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Molecular alterations associated with a BRAF inhibitor and an ER stress inducer

Present and possible future therapies of cutaneous melanoma

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The Examination is held online and starts at 11:00 on 18th May, 2021

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INTRODUCTION

Cutaneous malignant melanoma is the most serious type of skin cancer, and represent a high public health problem in different part of the world. Unfortunately, patient survival with melanoma is still very poor, especially for patients with metastatic lesions. The disease is caused by a combination of environmental (UV radiation) and inherited genetic factors.

Genetic landscape of human malignant melanoma

During the last two decades different molecular genetic methods, including fluorescence in situ hybridization, array comparative genomic hybridization, different next generation sequencing approaches were performed to define genetic/genomic alterations underlying melanoma development and progression. Different melanoma subtypes have different evolutionary routes that are associated with different genetic alterations. Based on aCGH data cutaneous melanomas with and without chronically sun-damaged skin and mucosal surfaces can be classified into multiple subtypes: (i) chronically-, (ii) intermittent-, (iii) minimal- sun exposure and (iv) protected from sun. Activating mutations in the *BRAF* oncogene are the most widespread genetic alterations observed in melanoma, with up to a 70 % incidence associated with intermittent sun exposure, the most frequent mutation consist of a single amino acid substitution of valine by glutamic acid at the 600-position ($BRAF^{V600E}$). This hotspot mutation leads to a ~500-fold increase in kinase activity, which constitutively activates the mitogen-activated protein kinase (MAPK) pathway. Targeted inhibition of the mutant $BRAF^{V600E}$ gene is one of the most promising therapeutic approaches for patients with unresectable or metastatic melanoma. Beside BRAF inhibitors, other successful treatment The advances in melanoma treatment have led to an increased median overall survival of patients with metastatic disease from ~ 9 months to over 2 years and, in some cases, have resulted in long-term remission. Even these new therapeutic approaches have significantly improved patient

survival, resistance to the BRAF inhibitors and the frequent side-effects of immunotherapies remain unsolved problems. It is urgently needed to discover the molecular background of drug resistance and find new therapeutic targets to improve the success of patient's survival.

Mitogen-activated protein kinase pathway

The mitogen-activated protein kinase (MAPK) pathway is one of the most important signalling cascade in eukaryotic cells. The pathway regulates molecular processes including cell proliferation, cell differentiation, apoptosis, cell survival, cancer cell dissemination, and resistance to different types of drug therapy. The activation of the pathway is triggered by growth factors after binding to their transmembrane receptor tyrosine kinases (RTKs) located on the cell surface in non-malignant cells. This binding will lead to the dimerization and auto-phosphorylation of these receptors. They will activate RAS which will activate RAF proteins; that will activate MEK. Activated MEKs phosphorylate and activate ERK1/2 cascade. ERK1/2 phosphorylate a variety of cytoplasmic translocate to the nucleus to control transcription of genes involved in cell proliferation

BRAF inhibitors

Numerous compounds were discovered since the discovery of the BRAF activating mutation, but only three inhibitor achieved clinical efficacy in melanoma: vemurafenib, dabrafenib, and encorafenib. The first type I inhibitor which entered to the clinical trials was vemurafenib. The development started at Plexxikon with a structure-guided drug identification and continued with the discovery of PLX4720 the tool compound of vemurafenib. At the same time another project led to the discovery of dabrafenib. The last in line type I inhibitor is encorafenib, an important properties of this compound compared to the others is having differential binding affinities for each protomer of asymmetric BRAF

dimers and a very slow off-rate from the BRAF enzyme. Currently, numerous "pan-RAF" inhibitors have entered the clinic (e.g. LXH254, TAK-632, etc.) which have broader activity on the RAF isoforms, in part through blocking dimeric RAF proteins. However, these compounds are all relatively early in clinical development. Further studies required to determine their efficacy, safety and the clinical applicability.

Resistance to BRAF inhibitors

Despite the significant response using selective BRAF mutant inhibitors most patients develop resistance and tumour regrowth. There are three chronologically distinct phases of resistance: (i) within a day, changing in cellular signalling leads to a new homeostasis; (ii) within a month, epigenetic, immuno-, and micro-environmental adaptation leads to tolerance; and (iii) after months (to years), genetic mutations result in outgrowth of resistant clones. BRAF inhibition direct consequence is the ERK dependent negative feedback can not be achieved, but as the tumour cells adapt to the presence of the inhibitor, and a new "steady state" is achieved.

Targeting ER stress in melanoma

Melanoma cells using numerous survival strategies, including induction of the unfolded protein response, which mediates resistance to endoplasmic reticulum (ER) stress induced apoptosis. These mechanisms can occur in many ways, like activation of oncogenes which suppresses ER stress induced apoptosis and/or upregulation of ER chaperones which increases the protein folding capacity of the cell. Both of them helps to avoid apoptosis for melanoma cells. Therefore, modulation of UPR could be a possible target in melanoma treatment and could be exploited in two ways. On one hand, blocking UPR components makes it impossible for the cell to restore its normal state. The other option is to overload

the already active UPR pathway. Both strategies drive the tumour cells to apoptosis.

Recently Cerezo et al. synthesized and characterized a new molecule family, thiazole benzensulfonamides (TZBs), which have anti-cancer properties. Based on their results, Cerezo et al. focused on one molecule of the family, named HA15, which was identified as the lead compound that induces elevated endoplasmic reticulum (ER) stress specifically in cancer cells without any adverse effects in normal cells. Cerezo et al. showed that the drug induces the death of all melanoma cells independently of the cell mutational status. Similar observations were reported for freshly isolated melanoma cells, independent of whether patients were sensitive or resistant to BRAF inhibitors. It was described that the ER protein BiP/GRP78/HSPA5 as being a specific target of HA15, describing the fact that interaction between the compound and BiP (binding immunoglobulin protein) enhances ER stress and leads to cell death via the concomitant induction of autophagy and apoptotic mechanisms in prostate, breast, colon, pancreas, glioma, cervical, and melanoma cells regardless of driver mutations or BRAF inhibitor resistance.

OBJECTIVES

The major focus of our study were to investigate the effect of two drugs targeting melanoma cells, with different molecular mechanisms. First we characterized the molecular background of a BRAF inhibitor: PLX4720 which is a vemurafenib analogue targeting the $BRAF^{V600E}$ mutated melanomas. We aimed to investigate effect of a recently synthesized anti-melanoma drug, named HA15, which (as was reported by Cerezo et al) displays anti-cancer activity not only against melanoma cells but also other liquid and solid tumours.

In details, during our study we aimed to

I. investigate the effect of a BRAF inhibitor (PLX4720) on two melanoma cell line pairs (primary tumour and metastasis-derived melanoma cell lines originated from the same patients),

- generate PLX4720-resistant cell lines,
- determine which genetic alterations, gene and protein expression are associated with the development of BRAFi resistance,
- compare the invasive properties of the parental and resistant cells
- examine the effect of drug withdrawal on cell proliferation in the resistant cell lines,

II. investigate the effect of the recently synthesized anti-melanoma drug (HA15) on the viability/proliferation of *BRAF*^{V600E}-mutant and resistant melanoma cells using different cell culture conditions,

- determine the gene expression differences of ER stress and autophagy markers under different cell culture conditions,
- generate HA15 resistant cell lines,
- compare the gene expression pattern of HA15 sensitive and resistant cell lines using RNA-seq analysis.

MATERIALS AND METHODS

Cultures of melanoma and melanocyte cell lines

All cell lines carried the BRAF^{V600E} mutation and were wild type for NRAS. Five cell lines (WM983A^{p1}/WM983B^{m1}; WM278^{p2}/WM1617^{m2} and A375) were obtained from the Coriell Institute for Medical Research. All cell lines were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum, 2 mmol/L glutamine, and 50 mg/mL gentamycin sulfate at 37°C. Melanocytes were

cultured as described by Godwin et al. Briefly, Plate at 30×10^4 cells per T25 flask and cultured in RPMI-1640 supplemented with 10% FBS, 200 nM 12-O-tetradecanoylphorbol 13-acetate (TPA), 100 pM cholera toxin (CT), 10 nM endothelin 1 (ET1), and 10 ng/ml human stem cell factor (SCF) and place the culture in a humidified, 37°C, 5% CO_2 incubator.

Development of PLX4720 and HA15 resistant cell lines and drug withdrawal experiments

The BRAF inhibitor (PLX4720) and HA15 resistant cell lines were developed in our laboratory using long term drug tereatment. These resistant cell lines were designated as: $WM983A^{RES}$, $WM983B^{RES}$, $WM278^{RES}$, $WM1617^{RES}$ and $WM983B^{HA15RES}$.

In drug withdrawal experiments cells from each of the resistant cell lines were seeded in a 24-well plate (in triplicate) and cultured in RPMI 1640 medium supplemented with 5 μM PLX4720 or 30 μM HA15 until cell attachment. Then, half of the cells from each cell line were switched from the drug-supplemented medium to vehicle control (DMSO, the solvent of drugs)-supplemented medium, while the other half of the cells remained in drug-supplemented medium for 72 hours. Viability was measured using WST-1 assay.

Cell viability assay

To define the viability of cells, the WST-1 cell proliferation reagent was applied according to the manufacturer's guidelines. Briefly, melanoma cells were seeded in 96-well and treated either with increasing concentrations of PLX4720 for 72 hours or with different concentrations of HA15 in triplicate; DMSO was used as a control during both study. Absorbance was measured at 440 nm using an Epoch™ Microplate Spectrophotometer. The reference absorbance was set at 700 nm. Cell viability was calculated by dividing the absorbance of the treated cells by that of the vehicle-treated (DMSO) control cells (considered 100%).

Matrigel in vitro invasion assay of the BRAFi sensitive and resistant cell lines

The invasive potential of melanoma cell lines was determined using BD BioCoat Matrigel Invasion Chambers. For the parental cell lines, the upper chamber of the insert was filled with 500 µl of cell suspension in serum-free medium. In the lower chamber 10% FBS containing medium was applied to as a chemoattractant. After a 24-hour incubation at 37°C, the cells in the lower chamber were fixed with methanol and stained with haematoxylin-eosin. The invaded cells were counted under a light microscope in 7 different visual fields at 200X magnification.

Flow cytometry

The effects of HA15 on WM983A cells were analysed by flow cytometry using Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit. Cells were treated with DMSO (control) and various concentrations of HA15 for 48 hours. Before drug treatment, we used starvation conditions for 14 hours. Cell pellets were resuspended in 1× Annexin-binding buffer. After adding 1 µL of Alexa Fluor 488 Annexin V and 1 µL of 100 µg/mL propidium iodide (PI) working solution to each 100-µL cell suspension, cells were incubated at room temperature for 15 min. After incubation, 400 µL of 1 × Annexin-binding buffer was added, and the stained cells were detected by flow cytometry measuring fluorescence emission at 530 and 575 nm.

Nucleic acid extraction, quality control, microarray hybridization, statistical analysis

RNA-based microarray experiments

RNeasy Mini Kit was used to isolate total RNA from the melanoma cell lines. Only samples with RIN value greater than 7.5 were considered in the hybridization to the Affymetrix Human Gene 1.0 microarrays. The labelling, hybridization and imaging setup were performed by UD-GenoMed Medical Genomic Technologies Ltd. The microarray data have been published in the Gene Expression Omnibus (GEO) repository under accession number GSE114443.

DNA-based microarray experiments

A G-spin™ Genomic DNA Extraction Kit was used to isolate DNA. The sample labelling, hybridization to Affymetrix CytoScan 750K microarrays and imaging setup were performed by UD-GenoMed Medical Genomic Technologies Ltd. The microarray data have been published in the GEO repository under accession number GSE114488.

Quantitative real -time PCR

The relative expression levels of the selected genes were determined by performing quantitative real-time PCR (qRT-PCR) using a LightCycler 480 Real-Time PCR System. Reverse transcription of the total RNA (600 ng) was performed using a High- Capacity cDNA Archive Kit. To perform qRT-PCR, SYBR Premix Ex Taq master mix was used. The qRT-PCR data were analysed using the Livak method (2DDCt), and glyceraldehyde-3-phosphate dehydrogenase (Hs9999 9905_m1) served as the reference gene.

Analysis of gene expression microarray data

In total, 9,652 of the 33,297 genes that passed the stringent filtering criteria were included in the analysis. Volcano plot filtering and paired t-tests with a random variance model were applied to reveal the differentially expressed genes among the parental and established resistant cell lines, and significance was considered at the nominal 0.05 level with at least a 2-fold change. The analysis was performed using BRB-ArrayTools and Hierarchical Clustering Explorer 3.5 (HCE) power analysis tool software.

DNA microarray analysis

DNA microarray data were analysed using Nexus Copy Number 6.1 software. To eliminate small copy number alterations (CNAs), the minimum number of probes per segment was set to 5. To detect the DNA copy number (CN) gains and losses, the following $\log_2$ ratio thresholds were set: ± 0.3 for gains and losses, 0.6 for high

CN-gains and -1.0 for homozygous deletions. Significantly different CN events between the parental and resistant melanoma cell lines were identified using a two-sided Fisher's exact test. To avoid sex bias, all probes on chromosomes X and Y were discarded.

RNA sequencing (RNA-Seq) and data analyses

Total RNA sample quality was determined using an Agilent BioAnalyzer with a Eukaryotic Total RNA Nano Kit according to the manufacturer's protocol. Samples with an RNA integrity number (RIN) were accepted for library preparation. cDNA li-braries for RNA-Seq analyses were prepared from 1 μg total RNA using an Ultra II RNA Sample Prep kit according to the manufactur-er's protocol. Library preparations and sequencing were performed at the Genomic Medicine and Bioinformatics Core Facility of University of Debrecen.

The RNA-Seq raw data have been deposited into the Sequence Read Archive database under accession number GSE164261.

Pathway analysis

The ToppFun tool ToppGene suite was used to detect functional enrichment of genes based on Gene Ontology pathways. The tool was used with default settings and a p-value cutoff of 0.05. For visualization of the molecular functional gene networks, we used the ClueGo tool kit of Cytoscape software with default settings and a p-value cut off of 0.05.

Protein expression analysis using Proteome Profiler Human XL Oncology Array Kit

A Proteome Profiler Human XL Oncology Array Kit was purchased from R&D Systems. The experiment was performed according to the manufacturer's protocol. The density of each duplicated spot was assessed using the ImageJ program and evaluated by subtracting the background. The density of the positive control was considered 100%.

Statistical analyses

SPSS 19.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The Shapiro-Wilk test was used to evaluate the normality of the data. Spearman's correlation coefficient was calculated to correlate the array comparative genomic hybridization (aCGH) and qRT-PCR data. To identify differentially expressed genes between conditions (parental-resistant, starved-not starved), we used the moderated t-test with the Benjamini-Hochberg FDR for multiple testing correction. A p-value less than 0.05 was considered statistically significant.

RESULTS

Growth inhibitory effect of PLX4720 on $BRAF^{V600E}$ mutant melanoma cell lines and effect of drug withdrawal on resistant cells

To define the growth inhibitory effect of a BRAFi, we treated two pairs of $BRAF^{V600E}$ mutant melanoma cell lines with the drug at various concentrations. A significant ($p < 0.001$) decrease in cell viability was observed above 500 nM PLX4720 in all cells.

The morphology of the developed resistant cells differed from that of the original cells. Withdrawal of PLX4720 from the cell cultures reduced cell proliferation in 3 resistant cell lines ($WM983A^{RES}$, $WM983A^{RES}$ and $WM983A^{RES}$). Interestingly, $WM278^{RES}$ cells exhibited growth promotion after drug withdrawal when compared to the continuously PLX4720 treated cells.

Invasive characteristics of the resistant cell line

The invasive properties of the sensitive cell lines were limited. In contrast, 3 of the four resistant cell lines ($WM983A^{RES}$ $WM983B^{RES}$ $WM278^{RES}$) showed increase invasiveness. Neither the WM1617 nor the resistant pair of this cell lines ($WM1617^{RES}$) were invasive based on the Matrigel assay. This observation was

further confirmed by the discovery of a significant correlation between well-known invasion/proliferation markers (*TCF4* and *LEF1* genes) using qRT-PCR (R=0.854; p=0.007).

Copy number alterations in the BRAFi sensitive and resistant melanoma cell lines

Copy number difference between the sensitive and resistant cell lines detected in all cell lines resistant to PLX4720 was found on a small part on chromosome 8q. This sequence contain the *EXT1* (8q24.11), *SAMD12* (8q24.12) and *REXO1L2P* (8q21.2) genes. In addition, we found increased copy number by aCGH on the 7q34 region in the $WM1617^{RES}$ cell line, this sequence harbour the BRAF oncogene.

Gene expression patterns of BRAFi sensitive and resistant cell lines

Unsupervised hierarchical clustering of the differentially expressed genes could distinguish between the resistant and sensitive cell lines. The number of unique genes with a > 2 fold change was 437 (204 genes were upregulated and 233 downregulated) in the resistant cell lines. The overexpressed genes are associated with growth factors, growth factor receptors, the extra cellular matrix (ECM), integrins and cell adhesion molecules. These genes play important roles in 151 biological processes based on Gene Ontology (GO) classification, including angiogenesis, blood vessel development, cell migration and wound healing. Downregulated genes were involved in pigmentation, melanocyte differentiation and lipid metabolic processes.

Protein array analysis of the BRAFi sensitive and resistant melanoma cell lines

Numerous differentially expressed proteins were observed in the resistant cell lines compared to their sensitive counterparts. Importantly, the increased expression of six proteins (ANGPLT4, EGFR, Endoglin, FGF2, Serpin E1, and

VCAM-1) and decreased expression of two proteins (OPN and Survivin) were consistently detected in all resistant cell lines.

Investigation the effect of HA15 on $BRAF^{V600E}$ mutated BRAFi sensitive and resistant melanoma cell lines

Effect of HA15 treatment on the viability of normal melanocytes

We first determined the effect of HA15 on normal human melanocytes using normal cell culture condition. Our results clearly show that the viability of melanocytes decreased significantly ($p \leq 0.05$) even after low-dose HA15 (10 μM) treatment, and increasing the drug concentration up to 100μM the cell viability further decreased. To simulate the cell culture conditions that were used in the original manuscript, melanocytes were cultured without FBS (altogether for 62 hours), after 14 hours starvation 10 μM HA15 was added and incubated for 48 hours. In contrast to normal cell culture conditions, the viability of the starved melanocytes decreased below 60% without any drug, and adding 10 μM HA15 the cell viability decreased below 45%.

Starvation for 14 hours before drug treatment resulted in a significant decrease in cell viability in all melanoma cell lines. However, if we replaced the medium with complete medium containing 10 μM HA15, we observed that the cells recovered completely after 48 hours. This clearly shows that the viability of cells were significantly influenced by the starvation condition and the drug had no effect under this condition. This observation was opposite to the published data. We observed that the viability of WM983A cells did not decrease significantly after 10 μM HA15 treatment for 48 hours but that WM983B cells were sensitive to both compounds, as we detected before. We observed the same effect for the compounds if those were originated from different companies. A dramatic decrease in cell viability was observed only at 50 M and 100 μM of HA15 independently from where the HA15 compound was originated from.

Effect of long-term starvation on A375 melanoma cell line viability

To investigate the effect of long-term starvation on cell viability, A375 melanoma cells were cultured without FBS for 14 hours and then with and without FBS for more 48 hours as well as with 10 μM HA15. Although the morphology of the cells was not influenced by drug treatment under normal culture conditions, after 62 hours of starvation, cell viability decreased significantly. In contrast, 10 μM HA15 treatment of the starved A375 cells resulted in a tremendous decrease in cell viability.

Effect of HA15 treatment on apoptosis induction in the WM983A melanoma cell line

Because HA15 concomitant induce apoptosis and autophagy, we determined the rate of the viable, apoptotic, dead and necrotic cells after HA15 treatment using the Annexin V-FITC apoptosis kit. Based on the flow cytometric data, it is clear that HA15 treatment did not influence either cell viability nor the rate of apoptosis at 10 μM compared to DMSO-treated control cells. These data show that HA15 did not induce apoptosis at a concentration of 10 μM HA15 and did not affect viability.

Effect of serum withdrawal and HA15 treatment on stress and autophagy marker expression in the A375 melanoma cell line

HA15 treatment induced ER stress and autophagy was determined under different experimental conditions at different time points in the A375 cell line. First the cells were starved for 14 hours and then i.) further cultured without FBS ii.) without FBS + 10 μM HA15 and iii.) and the gene expression level of three main stress markers (*CHOP*, *XBP1* and *BIP*) were determined. The relative gene expression of the three stress markers were similar to the control after 14 hours of FBS withdrawal. However, after 38 hours, expression of the markers increased in the starved cells without or with HA15 treatment; in contrast to the cells grown

and treated under normal cell culture conditions, only a slight increase was observed. The highest expressions were for the XBP1 gene in the starved cells (almost 50x fold change: FBS-), and expression increased significantly after 38 hours in the presence of 10 μM HA15 and was high at the endpoint.

Whether HA15 induces autophagy we choosed four autophagy associated markers (*DRAM1*, *P62*, *ATG5* and *ATG*) and determined the gene expression levels after HA15 treatment under normal and starved cell culture conditions. We found that pre-starvation (FBS-for 14 hours) did not induce significant expression changes in these genes. After 38 hours of starvation, the expression level of all four genes increased, the highest relative expression was measured for the DRAM1 gene. When incubating the starved cells with 10 μM HA15, the expression of two genes (*p62* and *ATG5*) increased significantly, at the same time notable expression changes of autophagy markers were not observed in cells grown in complete medium even after 48 hours of drug treatment. Overall, expression patterns of autophagy marker genes were similar in starved cells treated with HA15 at the time of 38 and 62 hours. In contrast, only *DRAM1* gene expression was elevated after HA15 treatment in cells growing in complete medium.

Development and characterization of HA15-resistant melanoma cell lines

We selected the most HA15-sensitive cell line (WM983B) to develop this drug resistant cell line and treated the cells continuously with 20 μM and 30 μM HA15 under normal cell culture conditions. After ~10 weeks, two resistant cell lines were developed. The viability of the resistant cells ($WM983B^{HA15RES30\mu M}$) was not affected by continuous HA15 treatment (30 μM HA15), except when concentration was increased to 50 μM. On the contrary, the sensitive WM983B cell line showed a significant decrease in viability at both concentrations.

We determined the expression level of the *BiP* gene in the sensitive WM983B and in both resistant cell lines ($WM983B^{HA15RES20\mu M}$ and $WM983B^{HA15RES30\mu M}$). The

HA15 resistant cell lines showed significantly increased *BiP* gene expression compared to the parental WM983B cells.

Effect of starvation on gene expression in the WM983B melanoma cell line using RNA-Seq analysis

We determined the gene expression signature of the starved cells and compared cells growing under normal cell culture conditions. RNA-Seq data revealed 6531 up- and 4890 downregulated transcripts in the WM983B melanoma cells growing under starved culture conditions. The highest expression difference was observed for the *RP11-134F2.8* gene (217-fold change). Two starvation-related genes, *SLCO4C1* (32-fold change) and *PIK3IP1* (14-fold change), were also highly expressed in the starved cell population. By molecular functional characterization of the upregulated genes genes were enriched in calcium ion binding, ion channel activity, and ion transmembrane transporter activity Pathways significantly associated with candidate genes in the WM983B melanoma cell line after 14 hours of FBS starvation are associated with plasma membrane structures and pathways involved in cholesterol and steroid biosynthesis, calcium signalling pathways and activation of gene expression by SREBF pathways.

Identification of differentially expressed genes in HA15-resistant melanoma cell lines using RNA-Seq analysis

RNA-seq analysis revealed 2,802 downregulated genes that are related to the extracellular matrix, integrin, collagen, microtubule, DNA replication origin binding and DNA helicase activity and are involved in the cell cycle, mitotic prometaphase, resolution of sister chromatid cohesion and DNA strand elongation pathways. The highest expression in the WM983B^{HA15RES} melanoma cell line was the protein-coding gene PAPPA2 (Pappalysin 2), and several highly expressed genes on the list are specifically linked to drug resistance, such as *ABCC9* and *IL13RA2*.

DISCUSSION

The discovery of mutations of the *BRAF* gene in different types of human malignancies has given fast improvement to the development of targeted therapies. The highest frequency of *BRAF* mutations (especially $BRAF^{V600E}$) is found in malignant melanoma, this mutation constitutes a therapeutic target for patients with advanced and metastatic tumours. These improvements have greatly increased the prognosis of patients with advanced melanoma; unfortunately, resistance to most of the drugs limits the number of patients with long-lasting responses. A large number of investigations have focused on identifying the molecular background of resistance, but regrettably, the leading mechanisms of resistance remain unclear.

The aims of our study were to investigate the growth inhibitory effect of a BRAF inhibitor (PLX4720) on melanoma cell lines, develop PLX4720-resistant cell lines and determine what kind of genomic alterations, proteins expression are associated with acquired BRAF inhibitor resistance. In parallel with these experiments, we have chosen a recently synthesized and characterized drug, named HA15, which was announced by the authors (Cerezo et al.) as an anti-melanoma drug. During our experiments we noticed that HA15 treatment conditions applied by Cerezo et al. was far from the optimal cell culture conditions, therefore we aimed to investigate the effect of HA15 on the viability/proliferation, gene expression on $BRAF^{V600E}$-mutant melanoma cells using different cell culture conditions. We successfully developed HA15 resistant cell lines that Cerezo et al failed and characterized gene expression differences between the HA15 sensitive and resistant cell lines.

The novelty of our study is that we developed BRAFi-resistant primary and metastatic melanoma cell lines originated from the same patients and analysed the alterations that arose during the development of drug resistance. Using a Proteome Profiler Human XL Oncology Array, we detected distinct protein signatures

associated with acquired BRAFi resistance. During our aCGH experiment, we found new, BRAFi resistance-associated CNAs that were characteristic of all resistant cell lines. We also observed that in addition to the development of drug resistance, melanoma cells developed BRAFi dependence. Three of the four resistant cell lines were more invasive than the original, sensitive cell lines. Melanoma and other types of tumour cells are known to display the EMT phenotype, which is adopted by cancer cells and is associated with increased invasiveness.

From a clinical perspective, drug dependency is an extremely important feature to consider during treatment. In our study, withdrawal of PLX4720 resulted in decreased cell proliferation in resistant cell lines, except for one. On the one hand, this finding is consistent with the hypothesis that resistant cells can develop drug dependency, which has high clinical relevance. Using aCGH we found new genomic alterations altered in all resistant cell lines on chromosomes 8q covering the EXT1, SAMD12 and REXO1L2P genes. All three genes exhibited elevated gene copy number. It was described that EXT1 regulates cancer cell stemness in doxorubicin-resistant breast cancer cells and promotes EMT-like behaviour.

We used a Proteome Profiler Human XL Oncology Array to analyse of the relative expression of 84 cancer-related proteins in all the cell lines. We found several differentially expressed proteins between the resistant and sensitive cell lines; however, more importantly, we observed that eight proteins (ANGPTL4, EGFR, ENDOGLIN, FGF2, SERPINE1, VCAM-1, Survivin and OPN) were differentially expressed in all resistant cell lines compared to the sensitive lines. One of the interesting findings of our study was that the expression of the OPN protein that was decreased in all resistant cell lines. OPN has recently emerged as a potentially valuable biomarker for diagnosing and treating cancers. Our findings are the first to suggest that decreased expression of OPN is clearly associated with acquired BRAFi resistance.

Beside the encouraging results which were obtained by different BRAF and MEK/ERK inhibitors, other drugs were also developed in order to avoid the development of drug resistance. Cerezo et al. developed the compound (HA15), which has high anti-cancer effect. Although the anti-cancerous effects of HA15 was well detailed by Cerezo et al., we noticed that the in vitro drug treatment conditions of melanoma cell lines were not optimal. Before drug treatment cells were starved (-FBS) for 14 h and this condition was kept during the whole experiment. We performed the experiment under normal and starved cell culture conditions using the same concentration of HA15 as it was published. We found that eight of our cell lines (including 4 BRAF inhibitor-resistant lines) were not sensitive to 10 μM HA15 treatment at all, in contrast, if cells were cultured without FBS, their viability decreased significantly after 14 hours of starvation In addition, we determined the expression of stress (CHOP, XBP1 and BIP) and autophagy (DRAM1, P62, ATG5 and ATG7) marker genes; and we were able to generate HA15-resistant cells, which Cerezo et al. did not.

FBS deprivation causes cell stress in many ways and is the most commonly used method of inducing cell stress. Nevertheless, the effectiveness of HA15 was determined after 14 hours of serum starvation, which would obviously alter the behaviour of cells and induce autophagy and apoptotic mechanisms; therefore, it is not an ideal experimental background to prove that these mechanisms are induced solely by HA15. We investigated the gene expression of 3 stress markers and noticed that the expression of the markers increased in the starved cells without or with HA15 treatment. Expression patterns of autophagy marker genes were similar in starved cells treated with HA15.

To determine the effect of 14 hours of starvation on gene expression, we performed RNA-Seq experiments and compared the gene expression patterns in WM983B cells growing in normal growth medium and after 14 hours of FBS starvation. The highest difference in expression was detected for the *RP11-*

134F2.8 gene (217-fold change). This gene encodes a novel DnaJ_C domain-containing protein of the ER that is involved in unfolded protein binding and is an important paralogue of DNAJB11 and a co-chaperone for BiP, which is a master regulator of ER.

Finally, yet importantly, contrary to the original publication, we were able to generate HA15-resistant cells. Interestingly, the HA15-resistant cell line shows characteristics similar to those of BRAFi-resistant cell lines. First, after withdrawal of the drug, the cells responded with decreased proliferation. Second, one of the resistance mechanisms to BRAF inhibitors is overexpression of the target BRAF gene. Similar to our previous results, we observed increased expression of the HA15 target gene *BiP* in a dose-dependent manner in HA15-resistant cell lines.

Finally, we hope our findings can assist in obtaining a more thorough understanding of the complex mechanisms of BRAFi resistance and helps clarify that HA15 may have a place in future therapy for melanoma**.**

MAIN FINDINGS AND RESULTS

The major focus of our study was to investigate the effect of two drugs targeting melanoma cells, with different molecular mechanisms.

Molecular alterations during the development of BRAF inhibitor resistance in melanoma cell lines

- We successfully established BRAFi-resistant primary and metastatic melanoma cell line models and characterized molecular alterations associated with acquired resistance.
- We described that drug withdrawal reduce cell proliferation in the resistant cell lines.

- Copy number alterations (gains of the *EXT1*, *SAMD12* and *REXO1L2P*) genes) in association with BRAFi resistance are new genetic alterations in melanoma.
- By proteome profiler analysis we described that the expression of 6 proteins (ANGPLT4, EGFR, Endoglin, FGF2, Serpin E1, and VCAM-1) increased in all resistant cell lines. One of the interesting findings of our study is that the expression of the OPN decreased in all resistant cell lines suggesting that decreased expression of OPN is clearly associated with acquired BRAFi resistance and may be unique, early marker of BRAFi resistance.

Is HA15 a selective anti-melanoma drug?

- We were able to generate HA15-resistant cell line and found that the HA15-resistant cells show similar features to BRAFi-resistant cell lines.
- In contrast to the published data, we did not detected significant melanoma cell death under normal cell culture conditions using HA15 treatment. The drug-induced anti-melanoma effect is due in part to the starvation of melanoma cells and not exclusively linked to the effect of the drug. Long-term starvation and HA15 treatment have synergistic effects on cell viability.
- By quantitative measurements of ER stress and autophagy markers we found that HA15 does not trigger stress alone but synergistically enhances ER stress under starvation conditions. We observed increased expression of the HA15 target gene BiP in a dose-dependent manner in HA15-resistant cell lines, suggesting that overexpression of the BiP gene might be involved to the development of HA15 resistance.
- After withdrawal of the drug, cells responded with decreased proliferation, probably because they developed not only resistance to HA15 but also drug addiction.

In conclusion, we successfully developed drug resistant melanoma cell lines using two drugs which acts through different molecular mechanisms. We found new genomic alterations and characterized the gene and protein expression patterns associated with the BRAF inhibitor resistant phenotype.

The new anti-melanoma drug HA15 and has only a moderate (not selective) effect on melanoma cell lines, In contrast with the published data, melanoma cells are able to develop resistance against HA15. Further studies are urgently need to clarify the anti-cancer effect of HA15.

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Registry number: DEENK/42/2021.PL
Subject: PhD Publication List

Candidate: István Szász
Doctoral School: Doctoral School of Health Sciences

List of publications related to the dissertation

1. **Szász, I.**, Koroknai, V., Patel, V., Hajdú, T., Kiss, T., Ádány, R., Balázs, M.: Cell Proliferation Is Strongly Associated with the Treatment Conditions of an ER Stress Inducer New Anti-Melanoma Drug in Melanoma Cell Lines.
Biomedicines. 9 (2), 1-19, 2021.
DOI: http://dx.doi.org/10.3390/biomedicines9020096
IF: 4.717 (2019)

2. **Szász, I.**, Koroknai, V., Kiss, T., Vízkeleti, L., Ádány, R., Balázs, M.: Molecular alterations associated with acquired resistance to BRAFV600E targeted therapy in melanoma cells.
Melanoma Res. 29 (4), 390-400, 2019.
DOI: http://dx.doi.org/10.1097/CMR.0000000000000588
IF: 2.75

List of other publications

3. Koroknai, V., **Szász, I.**, Hernandez, V. H., Fernandez, J. N., Cuenin, C., Herceg, Z., Vízkeleti, L., Ádány, R., Ecsedi, S., Balázs, M.: DNA hypermethylation is associated with invasive phenotype of malignant melanoma.
Exp. Dermatol. 29 (1), 39-50, 2020.
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Pathol. Oncol. Res. 26 (4), 2557-2566, 2020.
DOI: http://dx.doi.org/10.1007/s12253-020-00837-9
IF: 2.826 (2019)

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5. Balázs, M., Koroknai, V., **Szász, I.**, Ecsedi, S.: Detection of CCND1 Locus Amplification by Fluorescence In Situ Hybridization.
In: The Retinoblastoma Protein. Ed.: Pedro G. Santiago-Cardona, Springer Science+Business Media, New York, NY, 85-100, 2018.

6. Vízkeleti, L., Kiss, T., Koroknai, V., Ecsedi, S., Papp, O., **Szász, I.**, Ádány, R., Balázs, M.: Altered integrin expression patterns revealed by microarray in human cutaneous melanoma.
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Melanoma Res. 2, 100-107, 2016.
DOI: http://dx.doi.org/10.1097/CMR.0000000000000227
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