Warsaw, June 23rd, 2020

Anna Sobiepanek, M.Sc.
Chair of Drug and Cosmetics Biotechnology
Faculty of Chemistry, Warsaw University of Technology

Abstract of the PhD Thesis

„Application of label-free methods in the diagnostics and prognostics of metastatic melanoma"

Supervisor: Tomasz Kobiela, Ph.D., D.Sc.

Co-supervisor: Małgorzata Milner-Krawczyk, Ph.D.

Melanoma skin cancer develops from melanocytes, cells responsible for producing the pigment melanin, which in the human body protects the skin against solar radiation - the main risk factor of melanoma development. Taking into account that melanoma skin cancer quickly gains the ability to metastasize and there is no effective treatment for its advanced form, novel diagnostic and prognostic tools gain the highest attention. Especially due to the need to obtain a quantitative result of the analysis, which does not require the opinion of an experienced histopathologist. Nowadays, the popularity of label free-methods for the evaluation of all kinds of biological samples including whole cells and tissues has an upward trend. However, establishing a good measuring system with well-defined interactions is of a challenge. Thus, detailed knowledge about the properties of normal, cancerogenic and highly aggressive metastatic cells is a matter of consideration.

The application of specific cell structural components as biomarkers is an obvious selection. In the presented thesis the focus of interest was on glycans present on the surface of the cells. Melanoma undergoes the epithelial-to-mesenchymal transformation during the tumor progression and development of malignancy. This process is connected with abnormal glycosylation of several important proteins present on the cell membrane, which are responsible for cell adhesion, proliferation and migration. The properties of lectins to specifically recognize and bind to the surface glycans allow their use as molecular probes. This approach together with the usage of label-free methods can be used to estimate stages of malignancy and establishing the novel diagnostic procedures as well as developing treatment strategies.

The aims of the thesis were to (1) establish label-free methods for distinguishing cells at different stages of melanoma progression based on changes in their glycosylation profile, (2) verify if the established methods will enable the correct classification of cells from patients

with diagnosed melanoma metastasis, as well as (3) modulate the glycosylation profile of cells by using the selected biologically active compound (the endocannabinoid - Anandamide) as a potential therapeutic agent.

In the literature part of the thesis, the general characteristic of melanoma skin cancer was performed; which includes subtypes, epidemiology, risk factors, staging, traditional diagnostics and treatment. Next, several models of melanoma frequently used for studies were presented with some details concerning their establishment. Novel methodology concentrated on cancer research was also specified with the focus on labeling and label-free techniques. Furthermore, the important issues concerning the glycosylation of cells were described as well as the endocannabinoid system, which is considered as a potential therapeutic target.

The research part of the thesis, was divided into three parts to cover the aims of the thesis. Thus, in the first chapter regarding the results, the characterization of the commercial cell lines used as models was performed. It included the study of the cell growth rate, morphology and mechanical properties by means of the atomic force microscopy (AFM). The first attempt to differentiate cells from various progression stages of melanoma was performed with the lectin-blot technique. For the study, the lectin Concanavalin A (Con A), specific against mannosyl and glucosyl sugar residuals, was selected. To follow the lectin-glycan interaction in real-time, the quartz crystal microbalance with dissipation monitoring (QCM-D) was applied and two approaches of cell preparation for the measurements were established: the cell-based sensors and the suspension cell-based sensors. The crucial parameters influencing the lectin binding to glycans present on the cells' surface were defined and tested. The set procedures were then used for analyzing seven different commercial cell lines: the normal cells (melanocytes, HEMa-LP), cells from the primary sites (WM35 from the radial growth phase (RGP) and WM115 from the vertical growth phase (VGP)) as well as metastatic cells from the lymph nodes (WM266-4 and MeWo) and from the solid tumor site (A375-P and G-361). Cells from various progression stages of melanoma were distinguished based on the calculated affinity of lectin to glycans present on the cells as well as the newly defined parameter - the viscoelastic index (VI). Con A lectin presented a higher affinity to glycans on metastatic cells than to glycans on the normal cells and cells from the primary sites. The VI parameter describes the viscoelastic properties of the created lectin-glycan complexes on the cells and may show changes in the occurring interaction. The VI value was obtained from the raw data by plotting dissipation energy as a function of frequency (the so-called Df plots) and calculating the absolute value of the received slope curve. With this

simple calculation the distinction between normal, RGP, VGP and metastatic cells was received. Several other techniques were also included for the results comparison like: lectin-ELISA assay, the microscale thermophoresis and AFM, which in this case was adapted for measurements by the tip functionalization with Con A lectin. All the gathered results confirmed that the distinction of melanoma cells from different stages of progression due to the study of lectin interaction with glycans present on the cells was possible. Moreover, the established procedure which uses the suspension cell-based sensors was much quicker than the traditional one with cells grown on the surface of the sensor. Although the number of analyzed cell lines in general was limited, the received results show that the viscoelastic index value as well as affinity may be used as prognostics factors.

In the second chapter presenting the results, the required steps for the verification of the established method with the cell-based sensors were described. For the isolation of cells from two biopsies of patients with confirmed melanoma metastasis, the cooperation with scientists from the Medical University of Warsaw was established and a consent for this study was received from the Local Bioethics Committee. By adapting a novel non-enzymatic procedure of cell isolation, I was able to established cell lines from the lymph nodes (biopsy 1 - M1, M7, M16 and M28 cells; biopsy 2 - M9). These new cell lines presented different characteristics during the analysis of their growth rate, morphology and mechanical properties. In all cases, the typical melanoma biomarkers were confirmed by using the immunohistochemical staining (S-100 and HMB-45) and the qPCR (*MLANA*). Next, the lectin binding to glycans present on the self-isolated cells was performed with the QCM-D measurements and results were confirmed by the lectin-ELISA assay. The results obtained by measuring lectin-glycan interaction on the self-isolated melanoma cells confirmed the usability of the established procedure to classify the cells as melanoma with a different glycosylation profile (difference in VI and K_D values). Thus, they were classified as cells with a possibly different metastatic potential (low or high) what was also observed by the growth rate of these cells. That is why, the established label-free procedure may be recommended for practical use in the additional diagnostics and prognostics of melanoma.

The last chapter concerning the results, was focused on an attempt to modify glycosylation on metastatic melanoma cells with the applied compound, which could show its potential usability in cancer treatment. Anandamide (AEA) was selected as a compound with various effects on the cells including disturbance in the glucose uptake and inhibition of the cells' migration rate. However, its influence on the glycosylation profile of cells has not been

investigated yet. The influence of AEA (in DMSO as a solvent) on the commercial melanoma cell lines was evaluated by the MTT assay, cell staining and measurements of the mechanical properties of cells. The concentration of 1 μM AEA was selected, and next the interaction study of lectin to glycans on cells treated with anandamide were carried out. The results obtained with the QCM-D method suggest the AEA treatment may force the metastatic cells to change their glycosylation profile. To further investigate this result, cell migration rate after the AEA-treatment was studied as well as the influence on the expression of the genes encoding crucial enzymes of the glycan biosynthesis pathway (*GFAT-1* and *DMP1*).

Considering all the above, with the usage of the QCM-D method and the two established measurement procedures with the cell-based sensors and the suspension cell-based sensors, I was able to distinguish all the investigated types of the cells (melanocytes, the primary cells of the radial growth phase and vertical growth phase and metastatic cells) by means of the lectin-glycan interaction analysis. These results were confirmed by the lectin-glycan interaction measurements with AFM, the lectin-ELISA assay and microscale thermophoresis. Furthermore, the introduced parameter - the viscoelastic index, as well as affinity seem promising new prognostic factors of melanoma. The established model was verified with the use of cells isolated from patients with confirmed metastasis. The modulation of the metastatic melanoma cells' glycosylation profile was confirmed by means of the lectin-glycan on cell analysis with the QCM-D, migration assay and the gene expression analysis. The established model, which uses the label-free methods for measurements of the lectin interaction with glycans present on the cell surface, has a potential to become a novel diagnostic and prognostic tool.

Keywords:
melanoma, cell isolation, glycosylation profile, lectins, QCM-D, endocannabinoids