Accordingly, we studied HO-5114-induced ROS production in human breast cancer lines using the dihydrorhodamine 123 assay after 4 h treatment. At a 10 μM concentration, which lowered the viability of both human cancer lines to less than 10% of that of the untreated control, HO-5114 caused ROS production to the extent of about 1.7 and 2 times of the untreated control in the TNBC and HR+BC lines, respectively. Treatment with lower concentrations of HO-5114 that still induced a massive decrease in the viability of both cell lines caused no or only slight cellular ROS production, suggesting that the induction of oxidative stress was unlikely involved among the mechanisms of HO-5114′s cytotoxicity. Increasing HO-5114′s concentration to 20 μM elevated ROS production proportionally in both cell lines, indicating that ROS production was likely far from the saturation level under these conditions.

To investigate the role of oxidative stress induction in the anti-neoplastic effect of HO-5114, we studied how an antioxidant affects HO-5114′s cytotoxicity in BC cells. To this end, we treated the MCF7 and MDA-MB-231 cells with 1, 2.5, 5 or 10 μM HO-5114 for 24 h in the presence or absence of 1 mM N-acetylcysteine (NAC) and then measured the viability using the SRB assay. We could not observe any effect of NAC on HO-5114′s cytotoxicity in the case of the HR+BC line MCF7. In contrast, in the TNBC line MDA-MB-231, NAC significantly increased the viability of the control cells as well as the cells treated with up to 5 μM HO-5114; however, at a 10 μM HO-5114 concentration, there was no difference in viability between cells treated in the presence and absence of NAC.

5.4 Effect of HO-5114 on ΔΨm

HO-5114 is targeted to the mitochondria due to its diphenyl phosphonium component. Therefore, we studied whether it affects ΔΨm by measuring the JC-1 fluorescence. Based on its cationic properties, JC-1 is taken up by the mitochondria in a ΔΨm-dependent manner. In healthy mitochondria, it forms red fluorescent J-aggregates. Mitochondrial damage results in decreased ΔΨm, leading to a lower accumulation of JC-1 in the form of green fluorescent monomers, while the fluorescence disappears when the ΔΨm dissipates completely. After merely a 1 h treatment, HO-5114 at the concentration of 1 μM caused a significant drop in the ΔΨm of MCF7 cells, while increasing the drug’s concentration to 2.5 μM resulted in a massive ΔΨm loss indicated by the almost complete disappearance of the red fluorescence of

JC-1. The MDA-MB-231 line was more resistant to HO-5114; the same concentrations triggered basically the same changes in the ΔΨm that were observed for the MCF7 cells, but it necessitated 2.5 h of treatment rather than 1h only.

5.5 Effect of HO-5114 on mitochondrial energy production

Due to the increasing importance of energy metabolism among the pathomechanisms of cancer, we studied the effect of HO-5114 on the mitochondrial energy production of MDA-MB-231 and MCF7 lines using the Seahorse XFp Cell Mito Stress Test Kit. The device simultaneously measures the real-time cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), indicators of mitochondrial respiration and aerobic glycolysis, respectively. The cells were treated with 1 or 2.5 μM HO-5114 for 4 h, while OCR and ECAR were monitored during the last 75 min of treatment. Basal respiration was recorded for 15 min, and then the FoF1 ATPase inhibitor oligomycin was administered to assess ATP production. After another 20 min of recording, mitochondrial electron transport and ATP synthesis were uncoupled from each other by adding carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) to determine maximal respiration. After an additional further 20 min of recording, mitochondrial respiration was blocked by adding rotenone and antimycin A, inhibitors of Complex I and III of the mitochondrial respiratory chain, to determine proton leak and non-mitochondrial oxygen consumption.

From the recorded raw data, the Seahorse instrument generated multiple parameters of cellular energy metabolism that were all diminished by HO-5114 treatment except the proton leak, which was not affected in either cell line. Furthermore, coupling efficiency that indicates how tightly respiration is coupled to ATP synthesis was not affected in the MCF7 line but was decreased in the MDA-MB-231 line. The parameters of cellular energy metabolism associated with mitochondrial oxygen consumption, such as basal respiration, maximal respiration, and ATP production, were lower in the TNBC cells than in the HR+BC cells. Furthermore, 1 and 2.5 μM HO-5114 decreased these parameters to about the same extent for the latter cell line, while it affected them in a concentration-dependent manner for the former. Administration of the ATP synthase inhibitor oligomycin diminished OCR, which was accompanied by an elevation in ECAR in both cell lines. HO-5114 at a concentration of 1 and 2.5 μM reduced ECAR to about the same extent in the MCF7 line, while it increased and decreased ECAR compared to the untreated control at 1 and 2.5 μM, respectively.

Similar to the viability studies, we investigated the effect of NAC on the energy metabolism of untreated and HO-5114-treated BC cells. To this end, we included 1 mM NAC in a set of HO-5114-treated cells throughout the experiment. In the presence of NAC, the effect of HO-5114 on all parameters of cellular energy metabolism except the proton leak was reversed, in a higher extent for the MDA-MB-231 line than for the MCF7 line. In the MCF7 cells, HO-5114 decreased the proton leak that was further decreased in the presence of NAC. In contrast, HO-5114 increased the proton leak of the MDA-MB-231 cells that was further increased in the presence of NAC.

5.6 Effect of HO-5114 on colony formation

A colony formation assay was performed to assess the proliferation capacity of MCF7 and MDA-MB-231 cells treated with different concentrations of HO-5114. The cells were cultured in the presence of 50, 75, 100 or 250 nM of HO-5114 for seven days, and then the colonies were stained and counted. The drug effectively reduced colony formation in a concentration-dependent manner in both cell lines. Interestingly, the TNBC line was more sensitive to the treatment than the HR+BC line; 250 nM HO-5114 completely eradicated the MDA-MB-231 cells, while it allowed the survival of about 10 colonies of MCF7 cells.

5.7 Effect of HO-5114 on invasive growth

Cell proliferation, migration, and invasion are important in understanding tumor progression and metastasis formation. We used the xCELLigence Real-Time Cell Analysis method to assess the effect of HO-5114 on the invasive growth characteristics of MCF7 and MDA-MB-231 cells. The instrument measures electron flow transmitted between gold microelectrodes fused to the bottom surface of a microtiter plate in the presence of an electrically conductive culturing medium. Adherent cells cultured in the plates change the impedance expressed as arbitrary units called the cell index, the magnitude of which is dependent on number, morphology, size, and attachment properties of the cells. The cells were cultured in the presence of 75, 100 or 250 nM of HO-5114 for seven days, while the cell index was monitored in real-time. The drug effectively reduced the cell index in a concentration-dependent manner in both cell lines. At the highest concentration (250 nM), HO-5114 decreased invasive growth close to the detection limit in both cell lines. Similar to the colony formation experiments, the TNBC line was more sensitive to the treatment than the HR+BC line.

6. DISCUSSION & CONCLUSION II

TNBC is considered to have a poorer prognosis and a more limited targeted therapy repertoire than the HR+ subtype . Additionally, the energy metabolism of the two breast cancer subtypes differs profoundly, which is indicated by the opposite effect of mitochondrial rescue on glycolytically inhibited HR+BC and TNBC cells; it is negative for the former and positive for the latter. Accordingly, mitochondria-targeted compounds that compromise mitochondrial energy production may prove effective in the therapy of TNBC. Mito-CP was reported to deplete the cellular ATP level, to inhibit mitochondrial oxygen consumption, to affect mitochondrial morphology, and to dissipate ΔΨm. As a component-derivative of Mito-CP, HO-5114 was expected to have similar mitochondrial effects. The drug exceeded these expectations because 10 μM of HO-5114 suppressed viability to about the same extent as 50 μM Mito-CP during a 24 h exposure. In complete agreement with these previous results, in the present study, we found that even 1 μM of HO-5114 decreased the viability of both human breast cancer lines by more than 35%, while it almost completely suppressed it at a 10 μM concentration. At a longer exposure time (48 h), the drug's anti-proliferative effect became more pronounced in both the HR+ and the TNBC lines.

Mitochondria affect cancer cell survival through at least three major mechanisms: energy production, the intrinsic apoptotic pathway, and ROS generation. These three pathways are interrelated because apoptosis is an energy-dependent process, while energy shortage and the resulting decrease in ΔΨm leads to the release of pro-apoptotic intermembrane proteins, such as cytochrome c, an apoptosis-inducing factor, and endonuclease G. ROS damages the mitochondrial electron-transport chain and thus the ATP production, while the compromised electron-transport chain produces more ROS. ROS activates apoptosis via damaging macromolecules and interfering with the pro-apoptotic signaling pathways. This explains why a substantial induction of apoptosis after a 24 h exposure to 10 μM of HO-5114 in the TNBC line was observed, while lower concentrations of the drug were ineffective in this respect. In

contrast, and in full agreement with the widely accepted view that TNBC is more apoptosis-resistant than HR+BC, even 1 μM of HO-5114 induced massive apoptosis in the MCF7 line.

ROS participates in mediating cancer phenotype remodeling that manifests in apoptosis resistance and increased metastatic properties. The chronic hypoxia prevalent in solid tumors results in the constant activation of the HIF1α transcription factor that induces a malignant transformation associated metabolic remodeling; however, we found a very similar extent of HO-5114-induced ROS formation in the MCF7 and MDA-MB-231 lines, although the latter represents a higher stage of metabolic transformation than the former. The moderate increase in ROS accumulation in response to an increased HO-5114 concentration to 20 μM also indicated that ROS production in the BC lines was insensitive to HO-5114 treatment, contrary to the expectation. The difference in conditions between solid tumors and the cell culture, where uniform oxygen and fuel supply is provided, may account for the discrepancy between the expected and observed ROS production. Elevated ROS production is considered to be necessary for survival and growth of TNBC cells in vitro, therefore, antioxidants are expected to hinder their survival. However, we found that the antioxidant NAC increased the viability of control MDA-MB-231, while it did not affect MCF7 cells, indicating a higher ROS level that impeded proliferation in the former. The viability promoting effect of NAC overcompensated for the cytotoxic effect of HO-5114 at the concentration of up to 5 μM, but at 10 μM, it failed to do so. The absence of NAC's effect on HO-5114′s cytotoxicity in the HR+BC line indicated not only a reduced chronic oxidative stress in it compared to the TNBC line but also suggested differences in metabolic reprogramming between the two BC cell lines.

The driving force for ATP synthesis is provided by ΔΨm; however, it has additional essential roles, such as transporting nuclearly encoded mitochondrial proteins, transporting K^+, Ca^{2+}, and Mg^{2+}, generating ROS, mitochondrial quality control, and the regulation of pro-apoptotic intermembrane protein release. Cell survival essentially relies on the maintenance of ΔΨm. Accordingly, in ischemic situations, the F_oF_1 ATPase can operate in reverse mode and consume ATP to maintain ΔΨm to rescue the cell. The ATP is supplied by the substrate-level phosphorylation of non-glucose substrates under these conditions; however, considering the amount of the available non-glucose substrate pool, this survival attempt is often futile. In solid tumors, the cancer cells must adapt their metabolism to the chronic hypoxia and partially ischemic situation. In contrast to ROS induction, we observed a very sensitive response of ΔΨm loss to HO-5114 treatment. Even 1 μM of the drug induced significant changes in ΔΨm during as short a treatment as 1 h for the MCF7 line and 2.5 h for the MDA-MB-231 line.

Cancer cells face a double challenge in producing enough energy and a sufficient metabolic intermediate for proliferation in a predominantly hypoxic and partially ischemic environment. Mostly, they rely on glycolysis rather than mitochondrial oxidative phosphorylation, even if sufficient oxygen is available for the latter. Accordingly, increased glucose uptake is a characteristic feature of tumors that is used to identify them by ^{18}F-deoxyglucose positron emission tomography for diagnostic purposes. On the other hand, the most malignant cancer types, such as metastatic tumor cells, therapy-resistant tumor cells, and cancer stem cells, rely on mitochondrial ATP synthesis. The survival, proliferation, and metastasis of these cells depend on the oxidative phosphorylation and form the basis of their therapy resistance. Accordingly, for the most malignant cancer types, oxidative phosphorylation is an emerging therapeutic target, and drugs significantly affecting tumor cell metabolism may have therapeutic value. Considering its effects on energy metabolism in human breast cancer lines, HO-5114 fulfills this criterion. At a 1 and 2.5 μM concentration, it significantly diminished all OCR-related parameters in both cell lines except coupling efficiency. HO-5114 at a 2.5 μM concentration reduced ATP production that could contribute to the drug's anti-metastatic property. In complete agreement with its effect on the viability of BC lines, NAC counteracted the inhibitory effect of HO-5114 on the various parameters of cellular energy metabolism except the proton leak. These data support the conclusion that HO-5114 affects the energy metabolism of the BC lines. The proton leak can indicate damage to the mitochondrial respiratory chain or regulation of mitochondrial ATP synthesis via uncoupling proteins (UCPs). Indeed, the role of UCP2 in regulating the balance between substrate-level and oxidative phosphorylation has recently been reported. We found that both BC lines increased ECAR, i.e., substrate-level phosphorylation when oxidative ATP production was blocked by oligomycin. ECAR in the MDA-MB-231 line even returned to its initial rate when the oxidative phosphorylation was uncoupled by FCCP, demonstrating that the balance between the two ATP producing machinery is more responsive in the TN than in the HR+BC cells.

The hormone receptor status determines the cell proliferation, differentiation, and cancer progression properties of breast cancers. Accordingly, the MDA-MB-231 line represents a more aggressive, apoptosis- and therapy-resistant phenotype than the HR+ MCF7 line. The results of the aforementioned experiments that involved 1–24 h exposure to HO-5114 were in line with this view; however, in the colony formation and invasive growth experiments, where the cells were exposed to a 50–250 nM concentration of the drug for seven days, MDA-MB-231 proved to be more sensitive to the treatment than the MCF7 line. The reason for this

difference in sensitivity to HO-5114 treatment between short- and long-term exposure is not clear based on the experiments.

In conclusion, all data acquired in this study indicated that HO-5114 had a robust anti-neoplastic effect on cultured BC cells. Furthermore, resistance to HO-5114 treatment did not differ markedly between the HR+ and TNBC lines. The latter even seemed to be more sensitive to the drug in models involving long-term treatment; however, in vitro cell culture effects translate poorly to human therapy. Accordingly, to establish the therapeutic potentiality of HO-5114, follow up experiments have to be performed in animal models for determining its in vivo toxicity and anti-neoplastic effectiveness.

7. SUMMARY

Breast cancer is a major cause of death among women worldwide. Therapeutic options have broadened in the last decades, however there is a subtype of breast cancer which doesn't have targeted therapy. Triple-negative breast cancer is an aggressive phenotype with poorer prognosis.

In the first part of the study olaparib, oxaliplatin and PI3K inhibitors and their combination were examined. Olaparib is a frequently used PARP inhibitor for patients with BRCA mutations while oxaliplatin is a third generation platinum agent. Previous studies showed synergy of PARP inhibitors with platinum compounds, giving us the idea to investigate their effects on the two cell lines. In this study estrogen receptor-positive and progesterone receptor-positive MCF7 cells and triple-negative MDA-MB-231 cells were used to investigate their response to a new combination of drugs and to a novel Mito-CP derivative. The combination of these two compounds were augmented by PI3K inhibitor LY294002 to enhance the cytotoxic effect of oxaliplatin and to prevent the cytoprotective effect of olaparib. However our findings show a lack of synergy between them.

Therefore, in the second part of the study the novel Mito-CP derivative, HO-5114 was under investigation. There is a growing interest in targeting the mitochondria, as they are recognized targets for treating aggressive, metastatic and chemoresistant tumors. Mito-CP showed cytotoxic properties in various cancer cells, including breast cancer. Our study confirmed a significant anti-neoplastic effect of HO-5114 in both cell lines, interestingly the TNBC cell line was more sensitive to long-term treatment than the HR+ cell line.

PUBLICATIONS OF THE AUTHOR

Andreidesz K, Koszegi B, Kovacs D, Bagone Vantus V, Gallyas F, Kovacs K. Effect of Oxaliplatin, Olaparib and LY294002 in Combination on Triple-Negative Breast Cancer Cells. Int J Mol Sci. 2021 Feb 19;22(4):2056
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Andreidesz K, Szabo A, Kovacs D, Koszegi B, Bagone Vantus V, Vamos E, Isbera M, Kalai T, Bognar Z, Kovacs K, Gallyas F Jr. Cytostatic Effect of a Novel Mitochondria-Targeted Pyrroline Nitroxide in Human Breast Cancer Lines. Int J Mol Sci. 2021 Aug 20;22(16):9016
IF: 5.924

SCIENTIFIC ACTIVITIES

Presentations on international conferences

Kitti Andreidesz, Krisztina Kovács: *Investigating the effects of a PARP inhibitor (HO3089) on monocrotaline-induced pulmonary hypertension in rat lung tissue*. Medical Conference for PhD Students and Experts of Clinical Sciences, Pécs, 27th October 2018

Kitti Andreidesz, Krisztina Kovács: *The effect of a PARP inhibitor, Olaparib in combination with Oxaliplatin treatment on breast cancer cell lines* XII. International and XIX. National Interdisciplinary Grastyán Conference, Pécs, April 4-5 2019

Kitti Andreidesz, Krisztina Kovács, Balázs Sümegi, Ferenc Gallyas: *Investigating the effects of a PARP inhibitor (HO3089) on monocrotaline-induced pulmonary hypertension in rat lung tissue.* Interdiszciplináris Doktorandusz Konferencia, Pécs, 24-25 May 2019

Kitti Andreidesz, Krisztina Kovács: *Investigating the modifications of signaling pathways in monocrotaline-induced pulmonary hypertension in rat lung tissue by PARP inhibitor HO-3089.* International Student Congress, Graz, Austria, 30th May – 1st June 2019
Kitti Andreidesz, Krisztina Kovács: *The effects of olaparib and oxaliplatin on breast cancer cell lines.* Medical Conference for PhD Students and Experts of Clinical Sciences, Pécs, 9th November 2019

Krisztina Kovacs, Kitti Andreidesz, Dominika Kovacs, Balazs Koszegi, Viola Bagone Vantus, Antal Tapodi, Balazs Veres, Ferenc Gallyas: *New therapeutic approaches in the treatment of triple-negative breast cancer.* 17th International Conference on Cancer and Cancer Therapy, Webinar, 12th November 2021

Poster presentation on international conference

Kitti Andreidesz, Krisztina Kovács, Balázs Sümegi, Ferenc Gallyas: *Investigating the effects of a PARP inhibitor HO-3089 on signaling pathways in monocrotaline-induced pulmonary hypertension in rat lung tissue.* Hungarian Molecular Life Science Conference, Eger, 29-31 May 2019

Poster presentation on national conference

Andreidesz Kitti, Kovács Krisztina, Sümegi Balázs, ifj. Gallyas Ferenc: *A PARP inhibitor HO-3089 hatása jelátviteli útvonalakra pulmonáris hipertóniában.* 49th Membrane Transport Conference, Sümeg, 14-17 May 2019

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‘God doesn’t give you what you want… He creates the opportunity to do so.’