# Antitumoral effects of 9-cis retinoic acid and mitotane and evaluation of microRNAs in adrenocortical cancer xenograft models

Ph.D. Thesis

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Budapest
2016

## I. INTRODUCTION

Adrenocortical cancer (ACC, adrenocortical carcinoma) is a rare tumor type with very poor prognosis. Numerous problems are known in its diagnosis and treatment e.g. the lack of specific pre-operative biomarkers and the difficulty of the radiological differentiation. Considering its post-operative histological examination the differentiation between benign and malignant tissues is also difficult and requires great expertise. Furthermore, we are not aware of any effective treatment beside surgical treatment.

Currently, only one adrenal specific drug is known, the DDT derivate mitotane, which is used for more than 60 years in the clinical practice, without knowing its precise molecular way of action. In the chemotherapeutic treatment of metastatic ACC, the best result (14.8 months average survival) was reached with the combination of etoposid, doxorubicin, cisplatin and mitotane. Due to these reasons, intensive efforts are made to find novel effective agents.

In our previous *in silico* study, retinoid signaling via the retinoid X receptor (RXR) was identified as a major pathogenic pathway in ACC by functional genomic pathway analysis. Based on these results, an *in vitro* study

was performed with the specific ligand of RXR, with 9-cis retinoic acid, which decreased the hormone secretion and viability of NCI-H295R cells in a time and dose dependent manner and raised remarkable gene expressional changes. *In vivo,* in a small pilot NCI-H295R xenograft study, 9-cis retinoic acid decreased the growth of the tumor.

Our objective was to investigate the effect of 9-cis retinoic acid on ACC in a large-scale H295R xenograft model. Mice were treated with 9-cis retinoic acid, mitotane and their combination. After the treatment, pathological, genome, proteome and microRNA analysis were performed to find out the molecular mechanisms behind the anti-tumoral effect.

Expression of microRNAs are changed due to the treatments suggesting their potential use for treatment monitoring in future. This was investigated in an international collaboration, as well.

## II. OBJECTIVES

In my Ph.D studies, I analysed the correlation between adrenocortical cancer, antitumoral treatments and circulating microRNAs in several aspects. My objectives were:

1. How does the treatment of 9-cis retinoic acid, mitotane and its combination affect the tumor growth in NCI-H295R xenografts?
2. What kind of gene- and protein expressional changes are induced by the treatments?
3. Can circulating microRNAs be used for treatment monitoring?

## III. MATERIALS AND METHODS

### III.1. Cell culture

The human adrenocortical NCI-H295R cell line was obtained from the American Type Culture Collection. Cells were cultured in DMEM:F12 supplemented with $3{,}61*10^{-8}$ mol/l selenium, $1.92*10^{-5}$ mol/l linoleic acid, 0,001 mol/l insulin, $7{,}81*10^{-8}$ mol/l transferrin, $1.899*10^{-5}$ mol/l bovine serum albumin and adjusted to a final concentration of 1% HEPES, 1% Penicillin/Streptomycin, 2.5% Nu-Serum and 2.5% L-glutamine at 37 °C in a humidified 5 % $CO_2$ atmosphere. The medium was changed two or three times a week and subcultured once or twice a week.

### III.2. Xenograft model

43 male BALB/c SCID (severe combined immunodeficiency) mice aged 6-8 weeks with average weight between 21-23 g were injected subcutaneously with NCI-H295R cell suspension ($10^7$ cells/200 µl PBS). The 28 day long treatment was started when the solid tumor reached 3 mm mean diameter. Four groups were established: 1. control: 200 µl corn oil/day; 2. mitotane:

200 mg/kg/day; 3. 9-cis retinoic acid: 5 mg/kg/day; and 4. the combined form of mitotane and 9-cis retinoic acid. The tumors were measured twice a week by the same investigator. The volumes of the tumors were calculated by the following formula: (a*a*b*π)/6. At end of the treatments, the animals were killed by cervical dislocation in ether anesthesia. Tumors were removed and their weights were measured. One half of the tumor was fixed in formalin for histological and immunohistochemical examination, the other half was frozen in liquid nitrogen and stored at -80 $^{0}$C. Lungs, heart, kidneys, spleen and liver were also removed for histological analysis. Whole blood was collected, then plasma was isolated. All animal experiments were conducted according to the ethical standards of the animal Health Care and Control Institute, Csongrád County, Hungary, permit No. XVI/02037-2/2008.

### III.3. Histological and immunohistochemical analysis

Four μm sections of formalin-fixed paraffin-embedded tissues were dewaxed and processed either for hematoxylin-eosin (HE) staining or Ki-67 immunostaining. Ki-67 immunostaining. Different

regions of the viable xenograft tumor were annotated and Ki-67 positive and negative cells were individually marked on these regions of interests (ROIs). Proliferation index was given in percentages of positive cells. Ki-67 expression was scored by three independent pathologists in a blinded fashion.

### III.4. RNA isolation from tumor tissue

Total RNA was isolated from the frozen tumors with Qiagen miRNeasy Mini Kit according to the manufacturer's protocol. RNA concentration was measured with NanoDrop 2000 spectrophotometer. RNA integrity was determined by Agilent 2100 Bioanalyzer System.

### III.5. Messenger RNA (mRNA) expression profiling

Gene expression profiling was performed on 16 samples using a single-color array method by 4x44K Agilent Whole Genome Microarray slides according to the manufacturer's protocol.

### III.6. Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Seven genes were selected for validation by real-time RT-qPCR with Taqman gene expression assays: *MYC*, *APOA4*, *CXCR3*, *BAALC*, *PDE4A*, *PRDM1* and *TGFBI*. *ZNF625* was chosen as reference gene.

For the evaluation of the data we used the ΔΔCT method.

### III.7. Protein isolation

Tumor tissues were pulverized in liquid nitrogen and lysed in 1 ml of Lysis Buffer on ice for 30 minutes. Next, samples were centrifuged at 13000 rpm for 15 min. Supernatants were kept and protein concentrations were measured as described before by Bradford.

### III.8. Proteomics study

Equal amount of protein lysates were separated by SDS-PAGE on a 10% minigel and stained with colloidal Coomassie Brillant Blue. The gel lanes were cut to 10 pieces and their protein content were in-gel digested. The digestion reaction was stopped, and peptides were extracted from the gel and dried.

Samples were redissolved subjected to LC-MSMS analysis.

Mass spectrometry raw data were converted to MSMS peak list files using PAVA script. Peak list files corresponding to the same sample were zipped and searched against the human, mouse species specified UniProtKB random concat protein database. All database search were completed on our in-house ProteinProspector (ver. 5.14.1) (Baker, P.R and Clauser, K.R. http://prospector.ucsf.edu) search engine.

### III.9. Western-blot analysis

Based on our proteomic results and literature data, the SET protein was chosen for Western-blot analysis on 3 samples from each group of the xenograft study and on altogether 6 human adrenocortical samples (2 normal adrenal cortices, 2 adrenocortical adenoma and 2 adrenocortical cancer). Normal human adrenal cortices were obtained from patients operated for hypernephroma. The study on human samples was approved by the Ethical Committee of the Hungarian Health Council and informed consent was obtained from all patients involved. β-actin served as

loading control. Band density was measured by ImageJ software.

### III.10. Pathway analysis

To perform a functional pathway analysis the list of the significantly differentially expressed proteins were uploaded in the database of DAVID Bioinformatics Resources 6.7(https://david.ncifcrf.gov/).

### III.11. RNA isolation from plasma and exosome

Total RNA and exosome was isolated from 100 µl plasma samples for circulating and 130 with µl plasma samples for exosome microRNA analysis with Qiagen miRNeasy Serum/Plasma Kit and Total Exosome Isolation Kit according to the attached manufacturer's protocol amended with addition of a spike-in control microRNA *cel-miR-39*.

For this analysis, the blood samples of SW-13 and SJ-ACC3 xenograft model were also used, established by our international research partner (Dr. Constanze Hantel, Munich, Germany). In their study, the effectivity of EDP/M chemotheraputical treatment against its liposomal variant LEDP/M. were compared

### III. 12. RT-qPCR for circulating microRNA measurement

Based on our previous studies and literature data, by circulating microRNS isolated from NCI-H295R xenograft four, *hsa-miR-181b*, *hsa-miR-184*, *hsa-miR-210* and *hsa-miR-483-5p*, by exosomal microRNAs isolated from SW-13 and SJ-ACC3 xenograft two, *hsa-miR-210* and *hsa-miR-483-5p* was chosen for validation by qRT-PCR. *Cel-mir-39* was used as reference gene.

### III. 13. RT-qPCR for tissue microRNA measurement

Considering the results from the circulating microRNA measurements, tissue *hsa-miR-483-5p* (002338) was studied by RT-qPCR with Taqman miRNA assays according to the instructions of the manufacturer. Four reference genes were used: *U6*, *RNU6B*, *RNU44* and *RNU48*.

### III.14. Statistical analysis

For the statistical analysis of tumor growth, Mann-Whitney U test was applied (SPSS Statistics 20, IBM). The analysis of Ki-67 index was performed by One-way ANOVA followed by Tukey's post Hoc test or Kruskal-

Wallis ANOVA & Median Test (STATISTICA 7.0) depending on the results of the Saphiro-Wilks normality test. $P<0.05$ was considered to be significant.

The results from the microarray data were analyzed by Genespring 12.6 software (Thermo Fisher Scientific). A filter on expression at the $20^{th}$ percentile of raw signal values, then a 2-fold change filter was used, and One-way ANOVA was performed, followed by Tukey's post Hoc test without Benjamini-Hochberg false discovery rate calculation. RT-qPCR and proteomics data were analyzed by One-way ANOVA followed by Tukey's post Hoc test (STATISTICA 7.0).

Western-blot was evaluated by One-way ANOVA followed by Tukey's post Hoc test (STATISTICA 7.0).

## IV. RESULTS AND DISCUSSION

### IV.1. Analysis of xenograft tumor growth

The normalized tumor volume (relative to the first measured volume) was smaller in all treated groups than in controls during the whole experiment. At the end of our experiment, the average values of normalized tumor volumes were 22.48-fold, 9.3-fold, 12.3-fold and 8.22-fold higher in the control, mitotane treated, 9-cisRA-treated, and 9-cisRA+mitotane groups relative to the starting tumor volume, respectively. The reduction in tumor size has been significant in the mitotane only and 9-cisRA+mitotane groups.

### IV.2. Histology, Ki-67 scoring

Tumors in the 9-cis RA receiving groups appeared to be more differentiated compared to the control and mitotane groups by hematoxylin and eosin (H&E) staining.

The Ki-67 proliferation index was significantly lower in the 9-cisRA group in comparison to the control and mitotane groups. The lowest Ki-67 index was noted in the combined 9-cisRA+mitotane group (25.6 ± 4.09%) that was significantly smaller compared to all other groups

(p=0.00018 vs. control; p=0.00018 vs. mitotane; p=0.01684 vs. 9-cisRA).

### IV.3. Microarray analysis

We have found 483 significant gene expression changes after the statistical analysis, but only by omitting the Benjamini-Hochberg false discovery rate (FDR). By using FDR, only two significantly differentially expressed transcripts emerged whose biological relevance is unclear (LOC100996813, encoded a MATE2 variant, and LOC100268168, encoded an uncharacterized non coding RNA).

### IV.4. RT-qPCR validation

Seven genes have been selected for validation based on the microarray data chosen from genes with the highest positive and negative fold changes, taking into account literature data, as well. However, only two genes could be validated to be significantly differentially expressed. The well-known 9-cis retinoic acid target gene *APOA4* (apolipoprotein A4) turned out to be significantly overexpressed in the combined treated group relative to the control group (p=0.0045), whereas *PDE4A*

(phosphodiesterase 4A) appeared to be significantly underexpressed in the combined treatment group ($p=0.0024$). PDE4A, as a member of PDE (phosphodiesterase) family, act as crucial cAMP level regulator, and it is involved in the basic cell pathways and also takes part in cancer progression.

### IV.5. Proteomics analysis

47 significant protein changes have been found between the groups by mass spectrometry and bioinformatics.

### IV.6. Western blot

By considering literature data regarding the tumor biological relevance of these proteins, the SET protein was chosen for validation by Western-blotting. As an inhibitor of tumor suppressor PP2A (protein phosphatase 2A), SET has widespread impacts on the cellular functions including cell cycle, apoptosis and cell migration. Furthermore, it also influences ß-catenin, c-Myc and Akt pathways.

SET protein expression was smaller in all 9-cis retinoic acid treated groups relative to control, but only reached the level of significance in the combined treated group.

To look at the potential relevance of SET in human adrenocortical tumors, we have analyzed some human tissues in a preliminary study, and found that SET protein is undetectable in normal and ACA tissues, whereas it is expressed in ACC.

### IV.7. Functional pathway analysis

With functional pathway analysis we identified proteins in p53 and Wnt pathways, and several pathways connected to ribosomes or proteasomes.

### IV.8. MicroRNA expression

The expression of microRNAs were analyzed in three different xenograft models.

From the selected circulating miRNAs, *hsa-miR-483-5p* has been significantly underexpressed in the combined group relative to control ($p=0.028$) in the case of H295R xenograft. In SW-13 and SJ-ACC3 xenografts the exosomal microRNA *hsa-miR-210* was found to be significantly underexpressed in the LEDP/M treated group relative to control (in SW-13) or EDP/M treated (SJ-ACC3) group.

*hsa-miR-210* is the most significant hypoxamiR, whose expression levels increase during hypoxic conditions that are characteristic for many tumors including ACC. Its decreased expression might indicate treament efficacy.

Interestingly, we have not found significant changes in the expression of tissue *hsa-miR-483-5p.* The discrepancy in expression between tissue and circulating microRNAs is not unique, but its background is unclear.

## V. CONCLUSIONS

We have investigated the antitumoral effects of 9-cis retinoic acid alone and in combination with mitotane on adrenocortical cancer *in vivo* for the first time. We found that:

- tumor growth decreased significantly in the 9-cis retinoic acid treated groups, additionally, Ki-67 proliferation index was significantly smaller, as well.
- Only few gene expressional changes were found, and the overexpression of APOA4, and underexpression of PDE4A was validated in the combined treated group.
- By proteomics, 47 significant protein changes were found. Out of these, SET was chosen for validation by Western blot, and found underexpressed in combined treated group.
- The expression of *hsa-miR-483-5p* and *hsa-miR-210* decreased due to the combined treatment, raising their potential for treatment monitoring in the future.

## VI. PUBLICATIONS IN THE TOPIC OF THE THESIS

**Nagy Z**, Baghy K, Hunyadi-Gulyás E, Micsik T, Nyirő G, Rácz G, Butz H, Perge P, Kovalszky I, Medzihradszky KF, Rácz K, Patócs A, Igaz P. Evaluation of 9-cis retinoic acid and mitotane as antitumoral agents in an adrenocortical xenograft model. AMERICAN JOURNAL OF CANCER RESEARCH 5:(12) pp. 3645-3658. (2015) **IF: 3,425**

Jung S, **Nagy Z**, Fassnacht M, Zambetti G, Weiss M, Reincke M, Igaz P, Beuschlein F, Hantel C. Progress for a liposomal chemotherapy protocol against adrenocortical carcinoma. ENDOCRINE-RELATED CANCER 23:(10) pp. 825-837. (2016) **IF:4,472**

## IX. OTHER PUBLICATIONS

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