Targeting tumor hypoxia in combined therapeutic strategies in breast and ovarian cancer

PhD thesis

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1. Introduction

Breast cancer (BC) is the second most common subtype of cancer when considering the total population and first when taking into account female patients only. It is still the leading cause of death among women suffering from cancer. Comparing the incidence of ovarian cancer (OC) with breast cancer, a significantly lower incidence rate was observed (3.4%). However, the low incidence of ovarian cancer is responsible for nearly 5% of all cancer deaths in the female population.

Several factors interplay behind the high mortality of cancer. The major contributor to the lethal outcome of the disease is metastasis formation. It occurs upon tumor cells migrating from the primary tumor site to distant organs. This process is promoted by several factors, including environmental factors such as tumor hypoxia.

Tumor hypoxia is a common feature of solid tumors and a determining factor of the tumor environment. The rapid proliferation of tumor cells leads to elevated oxygen and nutrient demand. However, the inadequate vasculature of solid tumors is often incapable of supplying this. The key factor of the adaptation to poor oxygenation is the hypoxia-inducible factor 1 (HIF-1). In a low oxygenated environment, a transcriptionally active HIF-1 complex is formed (HIF-1α - HIF-1β), which binds

to the hypoxia-responsive elements of the promoter region of nearly 100 target genes, supporting their transcription. Consequently, HIF-1 is responsible for the activation of numerous gene cascades, involved in tumor progression. HIF-1 mitigates the immune system as well by enhancing the expression of programmed death ligand 1 (PD-L1).

According to the databases of The Cancer Genome Atlas (TCGA), the mRNA expression of HIF-1 is associated with the overall survival of BC and OC patients. Considering the underlying gene cascade of HIF-1 activations, the transcription factor is an attractive drug target. Therefore, several HIF-1 inhibitors have been developed. Even though the promising preclinical results, the HIF1- inhibitors serially failed in Phase II trials.

Targeting HIF-1 is essential to prevent metastasis formation and immune suppression, and due to the failure of HIF-1 inhibitor monotherapies, an absolute need for a strategic solution is inevitable. Combined therapeutic approaches were considered to overcome the limitations of HIF-1 inhibitor monotherapies.

Considering the severe public health impact of breast and ovarian cancers, we have opted to develop novel therapeutic strategies against these malignancies, focusing on tumor hypoxia targeting.

2. Objectives

The general objective of the research was to study the hallmark of hypoxia and the impact of an oxygen-deprived environment on tumor progression. Another goal was to circuit possible ways to target tumor hypoxia, the underlying pathways, and the microenvironment.

In this study, we aimed to:

(1) Study the impact of tumor hypoxia on crucial cellular processes and set up a suitable *in vitro* testing system for multidrug studies.

(2) Investigate the efficacy and safety of the combination of acriflavine (ACF) and paclitaxel (PTX) in *in vitro* and *in vivo* systems.

(3) Address hypoxia-related alterations and the compensatory effect of the drugs on tumor cell proliferation, migration, invasion, and metabolism.

(4) Observe the possibilities of indirect EMT targeting.

(5) Analyze the rationale behind simultaneously applying HIF-1 inhibitors and ICIs.

3. Methods

3.1. Cell culturing

MDA-MB-231, HS578T, 4T1, MDA-MB-453, OVCAR-8, SKOV-3 and OVCAR-3 cell lines were used. Cells were incubated at either atmospheric (normoxia, 21%) or at 1% oxygen level (hypoxia), at 37°C next to 5% CO_2 level.

3.2. Antiproliferative assessment of single and combinatorial drug effects and drug-drug interaction analysis

The antiproliferative activity of acriflavine and the acriflavine and paclitaxel drug combination was assessed by Sulforhodamine B (SRB) assay. The drug-drug interactions were assessed by SynergyFinder 3.0 according to the user guide of the software.

3.3. Western blotting

Acriflavine, paclitaxel, and cobalt (II)-chloride ($CoCl_2$) treated MDA-MB-231 cells and powdered frozen xenografts were fixed then precipitated protein was dissolved. The following antibodies were used: HIF-1α, E-cadherin (ECAD), and phosphorylated protein kinase B (p-AKT). Secondary anti-rabbit or anti-mouse antibodies were applied, then signal was

developed. The signal was analyzed with a densitometric approach by Image Lab software.

3.4. Sample processing for proteomic analysis

$2x10^5$ 4T1 cells/well were seeded and then treated with 0.5 μM acriflavine and incubated either at hypoxia or at normoxia. Protein was extracted by using a lysis buffer. The proteins were digested using S-Trap™ 96-well plate.

3.5. Nano-scale liquid chromatographic tandem mass spectrometry analysis (nLC-MS/MS)

The nLC-MS/MS analysis was performed on an Orbitrap Exploris 480 mass spectrometer coupled to a Vanquish Neo nano ultra-performance liquid chromatography system. MS raw files were analyzed using Spectronaut vs18.4. Pathway analysis was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8 tool. Pathway enrichment was assessed using the Reactome database.

3.6. Wound healing assay

MDA-MB-231 cells were seeded then wound was created. Acriflavine, paclitaxel, and rolipram mono- and combination therapies were applied. The cell migration was monitored by MuviCyte™ live-cell Imaging System.

3.7. *In vivo* hypoxia model establishment

Animal experimental procedures were approved and performed by the guidelines of the Institutional Animal Care and Use Committee at the National Institute of Oncology (Budapest, Hungary), under animal housing density regulations and recommendations from Directive 2010/63/EU of the European Parliament and the Council of the European Union. Permission license for breeding and performing experiments with laboratory animals: PEI/001/1738-3/2015 and PE/EA/1461-7/2020. NOD.CB17-Prkdcscid/NCrCrl (NOD SCID) mice were used in the study. To test the hypoxia-inducible impact of $CoCl_2$ mice were treated in different administration routes with the agent. Finally, kidneys were dissected and subjected to N-Myc downstream regulated 1 (NDRG1) mRNA expression analysis by qPCR method.

3.8. *In vivo* toxicity and efficacy

$1x10^6$ MDA-MB-231 cells were inoculated in the mammary fat pad of female NOD SCID mice. When tumors reached 50-70 mm^3, mice were randomized and allocated into eight groups – every combination of acriflavine, paclitaxel, and $CoCl_2$. $CoCl_2$ was mixed with drinking water ad libitum. Acriflavine and paclitaxel were administered intraperitoneally.

3.9. Immunohistochemistry (IHC) analysis of tumor microarrays (TMA)

Tumor microarray was produced from freshly diagnosed, non-metastasized, treatment naïve, breast tumors. To assess HIF-1α expression on TMA, the immunohistochemistry method was performed by using HIF-1α antibody. PD-L1 immunohistochemistry was conducted by the Ventana PD-L1 (SP142) Assay. Slides were evaluated by the QuPath 0.5.1. software.

3.10. Statistical analysis

The statistical analysis was performed by GraphPad Prism 6 software. One-way ANOVA and Tukey's multiple comparisons test were used for comparing mRNA and protein expressions. Two-way ANOVA and Sidak multiple comparison tests were applied to compare the effects of $CoCl_2$. *In vivo* data and wound healing assays were evaluated by using the Kruskal-Wallis test. The p-values lower or equal to 0.05 were considered statistically significant.

4. Results

4.1. Antiproliferative assessment of acriflavine

ACF functioned as a potent breast and ovarian cancer cell proliferation inhibitor, with the mean IC_{50} of 700 nM and 1200 nM at normoxia and hypoxia respectively. In the cases of MDA-MB-231 and HS578T triple-negative breast cancer (TNBC) cell lines, the activity of the drug was hypoxia-independent. Therefore, a murine cell line, 4T1 was also analyzed. Acriflavine efficiently inhibited the proliferation of 4T1 cells as well.

4.2. Proteomic profile alterations induced by hypoxic and acriflavine treatment

Hypoxic treatment caused upregulation in pathways involved in metabolic processes and ECM remodeling. On the contrary, it downregulated key pathways involved in global translation, suggesting the metabolic switch to an exclusive translation of essential proteins. The acriflavine-altered pathways showed a similar pattern under hypoxic conditions. The drug upregulated pathways of Rho GTPase signaling, mitosis, and metabolism of essential molecules, suggesting interference with migration, cell proliferation, and cellular metabolism. It inhibited RNA and protein metabolism and global translation.

4.3. Molecular analysis of *in vitro* hypoxia induction and inhibition

Protein expression analysis was conducted in the prolyl hydroxylase inhibitor, $CoCl_2$ and acriflavine treated MDA-MB-231 cell line. $CoCl_2$ served as a trustable hypoxia inducer, while acriflavine proved its HIF-1 inhibitory potential. Moreover, ACF reduced the level of p-AKT, a key component of the phosphoinositide 3-kinase (PI3K) pathway involved in cell proliferation, and reversed the effect of $CoCl_2$ on ECAD, an epithelial marker.

4.4. Interaction assessment of acriflavine and paclitaxel

The drug combination was tested on six breast and ovarian cancer cell lines under hypoxic and normoxic conditions. It was proven to be efficient in proliferation inhibition in each cell line. In the majority of the cases, we identified additive interactions, and the most favorable outcome, a synergistic interplay of acriflavine and paclitaxel was observed in the case of the MDA-MB-231 TNBC cell line.

4.5. Migratory assessment of the combination treatment

Wound-healing assay was conducted under normoxic and hypoxic conditions and the wound-closing inhibitory potential was assessed. The migration was inhibited by the monotherapies

under both conditions, with a more notable impact under hypoxia. The ACF+PTX combination efficiently inhibited migration under both conditions, with a more prominent effect under hypoxia with supplementary rolipram.

4.6. Assessment of the *in vivo* hypoxia-inducible impact of $CoCl_2$

To assess the *in vivo* HIF-1 stabilizing property of $CoCl_2$ mice were subjected to $CoCl_2$ treatment via different administration routes. NDRG1, HIF-1 target mRNA expression was measured. Considering the elevated expression of NDRG1 and the good tolerability of $CoCl_2$ application in drinking water, we opted for this administration route.

4.7. *In vivo* safety and efficacy evaluation of the drug combination

Regarding toxicity assessment, we concluded that the application of the drugs either in mono- or combination therapy is tolerable.

The *in vivo* tumor-inhibitory potential of the drug combination was studied on orthotopic breast MDA-MB-231 xenografts. The combination therapy efficiently suppressed the tumor development both with and without $CoCl_2$. Macrometastasis formation was inhibited as well.

4.8. Molecular analysis of the orthotopic xenografts

Protein expression alterations of ECAD, HIF-1α, and p-AKT were studied in the MDA-MB-231 xenografts. The ECAD expression was suppressed by $CoCl_2$ application, while additional ACF and ACF+PTX treatments increased the ECAD level. The elevated HIF-1α and p-AKT levels caused by $CoCl_2$, were diminished by the combination treatment.

4.9. HIF-1α and PD-L1 co-expression assessment

Considering our promising findings with HIF-1 inhibitor applications in combination regimens, we aimed to incorporate state-of-the-art immune checkpoint inhibitors (ICIs) into our studies. To find the clinical rationale behind combining HIF-1 inhibitors with ICIs, we studied the co-expression of HIF-1α and PD-L1 in *in silico* systems and TMAs. We observed a positive correlation between the mRNA expression of HIF-1α and PD-L1 *in silico* using the TCGA Breast database. Then, this finding was verified by analyzing HIF-1α and PD-L1 protein expressions using the TMAs of treatment-naive patients. We identified a positive correlation between the two proteins. Considering these, we are eager to develop suitable preclinical models to test the simultaneous efficacy of the inhibitors.

5. Conclusion

Our results indicate the following findings and achievements:

(1) An *in vitro* hypoxia modeling system, suitable for studying the mutual drug effects, was established. Tumor hypoxia significantly altered the proliferation, metabolism, transcription, and translation processes in TNBC cells.

(2) A synergistic drug combination with good tolerability, acriflavine and paclitaxel effectively inhibited the proliferation of MDA-MB-231 cells, the growth of cell line-derived xenografts, and the formation of macrometastases. Additionally, the drug combination presented an additive effect in the vast majority of the tested breast and ovarian cancer cell lines.

(3) Acriflavine modulated the protein expression of multiple pathways, effectively counteracting hypoxia-induced effects.

(4) Acriflavine inhibited EMT by altering EMT markers and Rho GTPase signaling of 4T1 TNBC cells. The administration of an additional EMT inhibitory compound, rolipram remarkably suppressed the migration of TNBC cells under hypoxia.

(5) We found the rationale behind combining HIF-1 inhibitors and ICIs in breast cancer treatment.

6. Bibliography of the candidate's publications

6.1. Publication related to the thesis:

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6.2. Other publications

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