Preclinical progress and first translational steps for a liposomal chemotherapy protocol against adrenocortical carcinoma

Sara Jung¹, Zoltan Nagy², Martin Fassnacht^{3,4}, Gerard Zambetti⁵, Max Weiss⁶, Martin Reincke¹, Peter Igaz², Felix Beuschlein¹ and Constanze Hantel¹

¹Endocrine Research Unit, Medizinische Klinik und Poliklinik IV, Ludwig-Maximilians-University, Munich, Germany

²2nd Department of Medicine, Semmelweis University, Faculty of Medicine, Budapest, Hungary

³Department of Internal Medicine I, Division of Endocrinology and Diabetes, University Hospital, University of Würzburg, Würzburg, Germany

⁴Comprehensive Cancer Center Mainfranken, University of Würzburg, Würzburg, Germany

⁵Department of Pathology, St Jude Children's Research Hospital, Memphis, Tennessee, USA

⁶Institute of Pathology, Ludwig-Maximilians-University, Munich, Germany

Correspondence
should be addressed
to C Hantel

Email

Constanze.Hantel@med.uni-muenchen.de

Abstract

Systemic therapy of adrenocortical carcinoma (ACC) is limited by heterogeneous tumor response and adverse effects. Recently, we demonstrated anti-tumor activity of LEDP-M (etoposide, liposomal doxorubicin, liposomal cisplatin, mitotane), a liposomal variant of EDP-M (etoposide, doxorubicin, cisplatin, mitotane). To improve the therapeutic efficacy and off-target profiles of the clinical gold standard EDP-M, we investigated liposomal EDP-M regimens in different preclinical settings and in a small number of ACC patients with very advanced disease. Short- and long-term experiments were performed on two ACC models (SW-13 and SJ-ACC3) *in vivo*. We evaluated the anti-tumoral effects and off-target profiles of EDP-M, LEDP-M and a novel regimen L(I)EDP-M including liposomal etoposide. Furthermore, the role of plasma *microRNA-210* as a therapeutic biomarker and first clinical data were assessed. Classical and liposomal protocols revealed anti-proliferative efficacy against SW-13 (EDP-M $P < 0.01$; LEDP-M: $P < 0.001$; L(I)EDP-M: $P < 0.001$ vs controls), whereas in SJ-ACC3, only EDP-M ($P < 0.05$ vs controls) was slightly effective. Long-term experiments in SW-13 demonstrated anti-tumor efficacy for all treatment schemes (EDP-M: $P < 0.01$, LEDP-M: $P < 0.05$, L(I)EDP-M $P < 0.001$ vs controls). The analysis of pre-defined criteria leading to study termination revealed significant differences for control ($P < 0.0001$) and EDP-M ($P = 0.003$) compared to L(I)EDP-M treatment. Raising its potential for therapy monitoring, we detected elevated levels of circulating *microRNA-210* in SW-13 after LEDP-M treatment ($P < 0.05$). In contrast, no comparable effects were detectable for SJ-ACC3. However, overall histological evaluation demonstrated improved off-target profiles following liposomal regimens. The first clinical data indicate improved tolerability of liposomal EDP-M, thus confirming our results. In summary, liposomal EDP-M regimens represent promising treatment options to improve clinical treatment of ACC.

Key Words

- ▶ adrenocortical carcinoma
- ▶ liposomal doxorubicin
- ▶ liposomal cisplatin
- ▶ liposomal etoposide
- ▶ Caelyx
- ▶ Lipoplatin
- ▶ Myocet
- ▶ NCI-H295R
- ▶ SJ-ACC3
- ▶ SW-13
- ▶ *microRNA-210*
- ▶ *microRNA-483-5p*

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Introduction

In recent years, genetic and molecular profiling of surgical tumor specimens has led to the identification of novel biomarkers with prognostic relevance for patients with adrenocortical carcinoma (ACC) (Assie *et al.* 2014). However, the complex interplay of underlying pathways that relate to treatment response remains largely unknown (Zheng *et al.* 2016). Accordingly, targeted therapies in the treatment of ACC patients have shown limited efficacy, and clinical management is mainly restricted to common cytotoxic agents (Berruti *et al.* 2012, Kroiss *et al.* 2012, Fassnacht *et al.* 2015). The current mainstay of treatment for advanced and metastasized ACC is defined as the EDP-M protocol (Fassnacht *et al.* 2012) consisting of classical formulations of the chemotherapeutic agents etoposide (E), doxorubicin (D), cisplatin (P) and mitotane (M). However, the overall therapeutic efficacy of this treatment scheme is still dissatisfactory, and the combination of these chemotherapeutic agents is associated with severe and dose-limiting adverse effects (Fassnacht *et al.* 2012). Moreover, therapeutic biomarkers are yet to be defined to identify the patients with relevant therapeutic benefit of this highly toxic regimen (Ronchi *et al.* 2009, Malandrino *et al.* 2010).

In the past years, encapsulation of doxorubicin and cisplatin in liposomes has been demonstrated as an important strategy for enhancing therapeutic efficacy and improving the off-target profiles of the parental drugs mainly regarding cardiotoxicity and nephrotoxicity (Boulikas 2009, Gabizon *et al.* 2012). Recently, our group has demonstrated furthermore an extraordinary uptake phenomenon of liposomes specifically by adrenocortical tumor cells (Hantel *et al.* 2010, 2012). Moreover, we provided evidence in a commonly used xenograft model for ACC (NCI-H295R) that an exchange of doxorubicin and cisplatin by liposomal variants (LEDP-M) was associated with enhanced therapeutic efficacy and reduced adverse effects (Hantel *et al.* 2014). Of note, liposomal formulations of doxorubicin (Myocet and Caelyx) or cisplatin (Lipoplatin) are already in clinical use for a wide range of malignancies including metastatic breast cancer, advanced ovarian cancer, progressive multiple myeloma, AIDS-related Kaposi's sarcoma or pancreatic cancer and non-small-cell lung cancer, respectively (Boulikas 2009, Marchal *et al.* 2015).

However, clinical translation of novel regimens for ACC emerged as challenging, particularly due to observed heterogeneity of ACC patients regarding tumor growth rate, treatment response and overall survival. Thus, we investigated LEDP-M in two further xenograft

models for ACC in this study: SW-13 and the recently introduced pediatric xenograft model SJ-ACC3 (Leibovitz *et al.* 1973, Pinto *et al.* 2013, Hantel & Beuschlein 2016). Moreover, we included liposomal etoposide resulting in a novel treatment scheme now named L(l)EDP-M. In addition, based on the detected differences in therapeutic responsiveness for both tumor models, we studied intratumoral and plasma microRNAs (miRs) as potentially interesting therapeutic biomarkers for ACC. Finally, we provide preliminary clinical data indicating that management of ACC patients, involving liposomal chemotherapies, might be a favorable clinical option to improve tolerability of the current gold-standard treatment for ACC.

Materials and methods

Cell culture

Material for cell culture was purchased from Gibco Invitrogen if not stated otherwise. SW-13 cells (obtained from ATCC and recently authenticated) were cultured in DMEM/F-12 medium (Dulbecco's Modified Eagle Medium) in a 5% CO₂-95% air atmosphere at 37°C. Cell culture medium was supplemented with 1% penicillin/streptomycin and 10% FBS (heat-inactivated fetal bovine serum).

Animal experiments

Animals and preclinical ACC tumor models

All animal experiments were approved by the Regierung von Oberbayern and are in accordance with the German guidelines for animal studies. Athymic NMRI *nu/nu* mice (female, 6–8 weeks old) were purchased from Harlan Winkelmann (Borchen, Germany) and stored under pathogen-free conditions. For SW13-xenografts, tumor cells were inoculated in 200 µL PBS (Gibco Invitrogen) for tumor induction. For therapeutic experiments, SW-13 cells were subcutaneously injected into the neck of each mouse (short term: 13×10^6 cells and long term: 11×10^6 cells/mouse). Cryopreserved SJ-ACC3 samples (Pinto *et al.* 2013) were subcutaneously engrafted as 2 mm³ tumor specimens; before implantation, they were transferred from liquid nitrogen to a 37°C water bath after which tumor tissue was rinsed several times in medium 199 (Gibco Invitrogen) supplemented with 1% penicillin/streptomycin (Gibco Invitrogen). Therapeutic treatments started for SW-13 in short-term experiments ($n=7-8$ mice) at day 14 and in the long-term setting ($n=14$ mice) at day 4 after tumor induction. For SJ-ACC3, short-term

($n=4-6$ mice) and long-term ($n=5$ mice) therapeutic treatments were started several weeks after implantation to enable successful tumor engraftment.

Therapeutic experiments For therapeutic experiments, a preclinically adapted EDP-M protocol (Fassnacht et al. 2012) was administered as already implemented in a recent study (Hantel et al. 2014). Mitotane powder (HRA-Pharma) was dissolved in sterile autoclaved corn oil and intraperitoneally injected at a daily dose of 300mg/kg body weight over three consecutive days before cytostatic treatment. All treatments were carried out at 24-h intervals. Cytostatic drugs were intravenously injected in a 2mg/kg dose of cisplatin and Lipoplatin (Regulon Inc, Athens, Greece) and 10mg/kg doses of doxorubicin, liposomal doxorubicin (Caelyx; Janssen-Cilag GmbH, Neuss, Germany), etoposide and liposomal etoposide (Encapsula NanoSciences LLC, Nashville, TN, USA) according to the treatment modalities outlined for one therapeutic cycle (Fig. 1).

For short-term experiments, tumor-bearing mice were treated with one therapeutic cycle. Forty-eight hours after

the last therapeutic intervention studies were terminated, the animals were killed. Then, the tumors were excised, immediately processed for paraffin embedding and in case of sufficient material, also, snap-frozen. Moreover, EDTA-blood was collected and centrifuged at 2000g at 4°C to obtain plasma samples. Frozen tumors and plasma samples were stored at -80°C.

For long-term therapeutic protocols, mice received repeated treatment cycles with a therapy-free interval of ten days. Animals were monitored daily, and tumor sizes were measured every second day (as tumor length $\times$ width (cm²)). Although for SJ-ACC3 the long-term study was terminated after the second cycle, SW-13 tumor-bearing mice received up to four therapeutic cycles to allow a more detailed investigation regarding the achievement of pre-defined endpoints (including tumor sizes and adverse effects) in the different treatment groups. In this setting, animals were killed when tumors reached a longest tumor diameter of 1.5cm or when specified side effects (as body weight loss or pathologically changed phenotype as abnormal body posture) occurred. For additional immunohistochemical and histological analysis,

Figure 1

Schematic illustration of the preclinical treatment schemes EDP-M, LEDP-M and L(l)EDP-M adapted from the clinical EDP-M protocol exemplified for one therapeutic cycle.

hearts and kidneys were also snap-frozen and paraffin-embedded to enable a more detailed investigation of these main off-target organs.

Histological and immunohistochemical evaluation About 4 µm sections (HM355E, Microm, Walldorf, Germany) of tumor tissue, kidneys and hearts were cut and rehydrated. Immunohistochemical staining of tumor tissue was performed after antigen retrieval using 10 mM sodium citrate buffer, which was followed by incubation with blocking buffer containing 3% BSA (Roche Diagnostics), 5 % goat serum (Jackson ImmunoResearch) and 0.5 % Tween 20 for 15 min. Proliferating cells were stained using the monoclonal rabbit anti-human Ki67 antibody (KI68R06, DCS innovative diagnostics, Hamburg, Germany) 1:200 in a blocking buffer. After overnight incubation at 4°C, slides were rinsed three times for 5 min in PBS. Secondary polyclonal antibody (BA-1000 goat anti-rabbit biotinylated IgG, Vector Laboratories, Burlingame, CA, USA) was applied for 30 min at room temperature. Vectastain ABC Kit (Vector Laboratories) according to the manufacturer's protocol visualized bound primary antibody after 30 min followed by 3,3'-diaminobenzidine (Sigma-Aldrich) incubation. For counterstaining, Vector Methyl Green nuclear counterstain (Vector Laboratories) was applied for 10 min at 60°C to enable cell number quantification of Ki67-positive and -negative cells. Apoptotic cells were detected using the colorimetric DeadEnd TUNEL System (Promega) as described in the manufacturer's protocol. Tumors were immunohistochemically evaluated by investigating six high-power fields (HPF, 0.391 mm², 400× magnification) per tumor. Histological evaluation of kidney and heart sections was performed using regressive hematoxylin/eosin (HE) staining (Harris Hematoxylin, Eosin Y, Sigma-Aldrich).

Molecular analyses

Based on a pilot experiment analyzing a panel of various miRs (miR-195, miR-210, miR-483-3p, miR-483-5p and miR-503) on NaCl, EDP-M and LEDP-M-treated NCI-H295R xenografts (data not shown) and data from the literature, we selected two miRs (miR-483-5p and miR-210) for detailed examination in our recent study. MicroRNA (miR) was analyzed with real-time quantitative PCR (RT-qPCR) using short-term plasma and tumor samples of SW-13 and SJ-ACC3 mice. Circulating RNA from plasma exosomes was extracted by processing the plasma samples according to the manufacturer's protocols (Total Exosome Isolation Kit and Total Exosome RNA

and Protein Isolation Kit, ThermoFisher Scientific) with addition of spike-in control cel-miR-39 (Ref. 2594091, Qiagen). Tumor RNA isolation, reverse transcription and RT-qPCR were performed as described previously (Nagy et al. 2015). Reactions were run in duplicates on a 96-well plate. Human primer assays and catalogue numbers (ThermoFisher Scientific) were as follows: Hsa-miR-210 (#000512) and hsa-miR-483-5p (#002338). For tumor and for plasma samples, RNU44 (#001094) and cel-mir-39 (#000200) were used as housekeeping genes, respectively (Brase et al. 2010, Nagy et al. 2015). miR levels were analyzed using the comparative ΔCt method (Schmittgen & Livak 2008). For evaluation of changes in miR level upon NaCl or LEDP-M treatment in individual mice, ratio of intratumoral to circulating miR level was calculated (miR ratio) after normalization to controls (% of controls):

$$\frac{\text{intratumoral miR (\%)}}{\text{circulating miR (\%)}} = \text{miR ratio (individual animal)}$$

A value of 1 indicates unchanged levels in tumor and plasma. A value >1 reveals an elevated expression of intratumoral miR, whereas a ratio <1 indicates elevated expression of circulating miR.

Clinical data

Six patients with very advanced ACC who have been benefited in the past from EDP-M, but experienced toxicity and/or desired experimental therapy, were offered liposomal doxorubicin or liposomal cisplatin (referred as liposomal EDP-M, lipEDP-M) on a compassionate-use basis (Table 1). This study was approved by the local ethic committee. All patients were informed about the experimental nature of this drug administration, and they gave informed consent in verbal and written forms.

Statistical analysis

Data are expressed as mean ± s.e.m. (standard error of the mean). Analysis was performed with Prism (Houston, TX, USA) using the unpaired *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison test (comparing all treatment groups) both including an adjustment of 95% CI (confidence interval). For the analysis of pre-defined endpoints leading to study termination, log-rank (Mantel-Cox) test was applied using SPSS statistics 23 software. Statistical significance is defined as *P* < 0.05 and is denoted as stars (**P* < 0.05; ***P* < 0.01; ****P* < 0.001) in the figures if not stated otherwise.

Table 1 Clinical data for the investigation of liposomally modified EDP-M (lipEDP-M) in six patients with very advanced ACC.

Patients		Liposomal EDP-M (lipEDP-M)									
		EDP-M	Δ of sum of target lesions					MDRD			Individual evaluation of lipEDP-M
ID	Age, sex	Cycles (n)	Reason for liposomal EDP-M	Cycles (n)	Treatment details	Prior lipEDP-M	Post lipEDP-M	Best objective response	Serious adverse event	Prior lipEDP-M	Post lipEDP-M
#1	45 years, ♀	12	Neurotoxicity and cardiotoxicity	5	c1-2: IP (100 mg/m ² , d1+2) c3-5: IP (200 mg/m ²)	NA	+30%	PD	–	90	95
#2	47 years, ♀	6 (only EP)	Individual wish	3	c1: IP (200 mg/m ²) c2-3: IP (200 mg/m ²) c3-5: IP (200 mg/m ²), ID (30 mg/m ²)	+40%	NA ^c	PD	Seizure, oculomotor nerve palsy ^d	88	87
#3	65 years, ♀	6	Renal function	1	Adjuvantly ED + IP (200 mg/m ²)	Radical surgery –10%	New lesions	PD	–	37	49
#4	65 years, ♂	3	Renal function	2	ED + IP (100 mg/m ² , d3+4)	+25%	+75%	PD	Sepsis, acute kidney failure	36	32
#5	43 years, ♂	8	Individual wish	2	E + ID (40 mg/m ²) + P	+26%	+25%	PD	–	68	65
#6	55 years, ♂	14 ^a	– ^b	2	E + ID (30 mg/m ²), carboplatin AUC 4	+21%	+21%	PD	Thrombocytopenia and anemia, both CTC 3	48	49

^aP was replaced by carboplatin; ^bno specific toxicity, but PD and high cumulative dosage of doxorubicin, ^cdue to palliative situation (increasing cerebral metastases) no formal tumor evaluation was performed, ^dmost likely due to newly diagnosed carcinomatous meningitis.

C, cycle; D, doxorubicin; E, etoposide; ID, liposomal doxorubicin (Myocet); IP, liposomal cisplatin (Lipoplatin); MDRD formula, mL/min/1.73 m²; n, number; NA, not available; P, cisplatin; PD, progressive disease. ♂: male, ♀: female, Δ: change.

Results

Investigation of short-term therapeutic efficacy on SW-13 and SJ-ACC3 xenografts

In the first step, short-term therapeutic efficacies of three different treatment schemes EDP-M, LEDP-M and L(l)EDP-M were tested on SW-13 and SJ-ACC3 xenografts. After administration of one therapeutic cycle (Fig. 1), anti-tumoral effects were investigated regarding tumor cell proliferation and induction of apoptosis using Ki67 and TUNEL immunohistochemistry, respectively (Fig. 2).

For both xenograft models, the highest total cell number (Ki67-positive and -negative cells/high-power field/tumor) was documented in the tumors of the control group (SW-13: 28.9 ± 2.2 ; SJ-ACC3: 35.9 ± 1.3 ; Fig. 2A and C). Although all treatments showed anti-proliferative effects against SW-13 (EDP-M: 20.5 ± 1.6 , $P < 0.01$; LEDP-M 17.2 ± 1.3 , $P < 0.001$ and L(l)EDP-M 14.7 ± 0.9 , $P < 0.001$, vs controls; Fig. 2A), for SJ-ACC3, only EDP-M led to a significant reduction in the number of tumor cells compared with controls (EDP-M: 30.3 ± 1.2 , $P < 0.05$; LEDP-M 31.5 ± 1.8 , $P > 0.05$ and L(l)EDP-M 32.5 ± 0.3 , $P > 0.05$ vs controls; Fig. 2C). Comparisons to other treatment groups were not significantly different ($P > 0.05$). For SW-13, the detected decrease in the number of tumor cells was furthermore accompanied by

a severe condensation of nuclei upon specific treatments compared with controls (Fig. 2F, G, H and I). Moreover, these anti-tumoral effects were confirmed histologically by a semi-quantitative analysis of hematoxylin/eosin-stained SW-13 tumors (Fig. 2J, K, L, M). Therefore, each tumor was histologically analyzed and categorized regarding necrotic areas (from low-to-high levels of necrosis with 0 to 3, respectively). As shown in Fig. 2E, although necrosis was rarely detected for the control group, the presence and grades of necrosis increased upon EDP-M over LEDP-M to L(l)-EDP-M administration on SW-13 xenografts.

Quantification of apoptosis revealed no significant alterations ($P > 0.05$) upon specific therapeutic treatments (SW-13: controls 2.4 ± 0.5 , EDP-M 3.0 ± 0.5 , LEDP-M 4.2 ± 0.7 and L(l)EDP-M 4.3 ± 0.9 , Fig. 2B; SJ-ACC3: controls 3.5 ± 0.8 , EDP-M 5.5 ± 0.8 , LEDP-M 5.1 ± 0.5 and L(l)EDP-M 5.4 ± 0.4 , Fig. 2D) for both tumor models.

Investigation of long-term therapeutic efficacy on SW-13 and SJ-ACC3 xenografts

Primary endpoint of the long-term study on SW-13 and SJ-ACC3 xenografts was the tumor size (expressed as length $\times$ width in cm^2) upon repeated treatment with EDP-M, LEDP-M or L(l)EDP-M (Fig. 3). This experiment confirmed the previously obtained results

Figure 2

Immunohistochemical and histological analysis of SW-13 (A, B and E) and SJ-ACC3 (C and D) xenografts derived from the short-term therapeutic experiment with quantification of Ki67-positive, Ki67-negative (A and C) and TUNEL-positive cells (B and D). Necrosis was analyzed semi-quantitatively using four categories ranked in ascending order from 0 (no necrosis) until 3 (extensive necrosis) for each SW-13 tumor, respectively (E). Representative pictures from Ki67 (F–I) and H&E staining (J–M) are presented for NaCl (F and J), EDP-M (G and K), LEDP-M (H and L) and L(l)EDP-M (I and M)-treated SW-13 tumors. Statistical significance vs controls is denoted as stars (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

from the short-term experiments for SW-13. The long-term study demonstrated anti-tumoral efficacy for all treatment modalities showing, in all regimens, a significant reduction in tumor size upon two therapeutic cycles compared with controls (day 29 after tumor cell injection, NaCl: 1.02 ± 0.08 ; EDP-M: 0.57 ± 0.10 , $P < 0.01$; LEDP-M: 0.62 ± 0.11 , $P < 0.05$; L(l)EDP-M: 0.48 ± 0.07 , $P < 0.001$, Fig. 3A). In contrast, for SJ-ACC3, no significant differences were detectable upon two cycles with the different therapeutic regimens (day 56 after implantation of tumor pieces, NaCl: 0.30 ± 0.14 ; EDP-M: 0.14 ± 0.06 , $P > 0.05$; LEDP-M: 0.12 ± 0.03 , $P > 0.05$; L(l)EDP-M: 0.17 ± 0.08 , $P > 0.05$; Fig. 3B).

Investigation of tolerability and off-target profiles on long-term treatment

In an attempt to investigate long-term tolerability of the different treatment modalities, we continued the

therapeutic treatment of SW-13 tumor-bearing mice up to four cycles. Following this approach, we monitored the achievement of pre-defined endpoints such as 1.5 cm long tumor diameter or the appearance of severe adverse effects (body weight loss and ascites) for study termination. As shown in Fig. 3C, following these criteria, L(l)EDP-M treatment led to significantly prolonged time to reach these pre-defined criteria in comparison with not only controls ($P < 0.0001$) but also EDP-M ($P = 0.003$). Assessing the reason for study termination, severe adverse effects occurred in 43% of animals treated with classical EDP-M, whereas a liposomal treatment led to 0% (LEDP-M) and only 7% (L(l)EDP-M) cases of adverse effects (Fig. 3D).

We further investigated kidneys and hearts from treated animals as these organs represent main targets for side effects of classical formulations of cisplatin and doxorubicin. Although HE staining of kidneys from

Figure 3

Effects on tumor size of SW-13 (A) and SJ-ACC3 (B) xenografts after two therapeutic cycles with the different treatment modalities. Statistical comparisons were performed using a one-way ANOVA followed by Bonferroni's Multiple Comparison Test (compare all groups). Stars (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) denote significant differences compared with controls, comparisons to other treatment groups were not significant ($P > 0.05$). SW-13 tumor-bearing mice that received up to four therapeutic cycles of NaCl, EDP-M, LEDP-M or L(l)EDP-M treatment were killed after achieving pre-defined endpoints. Regarding the achievement of study termination, the comparison of all treatment groups resulted in significant differences using a log-rank (Mantel-Cox) analysis. Significant differences are illustrated in (C) in comparison to L(l)EDP-M. Individual reasons for study termination are furthermore depicted in (D).

control mice ($n=14$) did not reveal any pathological finding, 5 of 14 kidneys in the EDP-M group displayed severe pathologies in the form of tubular casts (Fig. 4). However, such histological changes were not detectable in the therapeutic arms using liposomal formulations of cisplatin. Specifically, in none of the 14 animals treated with LEDP-M and only 1 of 14 mice treated with L(l)EDP-M, minor structural renal changes were found. Moreover, mild cardiotoxic effects were also detectable in the EDP-M arm exclusively. Although we observed vacuole formation and single-cell necrosis in 2 of 5 investigated hearts of the EDP-M group, no such effects were apparent in the other treatment groups (Fig. 4).

Preclinical investigation of circulating miR-210 as potential biomarker to monitor therapeutic efficacy

After one therapeutic cycle with NaCl or LEDP-M, miR ratio revealed no treatment-dependent changes for miR-483-5p expression in both tumor models

(SW-13: control 1.38 ± 0.52 , LEDP-M 0.81 ± 0.11 , SJ-ACC3: control 1.02 ± 0.12 , LEDP-M 1.74 ± 0.43 ; Fig. 5A). In contrast, miR-210 ratio was significantly altered in SW-13 after LEDP-M treatment (0.32 ± 0.06) compared with that in controls (1.20 ± 0.31), indicating elevated circulating miR-210 levels. Such an effect was not evident for SJ-ACC3 (control 1.26 ± 0.74 , LEDP-M 0.83 ± 0.32 ; Fig. 5B).

Clinical data

Six patients had been treated on a compassionate-use basis with a liposomally modified EDP-M scheme (lipEDP-M) including at least one liposomal drug. Although none of these heavily pretreated patients experienced an objective tumor response, the drug regimens were well tolerated. In three patients, kidney function was clearly impaired partly due to the renal toxicity of EDP-M, but the glomerular filtration rate did not further deteriorate for liposomal drug regimens. However, one patient experienced acute kidney failure after the second cycle

Figure 4

Pathological examination of HE-stained kidneys and hearts from the different treatment groups: control (NaCl), EDP-M, LEDP-M and L(l)EDP-M. Black arrows indicate vacuole formation and single-cell necrosis in hearts affected by EDP-M treatment.

Figure 5

Quantitative real-time PCR analysis of intratumoral and circulating miR 483-5p (A) and miR-210 (B) of control (NaCl) and LEDP-M-treated SW-13 and SJ-ACC3 tumor-bearing mice. For analysis of changes upon therapeutic treatment in individual animals, the ratio of intratumoral to circulating miR level was calculated after normalization to controls. A value of 1 indicates unchanged equal levels in tumor and plasma. A value of >1 reveals an elevated expression of intratumoral miR, whereas a ratio of <1 indicates elevated expression of circulating miR. Stars denote significant differences (* $P < 0.05$).

of lipEDP-M, which was most likely due to sepsis. Of note, three of the six patients judged the modified regimen better tolerable than the previously administered conventional EDP-M scheme. Two patients mentioned no difference and one had no subjective toxicity at all upon EDP-M and lipEDP-M treatment (Table 1).

Discussion

EDP-M is currently the medical standard treatment for advanced ACC not amenable for surgery. Nevertheless, therapeutic efficacy in clinics is considerably complicated by patient heterogeneity and especially by dose-limiting adverse effects of this multi-chemotherapeutic treatment (Berruti et al. 2005, Fassnacht et al. 2012, 2013). Recently, we brought preclinical evidence for promising anti-tumoral potential and improved off-target profiles for LEDP-M in the classical, preclinical tumor model for ACC NCI-H295R (Hantel et al. 2014). To obtain the preclinical results with the highest clinically predictive power, we investigated liposomal schemes of EDP-M in two further tumor models for ACC *in vivo*: hormonally inactive SW-13 (Leibovitz et al. 1973) and pediatric SJ-ACC3 xenografts (Pinto et al. 2013). In this context, it has to be mentioned that origin and applicability of SW-13 as ACC tumor model is controversial as SW-13 was originally reported as derived from a small-cell carcinoma metastasized to the adrenal gland. Thereby, its adrenocortical origin has been questioned. However, due to the lack of ACC tumor

models representing the sub-entity of non-secreting ACC in preclinical experiments, SW-13 cells have been used in the past and in this study to complement preclinical experiments.

Even though all treatment arms demonstrated therapeutic efficacy against SW-13 xenografts, this study revealed the highest anti-tumoral effects for L(I) EDP-M. Overall, for L(I)EDP-M we detected a highly significant reduction in the total number of tumor cells and induction of necrosis upon one therapeutic cycle as well as significantly reduced tumor sizes upon repeated treatment cycles. Thereby, our previous study on NCI-H295R xenografts (Hantel et al. 2014) and this study predict for adult ACCs at least comparable or improved therapeutic efficacies of liposomal EDP-M schemes compared with the classical regime. Furthermore, acute and long-term complications of EDP-M are of main impact for clinical tolerability and therapeutic benefit. EDP-M leads to typical off-target profiles characterized by hematological toxicities with dose-limiting leukopenia, gastrointestinal and other toxicities including cardiotoxic and nephrotoxic events (Berruti et al. 2005, Fassnacht et al. 2012). After cytostatic treatment with doxorubicin, approximately one of four patients experiences congestive heart failure when cumulative doses exceed 500 mg/m² (Lipshultz et al. 1991, Rahman et al. 2007). More recent data suggest the development of cardiomyopathy after anthracycline-based chemotherapy not only after much lower cumulative doses but also after 4–20 years in almost

25% of patients (Steinherz *et al.* 1991, Hequet *et al.* 2004). This is of decisive importance for adult patients as well as for children as cytotoxic effects of anthracyclines are thought to be irreversible (Rahman *et al.* 2007). Accordingly, classical EDP-M treatment leads in these organs to clearly detectable pathological alterations, which are known to be common adverse effects of doxorubicin and cisplatin treatment as they have been intensively studied (Mettler *et al.* 1977, Devarajan *et al.* 2004, Rahman *et al.* 2007, Stathopoulos & Boulikas 2012).

Consequently, we analyzed kidneys and hearts after multiple treatment cycles, as these organs are known to be the main off-target organs of cisplatin and doxorubicin treatment, respectively (Chlebowski 1979, Meyer & Madias 1994). In accordance with the clinical situation, we detected severe pathological alterations in these organs after multiple therapeutic cycles with EDP-M. In recent years, liposomal doxorubicin and cisplatin have been successfully applied in clinics for a wide range of malignancies to overcome the severe adverse effects of their parenteral drugs and to further enhance treatment efficacy. Several clinical studies demonstrated that liposomal doxorubicin and cisplatin significantly reduced cardiotoxicity and nephrotoxicity in a variety of malignancies (Boulikas 2009, Marchal *et al.* 2015). Thus, a liposomal EDP-M scheme was very likely to improve off-target profiles and could therefore lead in clinical use to a better tolerated and highly efficient treatment option for advanced ACC. In accordance with these assumptions, we detected no comparable cardiotoxic and nephrotoxic impairment upon LEDP-M or L(l)EDP-M treatments even after up to four treatment cycles. Moreover, evaluation of achievement of study termination including an observation of adverse effects after multiple treatment cycles confirmed these results by demonstrating significantly prolonged survival after L(l)EDP-M treatment compared with the classical EDP-M protocol.

In contrast to NCI-H295R (Hantel *et al.* 2014) and SW-13, pediatric SJ-ACC3 xenografts showed slight therapeutic responsiveness upon one therapeutic cycle exclusively with the classical EDP-M treatment. However, none of the investigated treatment schemes led to an effective and sustained therapeutic response in a subsequent long-term study. The contribution of the multi-chemotherapeutic EDP-M regimen to the overall disease response is anyway controversial, and a recent preclinical study of Pinto and coworkers (2013) revealed that cisplatin, but not etoposide and doxorubicin as single agents, had potent anti-tumoral effects. Anti-tumor effects upon cisplatin were obtained by monotherapy using

different dosing regimens and higher drug concentration. In addition, topotecan was identified as a potentially effective agent for pediatric ACC in this study. As outlined previously, liposomal cisplatin is already investigated in clinical studies (phase I, II and III trials); liposomal topotecan is also under preclinical evaluation for other malignancies (Tardi *et al.* 2000, Yu *et al.* 2012). Thus, a combinatorial approach of liposomal formulations of cisplatin and topotecan might be an interesting strategy to improve the therapeutic benefit and tolerability for pediatric ACC in the future.

However, not only prolonged survival time and general tolerability but also quality of life remains of pivotal interest for patients. ACC is a heterogeneous disease, which is often highly aggressive and progressive, but sometimes also appears more indolent and slowly proceeding. Responses upon EDP-M treatment can range from no anti-tumoral effect to high treatment efficacy (Berruti *et al.* 2005, Fassnacht *et al.* 2012). As biomarkers for treatment response do not exist yet, such high variability in therapeutic outcome makes it almost impossible to weigh up patient's long-term benefit and life quality against multiple chemotherapeutic cycles with putative severe side effects (Ferrari *et al.* 2016). Analysis of microRNA (miR) draws increasing attention as miR represents important regulators for pathological settings and tumorigenesis (Cherradi 2015). Aberrant miR expression patterns have been demonstrated for a variety of cancers including ACC, and circulating miRs have been correlated with cancer progression, therapy response and outcome of survival in blood samples (Brase *et al.* 2010, Cherradi 2015). Assessing the potential of miR-483-5p and miR-210 as therapeutic biomarkers, we evaluated the ratio of intratumoral to circulating miR to detect individual treatment-dependent changes. The *MIR483* gene is located in the second intron of the *IGF2* gene, and high miR-483-5p could be correlated with high expression of *IGF2* and malignancy in ACC (Patterson *et al.* 2011). Hypoxia-inducible miR-210 is involved in proliferation, mitochondrial respiration, DNA repair and angiogenesis (Huang *et al.* 2010), and high miR-210 levels were correlated with clinicopathologic parameters of aggressiveness as well as poor prognosis in ACC (Duregon *et al.* 2014) and other tumor entities (Greither *et al.* 2010, 2012, Hong *et al.* 2012, Qiu *et al.* 2013).

SW-13 and SJ-ACC3 tumors and plasma exosomes were analyzed after one therapeutic cycle upon NaCl or LEDP-M treatment. Our miR analysis was basically built on the hypothesis that tumor response to a given therapy might lead to specific changes in either intratumoral

or circulating levels of specific microRNAs. A few of publications describing the analysis of microRNA in patient tumors and plasma samples are available for ACC (Cherradi 2015), but to our knowledge a possible correlation of therapeutic efficacy has never been tested before. Existing miR data in ACC patients often describe synchronous upregulation or downregulation of miRs in tumor and plasma samples (Cherradi 2015). However, these results are based on the comparison of healthy individuals with tumor-bearing individuals. Regarding other tumor entities including breast, NSCLC, colorectal, prostate and gastric cancer, an assessment of therapeutic treatment efficacy and miR levels in serum or plasma samples has been initiated, and these publications conclude that a correlation of therapeutic efficacy with the circulating miR level might exist (Kim et al. 2011, Jung et al. 2012, Li et al. 2013, Kjersem et al. 2014, Lin et al. 2014, Muller et al. 2014). To take all putative regulations into account, we defined the miR ratio. A ratio of 1 indicates unchanged levels in tumor and plasma, a value of >1 reveals elevated levels of intratumoral and a ratio of <1 reveals an increase in circulating miR. In our experiments, we detected a significant decrease of miR ratio for miR-210 indicating a significant increase in circulating miR levels specifically in the LEDP-M-responsive SW-13 tumor model, whereas no comparable effects were detectable for therapy-resistant SJ-ACC3. Thus, our initial data provide evidence that an increase in circulating miR-210 might be an interesting candidate as marker for tumor response. However, to our knowledge, general interactions of circulating miRs in plasma and commercial liposomal formulations have not been evaluated yet. Additional investigations are needed to assess the general potential of miR-210 as a therapeutic biomarker for ACC.

Despite such promising research approaches for the future, liposomal EDP-M has great potential to instantly improve the therapeutic situation in clinics. Our first experience with liposomal cytotoxic drugs in ACC patients demonstrated that these drugs are overall very well tolerated despite the fact that all patients were heavily pretreated with standard EDP and other cytotoxic drugs. The fact that no patient experienced objective tumor response has to be seen in the context of very advanced disease and EDP-M pre-treatment, which both might make drug resistance more likely.

In summary, we conclude that liposomally modified EDP-M holds potential to instantly improve the current medical treatment of ACC. Although liposomal etoposide as applied in L(l)EDP-M has not been introduced in clinical practice yet, our liposomal treatment protocols

contain liposomal cisplatin (Lipoplatin) and doxorubicin (Caelyx and Myocet), which are for other tumor entities already in clinical use (Boulikas 2009, Marchal et al. 2015). Consequently, their implementation would allow a fast clinical translation. Thereby, our findings could improve the therapeutic efficacy, but mainly also tolerability and quality of life for advanced ACC patients.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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