Aus der

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Direktor: Prof. Dr. med. M. Reincke

Development and implementation of novel therapeutic options against adrenocortical carcinoma

Habilitationsschrift

vorgelegt von

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Introductory Summary

The current clinical gold standard Etoposide, Doxorubicin, Cisplatin and Mitotane (EDP-M/ Berruti scheme) is not satisfying for the treatment of adrenocortical carcinoma (ACC). However, even though a field of highly active research, clinical translation of preclinically promising therapies were unfortunately disappointing in the past, indicating that also utilized tumor models might inadequately predicted clinical applicability of novel pharmacological approaches in the past. This project aimed at the development and preclinical testing of a large panel of anti-cancer strategies to identify new therapeutic treatment strategies. Such studies included classical chemotherapeutic agents in common use, nano-technologially modified formulations of appropriate parental drugs, immunoliposomes and also various molecular inhibitors, In an attempt to better reflect patient heterogeneities, in addition to the classical and commonly applied gold-standard model in this field for many years, NCI-H295, next-generation tumor models such as SJ-ACC3, MUC-1 and ACC115m/TVBF-7 have been implemented, characterized and newly developed during this project.

Introduction & Objectives

Adrenocortical carcinoma (ACC) is a rare and highly metastatic malignancy with an estimated incidence of 0.7- 1 cases per million population per year [1]. Initial diagnosis of ACC patients could be asymptomatic or can present with symptoms of a large, locally invasive primary tumor. Surgery remains the mainstay for treatment with a curative approach in patients with early diagnosis. However, many patients with ACC are diagnosed at advanced stages of disease, with invading tumors and/or distant metastasis. Moreover, even after radical resection, the prognosis remains poor. Currently the only curative option for localized ACC tumors includes the complete tumor resection, followed by adjuvant treatment with mitotane [1]. In 2012 combination regimens including the cytostatic drugs etoposide, doxorubicin and cisplatin together with mitotane (EDP-M) have been demonstrated in a prospective randomized interventional trial to result in higher response rate and longer progression free survival in comparison with streptozotocin and mitotane [2]. Upon this trial EDP-M was defined as current gold-standard for the treatment of late stage ACC patients. However, overall survival and response rates for ACC are still disappointing with an average survival from diagnosis of 15 months and 5-year survival of around 20% [2, 3].

Up to now, also different molecular targeted therapies have been tested against ACC, including treatments targeting epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), multityrosine –kinases or the insulin-like growth factor 1 receptor (IGF-1R) inhibitor, among others. However, these treatments did not result in better overall survival or progression free rates vs. placebo [4, 5]. Although e.g. activity of anti-IGF1-receptor inhibiting approaches had been successfully demonstrated in preclinical and early clinical studies [6-8], later clinical trials with such compounds as single agents were disappointing [5, 9]. Instead, such clinical studies have supported that IGF-1R targeted therapies might be a promising strategy for a subgroup of ACC patients with specific clinical and functional properties [5, 8, 10].

In addition to molecular inhibitors, in recent years also different nanotechnological approaches have been used to improve classical chemotherapies by increasing therapeutic efficacy and conversely reduce adverse effects. One important development in this regard were liposomes, which have been widely used for encapsulation of doxorubicin, vincristine, and platinum derivatives among others [11-13]. Of note, with encapsulation, the overall particle size

compared to the free parental drug increases which leads to a decrease in volume of distribution and thereby often to improved patient tolerability. Moreover, depending of the specific sub-type, liposomes often display slower releasing rates and subsequently sustained bioavailability of the encapsulated drugs. These features lead in sum, for classical liposomes to passively increased intra-tumoral accumulation as multiple passages through the tumor vasculature together with appropriate high interstitial pressure facilitate liposomal entrapment in this compartment [11, 14]. Finally, so-called immunoliposomes have the capability to combine previously mentioned molecular targeting and first generation liposomal drugs, as they are designed to mediate- upon specific surface modifications- a directed interaction with a specific cell target.

Of note, molecular and genetic profiling of surgical tumor specimen led also to the identification interesting new and specific biomarkers for ACC [15]. However, functional relevance in tumorigenesis and therapeutic outcome is up to today still largely unknown. Thus, the development of novel treatment modalities is still impeded by the strong heterogeneity among patients with regards to pathology, secretion patterns, genomics and signaling pathway alterations and the lack of appropriate preclinical models matching these sub-groups [2, 16, 17]. However, important prerequisite for new developments in this regard are preclinical tumor models. For example, xenograft models are important tools for such functional studies as they bear the potential to mimic the complexity of solid tumors including tumor cells, stroma and blood vessels. Unfortunately, in recent years only two human cell lines (NCI-H295 [18], SW-13 [19]) and one recently established human pediatric xenograft model (SJ-ACC3 [20]) were available for the preclinical investigation of ACC. The broad implementation of SW-13 is in this context furthermore questionable, as it is suggested to be not derived from an ACC primary tumor or metastasis, but from surgical material of a small cell carcinoma, which then metastasized in the adrenal gland. Moreover, even though NCI-H295 and SW-13 were commonly available cell lines in this field and furthermore applicable as tumor-xenografts, they originate from cell suspensions following long-term culture and there is good evidence that extensive passaging changes biological properties compared to the original patient tumor. To overcome such limitations, patient-derived tumor xenografts (PDTX) have been established and tested for a variety of cancer types [21]. Following the same approach the pediatric tumor model SJ-ACC3 was established [20]. However, no cell line for complementary in vitro experiments could be derived from this xenograft. Thus, as outlined above, in recent years the number of available tumor models for ACC was very limited and not able to reflect the clinically observed patient tumor heterogeneity as well as specific therapeutic responses of individual patients.

Aims

The overall aim of this project was to optimize current systemic therapies for ACC by the development/ testing of novel therapeutic strategies in settings, which furthermore aimed to set new standards in terms of implemented preclinical models.

Specific sub-aims were:

• the development and preclinical testing of various anti-cancer drugs, new formulations and also treatment schemes to identify new systemic treatment strategies for ACC patients.

• the related molecular investigation of relevant signaling pathways.

• to develop and apply at each step of the project appropriate preclinical state of the art models for ACC.

Own Work

This cumulative habilitation thesis focusses on the following outlined articles:

I. Liposomal doxorubicin-based treatment in a preclinical model of adrenocortical carcinoma. **Hantel C***, Lewrick F*, Reincke M, Süss R, Beuschlein F. *Equally contributing first authors. J Endocrinol. 2012 May;213(2):155-61.

II. Liposomal polychemotherapy improves adrenocortical carcinoma treatment in a preclinical rodent model. **Hantel C**, Jung S, Mussack T, Reincke M, Beuschlein F. Endocr Relat Cancer. 2014 Apr 28;21(3):383-94.

III. Liposomal doxorubicin for active targeting: surface modification of the nanocarrier evaluated in vitro and in vivo: challenges and prospects. Jakoby J, Beuschlein F, Mentz S, **Hantel C***, Süss R*. *Equally contributing senior authors. Oncotarget. 2015 Dec 22;6(41):43698-711.

IV. IGF1-R inhibition and liposomal doxorubicin: Progress in preclinical evaluation for the treatment of adrenocortical carcinoma. Beuschlein F, Jakoby J, Mentz S, Zambetti G, Jung S, Reincke M, Süss R*, **Hantel C***. *Equally contributing senior authors. Mol Cell Endocrinol. 2016 Jun 15;428:82-8

V. Preclinical progress and first translational steps for a liposomal chemotherapy protocol against adrenocortical carcinoma. Jung S, Nagy Z, Fassnacht M, Zambetti G, Weiss M, Reincke M, Igaz P, Beuschlein F, **Hantel C**. Endocr Relat Cancer. 2016 Oct;23(10):825-37.

VI. Targeting heterogeneity of adrenocortical carcinoma: Evaluation and extension of preclinical tumor models to improve clinical translation. **Hantel C**, Shapiro I, Poli G, Chiapponi C, Bidlingmaier M, Reincke M, Luconi M, Jung S, Beuschlein F. Oncotarget. 2016 Nov 29;7(48):79292-79304.

VII. Targeting the multidrug transporter Patched potentiates chemotherapy efficiency on adrenocortical carcinoma in vitro and in vivo. Hasanovic A., Ruggiero C., Jung S., Rapa I., Volante M., Terzolo M., Beuschlein F., Poinsard C., **Hantel C.** *, Lalli E. * and Mus-Veteau I. *Equally contributing authors. International Journal of Cancer 2018 Jul 1; 143(1):199-211. doi: 10.1002/ijc.31296. Epub 2018 Feb 23.

VIII. Heat Shock Protein 90 as a Prognostic Marker and Therapeutic Target for ACC. Siebert C, Ciato D, Murakami, Frei-Stuber L, Perez-Rivas LG, Monteserin-Garcia JL, Nölting S, Maurer J, Feuchtinger A, Walch AK, Haak HR, Bertherat J, Mannelli M, Fasnnacht M, Korpershoek E, Reincke M, Stalla GK, **Hantel C**, Beuschlein F. Front Endocrinol (Lausanne) 2019 Jul 19.

IX. In vitro cytotoxicity of cabazitaxel in adrenocortical carcinoma cell lines and human adrenocortical carcinoma primary cell cultures. Fragni M, Palma Lopez LP, Rossini E, Abate A, Cosentini D, Salvi V, Vezzoli S, Poliani PL, Bosisio D, **Hantel C**, Tiberio GA, Grisanti S, Memo M, Terzolo M, Berutti A, Sigala S. Molecular and Cellular Endocrinology 2019 Dec 1; 498:110585.

X. Targeted Gene Expression Profile Reveals CDK4 as Therapeutic Target for Selected Patients With Adrenocortical Carcinoma. Liang R, Weigand I, Lippert J,

Kircher S, Altieri B, Steinhauer S, **Hantel C**, Rost S, Rosenwald A, Kroiss M, Fassnacht M, Sbiera S. and Ronchi CL. Front Endocrinol (Lausanne), 2020 April 16; 11; 219.

XI. Cytotoxic Effect of Trabectedin in human adrenocortical carcinoma cell lines and primary cells. Abate A, Rossini E, Bonini SA, Fragni M, Cosentini D, Tiberio G, Benetti D, **Hantel C**, Lagana M, Grisanti S, Terzolo M, Memo M, Berutti A, Sigala S. Cancers (Basel), 2020 Apr 9; 12(4):928.

XII. Liver X Receptor Inhibition potentiates Mitotane-Induced Adrenotoxicity in ACC. Warde KM, Schoenmakers E, Martinez E, Lim YJ, Leonard M, Lawless, SJ, O`Shea P, Chatterjee KV, Gurnell M, **Hantel C**, Dennedy MC. Endocr Rel Cancer, 2020 Jun; 27(6):361-373.

XIII. Cytotoxic effect of progesterone, tamoxifen and their combination in experimental cell models of human adrenocortical cancer. Sigala S., Rossini E., Tamburello M., Abate A., Beretta S., Fragni M., Cominelli M., Cosentini D., **Hantel C**., Bono F., Grisanti S., Poliani PL, Tiberio GAM, Memo M., Berruti A. Frontiers Endocrinology 2021 Apr 26;12:669426.

XIV. Novel insights into the Molecular Regulation of Ribonucleotide Reductase in Adrenocortical Carcinoma Treatment. Bothou C, Sharma A, Oo A, Kim B, Perge P, Igaz P, Ronchi CL, Shapiro I, **Hantel C**. Cancers (Basel). 2021 Aug 20;13(16).

XV. Stimulated Expression of CXCL12 in Adrenocortical Carcinoma by the PPARgamma Ligand Rosiglitazone Impairs Cancer Progression. Cantini G., Fei L., Canu L., Lazzeri E., Sottili M., Francalanci M., Angelotti ML, De Filpo G, Ercolino T, Gelmini S, Mangoni M., Nesi G., **Hantel C**., Mannelli M., Maggi M., Luconi M. J Pers Med. 2021 Oct 27;11(11):1097. doi: 10.3390/jpm11111097.

Studies carried out

In an attempt to optimize the existing treatment modalities for ACC patients, the initial aim of the project was the investigation of a novel combinatory approach targeting the IGF1-receptor for the treatment of ACC. Previously, we had established an immunoliposomal agent by coupling an anti-IGF1-receptor inhibiting antibody (1H7) to the surface of liposomal doxorubicin [22]. In these experiments, we had provided first evidence for a putative therapeutic applicability of such 1H7-Liposomes against neuroendocrine tumors of the gastroenteropancreatic system (GEP-NETs), breast cancer, neuroblastoma and prostate cancer [22].

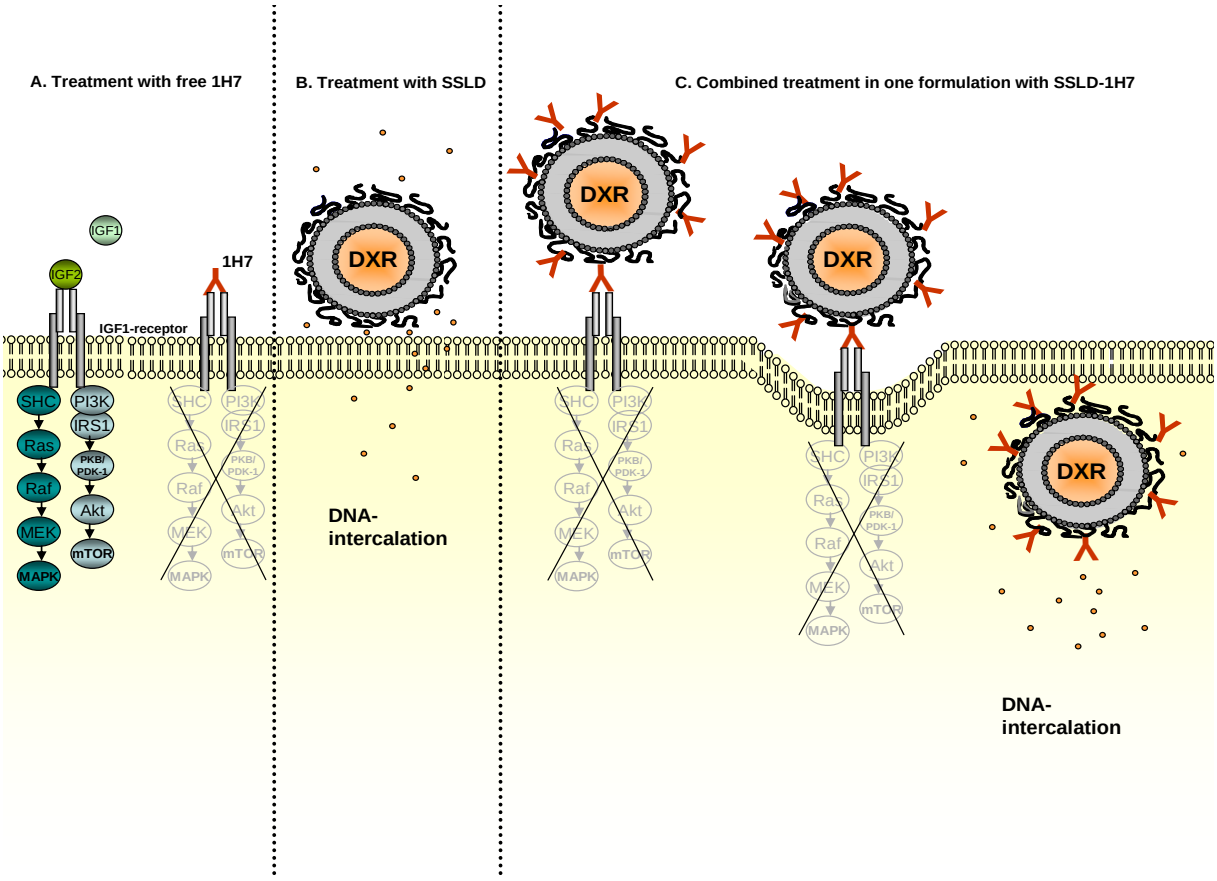

Figure 1: Schematic illustration of 1H7-Liposomes [22].

In two further studies, we extended our investigations to ACC. Firstly, NCI-H295R cells were used for in vitro association studies with different liposomal drugs and the appropriate formulations were then subsequently investigated on NCI-H295R tumor xenografts in pharmacokinetic and therapeutic experiments. A significant reduction in tumor size was detectable in NCI-H295R tumor-bearing mice after a single treatment with specifically anti-IGF1-receptor targeted immunoliposomes and – even though diminished- also for not-targeted sterically stabilized liposomal doxoribicin [23]. In a second study we used as drug-basis commercially available liposomal doxorubicin (Caelyx®) which was by us specifically post-modified with 1H7 [24]. Long-term therapy with multiple treatments led against NCI-H295R to significantly reduced tumor sizes for immunoliposomal formulations, but also for treatments which combined the single agents. As for the separate therapeutic arms preparations were clinically already available, these findings indicated that a clinical evaluation of a combined approach might be of interest for ACC patients.

Interestingly, the above mentioned latest generation of 1H7-Liposomes involved a technique, which allowed specific post-modifications of liposomal surfaces by pre-synthesized antibody-anchor conjugates. Such anchors could be furthermore utilized to facilitate personalized approaches for already commercially available liposomal drugs and could be also applied for a wide range of targets. In a next study, the applicability of this technique was investigated in more detail including the influence of different modification techniques, lipid composition and buffers, generally applied for liposomal preparations. These experiments provided detailed evidence that post-modification of commercially available liposomal agents for active tumor targeting is indeed possible and furthermore that lyophilisation represents an applicable method to obtain storable surface modifying antibody-anchor conjugate [25].

(**Hantel C***, Lewrick F*, Reincke M, Süss R, Beuschlein F. *Equally contributing first authors. *J Endocrinol. 2012 May;213(2):155-61*. Liposomal doxorubicin-based treatment in a preclinical model of adrenocortical carcinoma [23].

Beuschlein F, Jakoby J, Mentz S, Zambetti G, Jung S, Reincke M, Süss R*, **Hantel C***. *Equally contributing senior authors. *Mol Cell Endocrinol. 2016 Jun 15;428:82-8*. IGF1-R inhibition and liposomal doxorubicin: Progress in preclinical evaluation for the treatment of adrenocortical carcinoma [24].

Jakoby J, Beuschlein F, Mentz S, **Hantel C***, Süss R*. *Equally contributing senior authors. *Oncotarget. 2015 Dec 22;6(41):43698-711*. Liposomal doxorubicin for active targeting: surface modification of the nanocarrier evaluated in vitro and in vivo: challenges and prospects [25].

Moreover, we identified during the above outlined immunoliposomal studies an extraordinary uptake phenomenon of plain liposomes specifically by adrenocortical tumor cells [23]. These findings indicated that also the implementation of untargeted liposomal preparations could represent treatment modalities with particular advantage for tumors of adrenocortical origin.

Consequently, we tested in the NCI-H295R tumor model the systemic gold-standard for ACC (EDP-M) in comparison with regimens replaced with liposomal formulations of the parental drugs. In a first study, we provided preclinical evidence for enhanced and sustained anti-tumoral potential for LEDP-M (etoposide, liposomal doxorubicin, liposomal cisplatin plus mitotane) [26]. Short-term therapeutic treatment with LEDP-M led to a significant decrease in the total number of tumor cells and increase in the number of apoptotic cells in NCI-H295R xenografts, while no therapeutic effect was evident in animals treated with the classical EDP-M scheme. Long-term treatment with two therapeutic cycles indicated furthermore highly sustained anti-tumoral efficacy as well as improved off target profiles for the novel LEDP-M scheme [26].

Next, to obtain preclinical results with highest clinically predictive power, we investigated liposomal schemes of EDP-M in two further tumor models in vivo: the hormonally inactive SW-13 and the to that date recently established pediatric SJ-ACC3 tumor model [27]. Furthermore, we included liposomal etoposide resulting in a novel treatment scheme called L(l)EDP-M. Even though all treatment arms demonstrated therapeutic efficacy against SW-13 xenografts, this study revealed upon 1, 2 and 4 therapeutic cycles and by the analysis of different endpoints, highest anti-tumoral effects for L(l)EDP-M.

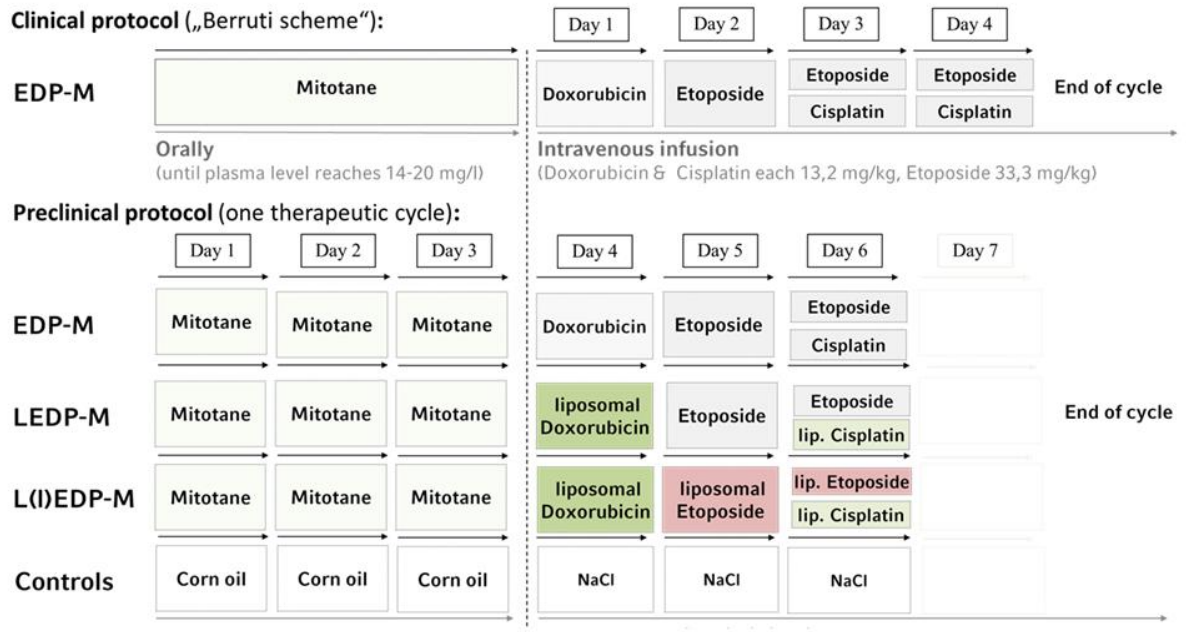

Figure 2: Schematic illustration of the novel chemotherapeutic regimens LEDP-M and L(l)EDP-M which were derived from the current clinical gold-standard EDP-M / Berruti Scheme [27].

Thereby, our previous study on NCI-H295R xenografts [26] and the subsequent study on SW-13 xenografts [27] predicted for adult ACCs at least comparable or improved therapeutic efficacies of liposomal variants compared with the current clinical gold-standard. In contrast, none of the investigated treatment schemes led to effective and sustained therapeutic effects against pediatric SJ-ACC3 xenografts.

However, of main impact for clinical benefit and furthermore rather ACC subtype-independent are of course also acute and long-term complications of systemic treatments. In accordance with the clinical situation, we detected nephrotoxic and cardiotoxic alterations in animals treated with multiple therapeutic cycles of the classical EDP-M scheme. However, no such effects were apparent upon treatment with the liposomal regimens LEDP-M or L(l)EDP-M. A subsequent study with a small number of ACC patients confirmed that administered liposomal drugs were overall very well tolerated [27]. Thus, with these experiments we provided successfully first translational steps for a clinical translation.

(**(Hantel C**, Jung S, Mussack T, Reincke M, Beuschlein F. *Endocr Relat Cancer*. 2014 Apr 28;21(3):383-94. Liposomal polychemotherapy improves adrenocortical carcinoma treatment in a preclinical rodent model [26].

Jung S, Nagy Z, Fassnacht M, Zambetti G, Weiss M, Reincke M, Igaz P, Beuschlein F, **Hantel C.** *Endocr Relat Cancer*. 2016 Oct;23(10):825-37. Preclinical progress and first translational steps for a liposomal chemotherapy protocol against adrenocortical carcinoma [27].

→ This work was awarded in 2017 by the German Endocrine Society with the "Novartis Young Investigator Award" to Sara Jung.)

As described above, the predictive clinical value of the implemented SW-13 tumor model was highly debated in the past as it lacks features of adrenocortical differentiation. Thus, in an attempt to improve the availability of preclinical models, also a novel tumor model for ACC was

established in this context [28]. Upon surgical implantation of ACC derived patient tumor specimen in athymic nude mice, one xenograft (MUC-1) showed particular engraftment properties and sustained tumor growth. MUC-1 tumor analysis revealed highly vascularized, proliferating and SF-1 positive xenografts.

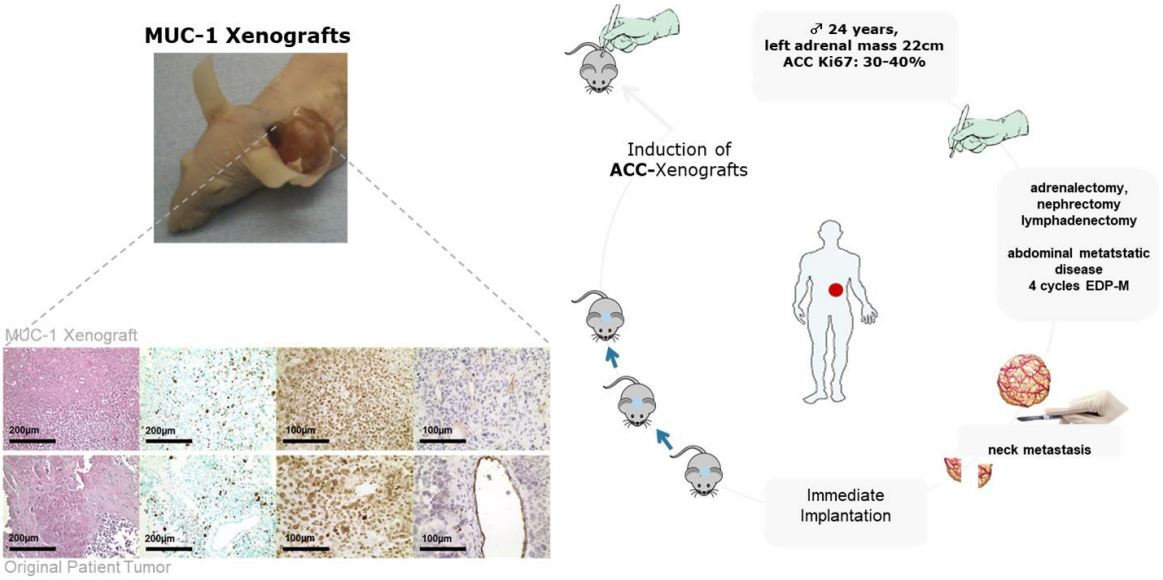

Figure 2: Schematic illustration of the induction of MUC-1 xenografts (modified from [28]).

In a next step, we characterized NCI-H295R, SW-13 and SJ-ACC3 xenografts for Ki67, IGF-1R, IGF2, SF-1 and EGF-receptor status in comparison to MUC-1 [24, 28]. In addition, we established a cell line, which demonstrates hormonal activity and specific phenotypical characteristics of ACC. With regards to therapeutic responsiveness, our experiments revealed drug resistance for MUC-1 cells against the clinical gold standard EDP-M, which was not observed for NCI-H295R cells [28]. This finding was not unexpected as the patient, from which MUC-1 was obtained, had received several cycles of EDP-M before development of metastases, while such treatment is not reported for NCI-H295R. Moreover, also therapeutic responsiveness upon 1H7-liposomal treatments was tested against NCI-H295R-, SJ-ACC3- and MUC-1- Xenografts, which all displayed different levels of IGF-1R and IGF-2 expression [24]. Overall, this study provided in accordance with these characteristics therapeutic efficacy of IGF-1R inhibiting approaches together with liposomal doxorubicin for NCI-H295R and SJ-ACC3, but not for MUC-1 thereby indicating highly important tumor heterogeneities as also frequently observed in clinical observations for ACC [24].

(**Hantel C**, Shapiro I, Poli G, Chiapponi C, Bidlingmaier M, Reincke M, Luconi M, Jung S, Beuschlein F. *Oncotarget. 2016 Nov 29*;7(48):79292-79304. Targeting heterogeneity of adrenocortical carcinoma: Evaluation and extension of preclinical tumor models to improve clinical translation [28].

→ This work was awarded in 2016 by the german endocrine society with the “Schoeller-Junkmann-Award” to Constanze Hantel.

Beuschlein F, Jakoby J, Mentz S, Zambetti G, Jung S, Reincke M, Süss R*, **Hantel C***. *Equally contributing senior authors. *Mol Cell Endocrinol. 2016 Jun 15*;428:82-8. IGF1-R inhibition and liposomal doxorubicin: Progress in preclinical evaluation for the treatment of adrenocortical carcinoma [24])

In further studies therapeutic responsiveness of NCI-H295R vs. MUC-1 was tested under various conditions involving the hedgehog receptor patched, heat-shock-proteins, CDK4 and 6, Liver-X-receptor, hormonal receptors, Cabazitaxel, Trabectedin and Rosiglitazone. These experiments revealed under all tested conditions a drug-resistant phenotype of MUC-1 compared to NCI-H295R and indicated thereby that MUC-1 might represent a clinically frequently observed subgroup of ACC patients with high drug tolerance/resistance.

(Hasanovic A., Ruggiero C., Jung S., Rapa I., Volante M., Terzolo M., Beuschlein F., Poinsard C., **Hantel C.** *, Lalli E. * and Mus-Veteau I. *Equally contributing authors*. International Journal of Cancer 2018 Jul 1; 143(1):199-211.* doi: 10.1002/ijc.31296. Epub 2018 Feb 23. Targeting the multidrug transporter Patched potentiates chemotherapy efficiency on adrenocortical carcinoma in vitro and in vivo [29].

Siebert C, Ciato D, Murakami, Frei-Stuber L, Perez-Rivas LG, Monteserin-Garcia JL, Nölting S, Maurer J, Feuchtinger A, Walch AK, Haak HR, Bertherat J, Mannelli M, Fasnnacht M, Korpershoek E, Reincke M, Stalla GK, **Hantel C**, Beuschlein F. *Front Endocrinol (Lausanne) 2019 Jul 19.* Heat Shock Protein 90 as a Prognostic Marker and Therapeutic Target for ACC [30].

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Abate A, Rossini E, Bonini SA, Fragni M, Cosentini D, Tiberio G, Benetti D, **Hantel C**, Lagana M, Grisanti S, Terzolo M, Memo M, Berutti A, Sigala S. *Cancers (Basel), 2020 Apr 9; 12(4):928*. Cytotoxic Effect of Trabectedin in human adrenocortical carcinoma cell lines and primary cells [33].

Warde KM, Schoenmakers E, Martinez E, Lim YJ, Leonard M, Lawless, SJ, O`Shea P, Chatterjee KV, Gurnell M, **Hantel C**, Dennedy MC. *Endocr Rel Cancer, 2020 Jun; 27(6):361-373*. Liver X Receptor Inhibition potentiates Mitotane-Induced Adrenotoxicity in ACC [34].

Sigala S., Rossini E., Tamburello M., Abate A., Beretta S., Fragni M., Cominelli M., Cosentini D., **Hantel C.**, Bono F., Grisanti S., Poliani PL, Tiberio GAM, Memo M., Berruti A. *Frontiers Endocrinology 2021 Apr 26;12:669426*. Cytotoxic effect of progesterone, tamoxifen and their combination in experimental cell models of human adrenocortical cancer [35].

Cantini G., Fei L., Canu L., Lazzeri E., Sottili M., Francalanci M., Angelotti ML, De Filpo G, Ercolino T, Gelmini S, Mangoni M., Nesi G., **Hantel C.**, Mannelli M., Maggi M., Luconi M. *J Pers Med. 2021 Oct 27;11(11):1097.* doi: 10.3390/jpm11111097. Stimulated Expression of CXCL12 in Adrenocortical Carcinoma by the PPARgamma Ligand Rosiglitazone Impairs Cancer Progression [36].)

We complemented these studies with an extensive drug screen including a large panel of classical chemotherapies (doxorubicin, etoposide, cisplatin, mitotane, gemcitabine, paclitaxel), phytochemicals (9-cis-retinoic-acid, Isoquercitrin) and molecular targeted inhibitors (Erlotinib, Linsitinib, Sorafenib, Sunitinib, XAV-939, VE822, COH29, adavosertib, prexasertib and AZD0156).

In this study, we provided strong evidence for anti-tumoral efficacy of the classical combination gemcitabine and cisplatin against NCI-H295R and MUC-1. Of note, the general therapeutic potency of gemcitabine and cisplatin combination is itself not new or limited to ACC. However, even though combined treatments are in many cases highly potent compared with gemcitabine treatment alone, additional treatments are often still required. Indeed, accompanying elevated expression of ribonucleotide reductase subunit M1 (encoded by RRM1 gene) and the small regulatory dimers RRM2 or p53R2 (encoded by RRM2 and RRM2B) also indicated in our preclinical settings strongly developing Gemcitabine resistance, a frequent side effect in clinical patient care. MUC-1 represented in these studies again the less responsive model to this treatment. Interestingly, for both models the developing resistance effect was partially reversed upon addition of Cisplatin.

We confirmed our findings for RRM2 protein, RNR-dependent dATP levels, and modulations of related ATM/ATR signaling. Next, to potentiate the therapeutic effects of gemcitabine and cisplatin on a molecular level, we screened for complementing inhibitors of the DNA damage/repair system targeting Ribonucleotidreductase (RNR), Wee1, CHK1/2, ATR, and ATM. Notably, the combination of Gemcitabine, Cisplatin and the dual RRM1/RRM2 inhibitor COH-29 resulted in previously unreached total cell killing for both models.

(Bothou C, Sharma A, Oo A, Kim B, Perge P, Igaz P, Ronchi CL, Shapiro I, **Hantel C**. *Cancers (Basel). 2021 Aug 20*;13*(16)*. Novel insights into the Molecular Regulation of Ribonucleotide Reductase in Adrenocortical Carcinoma Treatment [37].

→ This work was awarded in march 2022 by the German Endocrine Society with the “Anke-Mey-Award” to Christina Bothou and Ashish Sharma.)

Summary, further developments and Outlook

In summary, with the projects herein, various novel therapeutic approaches have been developed and/or tested in a preclinical setting.

While a combination of clinically available liposomal doxorubicin and anti-IGF1R inhibitor might be promising for a sub-group of ACC patients, an immunolipsomal approach did not bring further benefit in this context, thereby differing to previous results from neuroendocrine tumors of the gastroenteropancreatic system. As we observed during these studies an extraordinary uptake of plain liposomes specifically in ACC cells, this effect seems to be most likely to explain these ACC-specific observations. Accordingly, we went on with plain/sterically stabilized liposomes for the treatment of ACC. Of note, as the parental drugs from the clinical gold-standard were still included and only exchanged by liposomal variants a completely different therapeutic profile would be not expected in clinical studies. Accordingly, our findings revealed depending on the applied tumor models, better or at least the same therapeutic efficacy compared to the current clinical gold standard, but model-independent in all studies in any case highly improved tolerability profiles.

Consequently, In 2016, as part of the European Congress of Endocrinology 2016 in Munich, Germany, an international consortium of ACC experts came together and decided on basis of the previously described preclinical data to initiate a clinical translation of the results obtained for liposomal therapies against ACC. Despite the candidates instituitions (coordinated from the preclinical side by the candidate and from the clinical side by Prof. Dr. Felix Beuschlein, current director of the Clinic for Endocrinology, Diabetology and Clinical Nutrition, UniversitätsSpital Zurich) the centers/ institutions which were furthermore involved in these activities, can be found below

Massimo Terzolo	Universitá degli Studi di Torino (UNITO)	Italy
Eric Baudin	Institut Gustave Roussy (IGR)	France
Martin Fassnacht	Universitätsklinikum Würzburg - Klinikum der Bayerischen Julius Maximilians Universität (UKW)	Germany
Wiebke Arlt	The University of Birmingham (UOB)	U.K.
Harm Haak	Máxima Medisch Centrum, Eindhoven (MMC)	The Netherlands
Britt Skogseid	Department of Medical Sciences, Uppsala University (UOU)	Sweden
Mouhammed A. Habra	The University of Texas MD Anderson Cancer Center (UOT)	U.S.A.
Guillaume Assie	Institut Cochin Paris, Institut National de la Santé et de la Recherche Médicale (INSERM U1016)	France
Barbara Jarzab	Maria Sklodowska-Curie Memorial Cancer Centre and Institute of Oncology, Gliwice Branch, Gliwice (MSCG)	Poland
Darko Kastelan	University of Zagreb, School of Medicine (UZSM)	Croatia
Alfredo Berruti	Università degli Studi di Brescia (UNIB)	Italy
Martin Fassnacht	European Network for the Study of Adrenal Tumours (ENSAT)	Europe / France
Jérôme Bertherat	European Society of Endocrinology (ESE)	Europe / U.K.
Teni Boulikas	Regulon AE (REG)	Greece

Upon many expert communications about the best drug combination for such an initial trial, the expert clinicians selected liposomal cisplatin (Lipoplatin^{TM}) and mitotane in an adjuvant setting. A respective clinical protocol was set up and submitted together, by the consortium and the manufacturing company Regulon Inc., to the Europeans Medicines Agency (EMA) to apply in a first step for orphan drug status designation of Lipoplatin for the treatment of ACC patients. Even though in June 2007, Lipoplatin^{TM} was provided with orphan drug status for the treatment of pancreatic cancer (EU/3/07/451), this first application for the extension of this indication to ACC treatment has been meanwhile unfortunately rejected by the EMA with the justification that the preclinical results were obtained for the complete schemes LEDP-M and L(l)EDP-M. These schemes included further liposomal drugs (liposomal doxorubicin and

liposomal etoposide) and not only liposomal cisplatin as proposed in the application. However, from the clinical point of view there were at least two reasons why these experiments should/ can not be clinically translated as whole in one step: 1. Translation of several drugs bares higher risks for the patients and 2. Liposomal Etoposide was manufactured specifically for the preclinical experiments and is currently as single agent in this form commercially not available. However, the EMA invited to submit new preclinical studies on Lipoplatin™ and mitotane as necessary basis for a potential Lipoplatins orphan drug status designation in combination with mitotane for the treatment of ACC patients. Such studies are currently under consideration to be performed by the candidates group. Overall, these developments regarding clinical translation clearly reflect the relevance of our studies on liposomal EDP.

Moreover, with the development of MUC-1 the preclinical landscape drastically changed in terms of tumor models in the field of ACC after a long lack of new tumor models for more than 25 years. Fortunately, beginning with MUC-1 in 2016 the community was able to develop further models such as CU-ACC1/2 [38] in 2018, JIL-2266 in 2021 [39] and most recently a new cell line named TVBF-7 (renamed from the original primary culture ACC115m [35, 40]). Our manuscript of first description and full characterization of TVBF-7 has been just recently accepted for publication in **Cells" (2022 Apr 24;11(9):1439**:

"A Comprehensive Investigation of Steroidogenic Signaling in Classical and New Experimental Cell Models of Adrenocortical Carcinoma." authored by Sandra Sigala *, Christina Bothou*, David Penton, Andrea Abate, Mirko Peitzsch, Deborah Cosentini, Guido A.M. Tiberio, Stefan R. Bornstein, Alfredo Berruti* and **Constanze Hantel***

This most recent publication describes the extensive investigation of specific driver genes, steroidogenesis and electrophysiological responsiveness, as well as an extraordinary androgen/androgen receptor upregulating phenotype for MUC-1 and a profile of impressive autonomous cortisol secretion for TVBF-7. Together with the physiologically broad stimulable hormonal phenotype of NCI-H295, this panel of models further strongly improves the available preclinical armamentarium in the field.

Of note, meanwhile we were furthermore able to demonstrate in Zebrafish models that MUC-1 and TVBF-7 retain their specific clinical features also regarding metastatic properties in comparison to the primary tumor derived NCI-H295 which do not demonstrate signs of metastasization.

Moreover, 3D-models have been recently established and characterized based on NCI-H295R, MUC-1, TVBF-7, but also freshly obtained patient-tissue derived from benign and malignant adrenocortical and medullary tumors. The appropriate manuscript was published in «Cell Death and Disease»:

Innovative multidimensional models in a high-throughput-format for different cell types of endocrine origin.

Bornstein S*, Shapiro I*, Malyukov M, Züllig R, Luca E, Gelfgat E, Beuschlein F, Nölting S, Berruti A, Sigala S, Peitzsch M, Steenblock C, Ludwig B, Kugelmeier P, **Hantel C**.

Cell Death Dis. 2022 Jul 25;13(7):648.

→ This work was awarded in oct 2022 by the European Network for the Study of Adrenal Tumors with the Bruno Allolio Award for Adrenocortical Cancer Research 2022 (ENSAT ACC Award)

Further developments in terms of surrounding tumor-microenvironment and optimizations of the appropriate tumor models are ongoing as well as further preclinical and clinical testings based on the above described studies.

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