

Liposomal polychemotherapy improves adrenocortical carcinoma treatment in a preclinical rodent model

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Abstract

Owing to high relapse rates and early metastatic spread, prognosis in adrenocortical carcinoma (ACC) patients remains poor, highlighting the importance of developing new treatment alternatives for them. Recently, polychemotherapy regimens including etoposide, doxorubicin, and cisplatin together with mitotane (EDP-M) have been defined as the standard treatment for late-stage disease patients. Nevertheless, the administration of conventional cytostatic drugs is associated with severe and dose-limiting side effects. In an attempt to optimize existing clinical treatment regimens, in this study, we investigated the therapeutic efficacy of EDP-M in comparison with that of a paclitaxel-modified scheme (paclitaxel, doxorubicin, cisplatin plus mitotane (PDP-M)) in preclinical *in vitro* and *in vivo* models. In addition, based on an extraordinary uptake phenomenon of liposomes in ACC cells, we further evaluated liposomal variants of these protocols (etoposide, liposomal doxorubicin, liposomal cisplatin plus mitotane (LEDP-M) and nab-paclitaxel, liposomal doxorubicin, liposomal cisplatin plus mitotane (LPDP-M)). *In vitro*, PDP-M was more potent in the induction of apoptosis and inhibition of cell viability as well as cell proliferation than EDP-M. Following the administration of a single therapeutic cycle, we further demonstrated that LEDP-M and LPDP-M exerted significant antitumoral effects *in vivo*, which were not as evident upon EDP-M and PDP-M treatments. These results were confirmed in a long-term experiment, in which the highest and sustained antitumoral effects were observed for LEDP-M. In summary, liposomal cytostatic substances could represent a promising option that deserves testing in appropriate clinical protocols for the treatment of ACC patients.

Key Words

- ▶ adrenocortical carcinoma
- ▶ chemotherapy
- ▶ preclinical animal model
- ▶ liposomes
- ▶ lipoplatin
- ▶ adrenal cortex

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Introduction

Adrenocortical carcinoma (ACC) represents a rare but highly malignant tumor entity. In a large proportion of cases, the high growth rate of this endocrine neoplasm results in late diagnosis not amendable to surgical

resection. However, even when diagnosed at an early stage, radical surgery is afflicted by high relapse rates (Terzolo *et al.* 2007). Thus, overall prognosis in ACC patients is still poor, and innovative treatment options are

urgently required (Abiven *et al.* 2006, Libe *et al.* 2007). Medical treatment of ACC is limited to common cytotoxic agents, which are usually given in combination with the adrenolytic substance mitotane. However, these therapeutic agents in general result in only partial responses and complete remissions are rarely observed. Only recently, a prospective randomized interventional trial for late-stage ACC patients has been finalized to compare the most frequently administered therapeutic treatment schemes in a cross-over design: streptozotocin plus mitotane (Sz-M) vs etoposide, doxorubicin, and cisplatin plus mitotane (EDP-M) (Fassnacht *et al.* 2012). Within this study design, EDP-M resulted in higher response rates and longer progression-free survival in comparison with Sz-M. Nevertheless, overall therapeutic efficacy was still dissatisfactory and treatment regimens were associated with severe and dose-limiting adverse effects (Fassnacht *et al.* 2012). In recent years, different nanotechnological modifications have been used to improve classical chemotherapies by increasing therapeutic efficacy and diminishing side effects. As an example, liposomes have been widely used for the encapsulation of doxorubicin, vincristine, and platinum derivatives, among others (Harrington *et al.* 2002, Immordino *et al.* 2006). The rationale for using liposomes is mainly based on three important observations: first, liposomes display slower releasing rates and subsequently sustained bioavailability of the encapsulated drugs; second, increase in particle size results in modified biodistribution patterns; and third, liposomes are characterized by passive intratumoral accumulation through an enhanced permeability and retention effect, which largely depends on a prolonged blood circulation time. Multiple passages through the tumor vasculature together with high interstitial pressure further facilitate liposomal entrapment in this compartment (Drummond *et al.* 1999, Gabizon 2002, Harrington *et al.* 2002). The previously described effects of conventional liposomes are markedly improved by PEGylation of the liposomal surface, which leads to the development of the so-called sterically stabilized or stealth liposomes (Papahadjopoulos *et al.* 1991, Drummond *et al.* 1999, Gabizon *et al.* 2003). Sterically stabilized liposomal variants of doxorubicin and cisplatin are commercially available and have been shown to exhibit higher therapeutic efficacy or at least better tolerability than the free drugs. Both agents have so far been successfully tested against various solid tumors including breast cancer (Rivera 2003, O'Brien 2008), Kaposi's sarcoma (Coukell & Spencer 1997, Sharpe *et al.* 2002), non-squamous non-small-cell lung cancer (Mylonakis *et al.* 2009, Stathopoulos *et al.* 2011), and

pancreatic cancer (Stathopoulos *et al.* 2006), among others. Recently, we have demonstrated an extraordinary uptake phenomenon of liposomes, specifically in adrenocortical tumor cells (Hantel *et al.* 2010, 2012). These findings indicated that liposomal encapsulation could represent a treatment modality with particular advantage for treating tumors of adrenocortical origin resulting in the enhancement of therapeutic efficacy in comparison with free cytotoxic agents.

In an attempt to optimize the existing treatment modalities for ACC patients, we tested in preclinical tumor models the established treatment scheme (EDP-M) in comparison with a liposomal variant of this regimen with free doxorubicin and cisplatin replaced with their liposomal formulations (etoposide, liposomal doxorubicin, liposomal cisplatin plus mitotane (LEDP-M)). Although successful liposomal encapsulation has also been described for etoposide with reduced volume of distribution and decreased plasma clearance (Sengupta *et al.* 2000, Sistla *et al.* 2009), this formulation is not yet available as a commercial preparation. As doxorubicin and cisplatin have been shown to result in good therapeutic responses together with paclitaxel (Stathopoulos *et al.* 2006, 2011, Leonardi *et al.* 2010), we included two further study arms where etoposide was replaced either with conventional paclitaxel (paclitaxel, doxorubicin, cisplatin plus mitotane (PDP-M)) or with a novel nanotechnologically optimized variant of this drug (nab-paclitaxel, LPDP-M (nab-paclitaxel, liposomal doxorubicin, liposomal cisplatin plus mitotane)).

This modified preparation has been shown to possess superior antitumor activity and lower toxicity in comparison with paclitaxel (Gradishar 2006, Miele *et al.* 2009), and recently preclinical evidence has demonstrated its potential suitability for treating ACC patients (Demeure *et al.* 2012).

The aim of this study was to assess the antitumoral effects of the novel treatment schemes PDP-M, LPDP-M, and LEDP-M in comparison with those of the classical EDP-M protocol in preclinical tumor models of ACC.

Subjects and methods

Cell culture

NCI-H295R cells (obtained from ATCC, c/o LGC Standards, Wesel, Germany) were cultured in a 1:1 mixture of DMEM and Ham's F-12 medium (DMEM-F12 and supplements, Gibco Invitrogen) at 37 °C in a 5% CO₂-95% air atmosphere. The medium was supplemented

with insulin (10 mg/ml), transferrin (5.5 mg/ml), and selenium (5 ng/ml), L-glutamine (2.5 mm), penicillin (100 U/ml), streptomycin (100 µg/ml), and 2% ultrosor G (Cytogen, Sinn, Germany). For all the applied assays, 40 000 NCI-H295R cells were seeded per well in 96-well plates and incubated overnight before the addition of therapeutic substances. The tested concentrations were based on the individual IC₅₀ (half maximal inhibitory concentration) of each drug with reference to cell proliferation: etoposide, 1.2 µM; doxorubicin, 11 µM; cisplatin, 9.6 µM; paclitaxel, 0.33 µM; and mitotane, 15.9 µM. In each case, drugs were tested from their lowest to their highest concentration (0.25×IC₅₀, 0.5×IC₅₀, 1×IC₅₀, and 2×IC₅₀) as a combination of EDP-M or PDP-M.

Caspase-3/-7 assay NCI-H295R cells were seeded in a 96-well white polystyrene plate (MaxiSorp, Nunc, Langensfeld, Germany) and incubated with the different cytostatic combinations for 8 h. For the quantification of caspase-3 and caspase-7 activities, the luminescent assay Caspase-Glo 3/7 (Promega) was used. Detection was carried out in a Victor³ 1420 multilabel counter from Perkin Elmer (Rodgau, Germany) following the manufacturer's protocol.

Cell viability assay (MTT) and cell proliferation assay (5-bromo-2'-deoxyuridine) NCI-H295R cells were seeded in a 96-well plate and cultivated for 24 h either with EDP-M or with PDP-M. A colorimetric immunoassay (Roche) based on the measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporation during DNA synthesis was used for the quantification of cell proliferation and a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Sigma-Aldrich) was used for the investigation of cell viability following the manufacturers' protocols. Measurements were made in a SPECTRA microplate reader from Tecan (Crailsheim, Germany).

Animal experiments

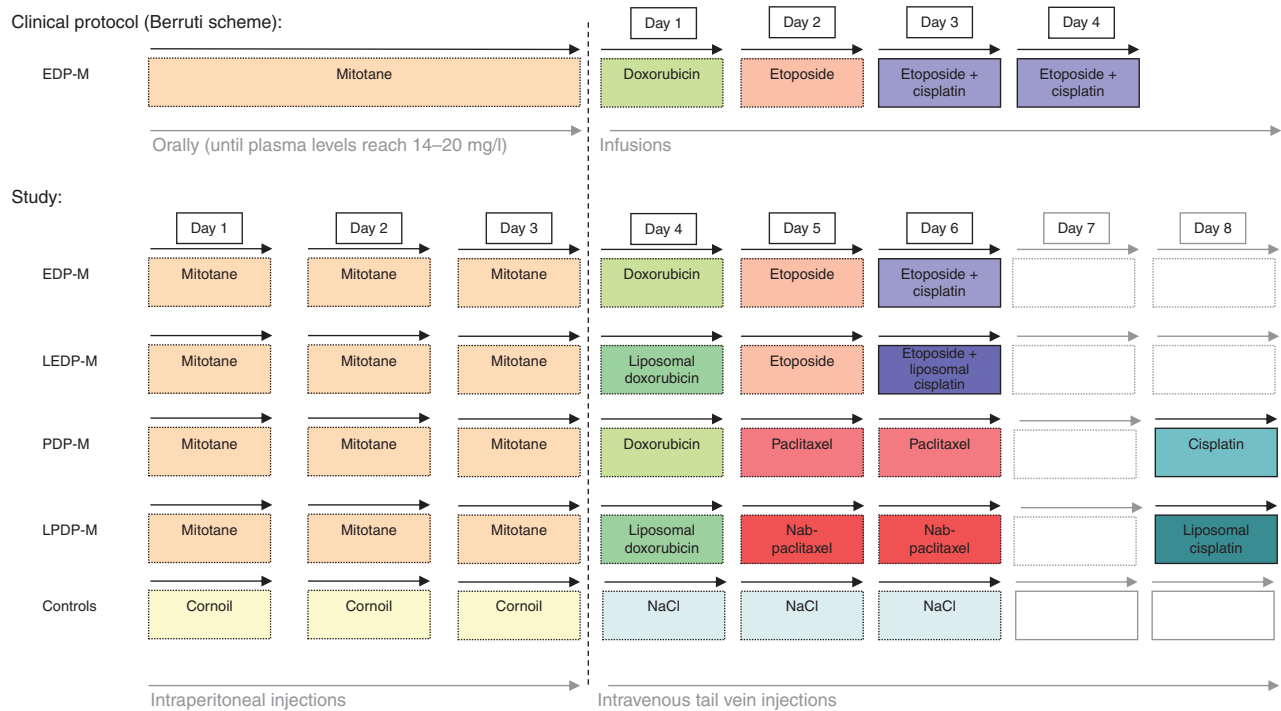
Animals and tumor models Female athymic NMRI *nu/nu* mice (6–8 weeks) were purchased from Harlan Winkelmann (Borchen, Germany) and housed under pathogen-free conditions. For preparing NCIh295 xenografts, 15×10⁶ tumor cells in a volume of 200 µl PBS were inoculated for tumor induction (*n*=7 mice per treatment group). Patient-derived ACC xenograft pieces (~2×2 mm) of excised tumor specimens (patient 1: ACC primary tumor, *n*=4 mice per treatment group and patient 2: ACC metastasis, *n*=5 mice per treatment group) were implanted

s.c. into the neck of individual mice. In the short-term experiment, therapeutic treatments were started on day 20, and in the long-term experiment, treatments were started 11 days after NCIh295 tumor inoculation. To ensure successful engraftment of implanted surgical tumor samples in these animals, the therapeutic treatment protocol was initiated several weeks after implantation. All the experiments were carried out following protocols approved by the Regierung von Oberbayern and in accordance with the German guidelines for animal studies. Furthermore, experiments involving patient biomaterial were approved by the Local Ethics Committee.

Utilized drugs Mitotane powder (HRA Pharma, Paris, France) was dissolved in autoclaved corn oil and injected i.p. at a daily dose of 300 mg/kg body weight over 3 consecutive days. Unless stated otherwise, cytostatic drugs were injected i.v. at a 2 mg/kg dose of cisplatin and lipoplatin (Regulon, Inc., Athens, Greece) and 10 mg/kg doses of doxorubicin, liposomal doxorubicin (caelyx, Essex Pharma GmbH, Munich, Germany), etoposide, paclitaxel, or nab-paclitaxel (abraxane, Celgene GmbH, Munich, Germany) according to the treatment schemes described for the different experiments in detail below.

Therapeutic experiments In an attempt to reflect the clinical situation as closely as possible, we adapted the classical EDP-M scheme (Berruti protocol, Berruti *et al.* (2005)) for a preclinical mouse model (Fig. 1 and Supplementary Table 1, see section on supplementary data given at the end of this article). Unless stated otherwise, all treatments were administered at 24-h intervals. Mitotane treatment was standardized to a duration of 3 days before the administration of the cytostatic drugs. Furthermore, as the cytostatic drugs were given as bolus tail vein injections, the i.v. administration was reduced to a maximum of four injections per mouse per 3 days. In addition, as paclitaxel is recommended to be administered 48 h before cisplatin administration, the paclitaxel-based schemes were modified compared to the etoposide-based regimens (Milross *et al.* 1995). In the short-term experiment, 48 h after the last treatment, the mice were killed and EDTA-blood samples were collected. Furthermore, tumors, hearts, and kidneys were collected and paraffin-embedded for histological and immunohistochemical analyses.

In the long-term therapeutic experiment, the mice were subjected to two cycles of the previously described treatment schemes with a therapy-free interval of 10 days in between. The mice were monitored daily, and the

**Figure 1**

Schematic illustration of the preclinical treatment modalities adapted from the clinical EDP-M (Berruti protocol) exemplified for one therapeutic cycle.

observation period was set to the period when the diameter of the first tumor reached a value of 1.5 cm. Antitumoral effects were recorded by measuring the tumor size (length \times width (cm²)).

Pathological and immunohistochemical examination Histological examination of tissue slides was done using Masson–Goldner (MG) trichrome (Carl Roth, Karlsruhe, Germany), hematoxylin–eosin (H&E), or picosirius-red (Sigma-Aldrich) staining. Immunohistochemical analysis was carried out by rehydrating the paraffin-embedded sections followed by incubation with a blocking buffer containing 3% BSA (Roche Diagnostics), 5% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and 0.5% Tween 20 for 15 min. Monoclonal mouse anti-human Ki67 (DakoCytomation, Glostrup, Denmark; 1:200 in blocking buffer) antibody was used to stain for specific Ki67. Incubation was carried out overnight at 4 °C. After rinsing for 15 min in PBS, secondary antibody (goat anti-mouse biotinylated IgG, Santa Cruz Biotechnology, Santa Cruz, CA, USA; biotin-SP-conjugated AffiniPure goat anti-rat, Jackson ImmunoResearch Laboratories; or biotinylated anti-rabbit, Vector Laboratories, Burlingame, CA, USA) was applied for 30 min at room temperature. Bound primary antibody was visualized using

the VECTASTAIN ABC Kit (Vector Laboratories) according to the manufacturer's protocol with incubation for 30 min followed by 3,3'-diaminobenzidine (Sigma-Aldrich) staining. After Ki67 immunohistochemistry, tumor slides were further stained with Vector Methyl Green Nuclear counterstain (Vector Laboratories). For the detection of apoptotic cells, the DeadEnd Colorimetric TUNEL System (Promega) was used following the manufacturer's protocol.

For quantification, six high-power fields (HPFs, 0.391 mm², 400 \times magnification) per NCIh295 tumor were investigated and quantified for Ki67-positive cells, Ki67-negative cells, or TUNEL-positive cells. For explanted patient xenografts, complete slides were analyzed, and the quantified cells were indicated as the number of positive cells per HPF (ki67) or per mm² (TUNEL).

Statistical analysis

All results are expressed as means \pm s.e.m. Statistical significance was determined using one-way ANOVA with Bonferroni's multiple comparison test or the unpaired *t*-test (Prism Software, Houston, TX, USA). Statistical significance was defined as $P < 0.05$, and it is denoted as stars ($*P < 0.05$, $**P < 0.01$, and $***P < 0.001$) in the figures, unless stated otherwise.

Results

Investigation of the antitumoral effects of EDP-M and PDP-M *in vitro*

The effects of EDP-M or PDP-M treatment on the apoptosis, proliferation, and viability of NCI-H295R cells were investigated using caspase (Fig. 2A), MTT (Fig. 2B), and BrdU (Fig. 2C) assays respectively. These experiments demonstrated the superiority of PDP-M in comparison with EDP-M regarding all the investigated endpoints and under various concentrations as exemplified for the induction of apoptosis (percentage of 100% basal; IC_{50}) EDP-M: $473.3 \pm 11.7\%$ vs (IC_{50}) PDP-M: $629 \pm 16\%$; $P=0.001$), viability (IC_{50}) EDP-M: $69.7 \pm 0.9\%$ vs (IC_{50}) PDP-M: $40.7 \pm 1.7\%$; $P=0.0001$), and inhibition of proliferation ($0.5 \times IC_{50}$) EDP-M: $105.3 \pm 9.4\%$ vs ($0.5 \times IC_{50}$) PDP-M: $14.5 \pm 4.4\%$; $P=0.0009$).

Investigation of short-term therapeutic efficacy in tumor xenografts

In vivo, short-term therapeutic efficacy of the four different treatment schemes, EDP-M, PDP-M, LEDP-M, and LPDP-M, was tested in NCIh295 xenografts. After the administration of one therapeutic cycle (according to Fig. 1), antitumoral effects were first investigated using Ki67 immunohistochemistry (Fig. 3A). The highest number of Ki67-positive and subsequently proliferating cells (cells/HPF/tumor) was documented in controls (42.0 ± 3.0). While treatment with EDP-M (38.5 ± 2.5), LEDP-M (37.9 ± 1.1), and PDP-M (33.6 ± 1.6) did not result in significant differences compared with controls, LPDP-M treatment was followed by a significant decrease in the fraction of proliferating cells (31.3 ± 1.0 , $P < 0.01$; Fig. 3A). Notably, the liposomal chemotherapeutic regimens also affected the non-proliferating tumor fraction and led to a significant reduction in the number of Ki67-negative tumor cells upon LPDP-M (21.0 ± 2.3 ; $P < 0.01$) and LEDP-M (24.0 ± 1.3 ; $P < 0.01$) treatments compared with controls (42.5 ± 3.6), while this effect was not detected in the EDP-M-treated (33.2 ± 3.6) and PDP-M-treated (29.3 ± 1.5) NCIh295 tumors (Fig. 3B). On combining both proliferating and non-proliferating cellular fractions in our analysis, not only LEDP-M (62.0 ± 1.6 ; $P < 0.05$) and LPDP-M (52.3 ± 3.0 ; $P < 0.001$) treatments, but also PDP-M treatment (63.0 ± 1.4 ; $P < 0.05$) was found to be associated with a significant reduction in the total number of tumor cells in comparison with the untreated controls (85.6 ± 5.8), while the classical EDP-M treatment did not result in similar antitumoral effects (71.8 ± 8.6 ; $P > 0.05$; Fig. 3C).

Furthermore, we quantified the induction of apoptosis by the different treatment schemes where liposome-based treatment regimens were also associated with a more pronounced effect for TUNEL-positive cells. The administration of LEDP-M (37.1 ± 2.0 ; $P=0.004$ vs controls and

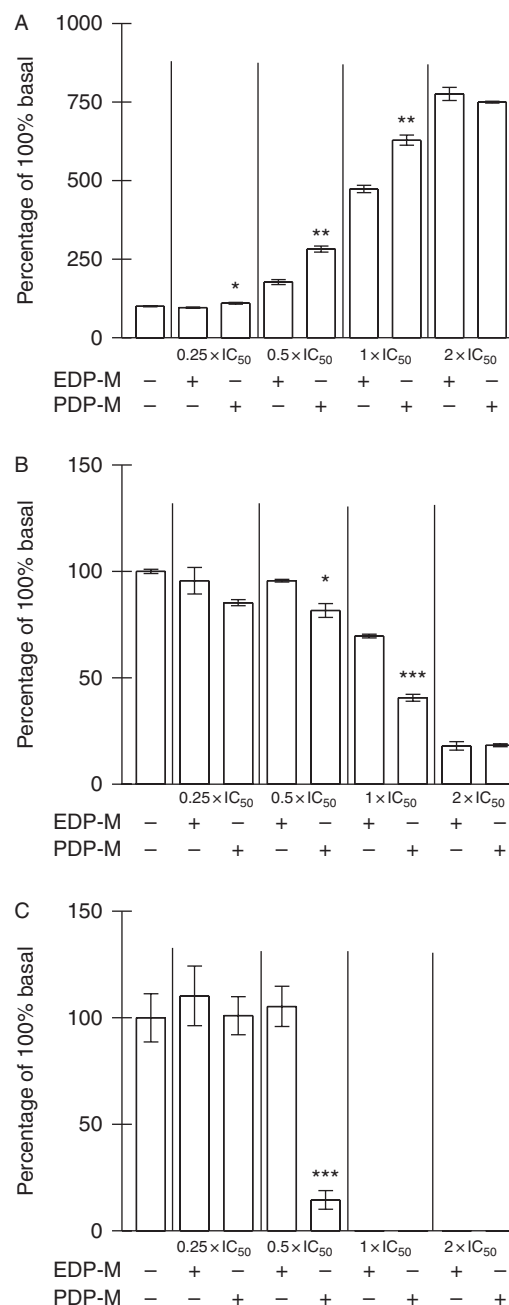
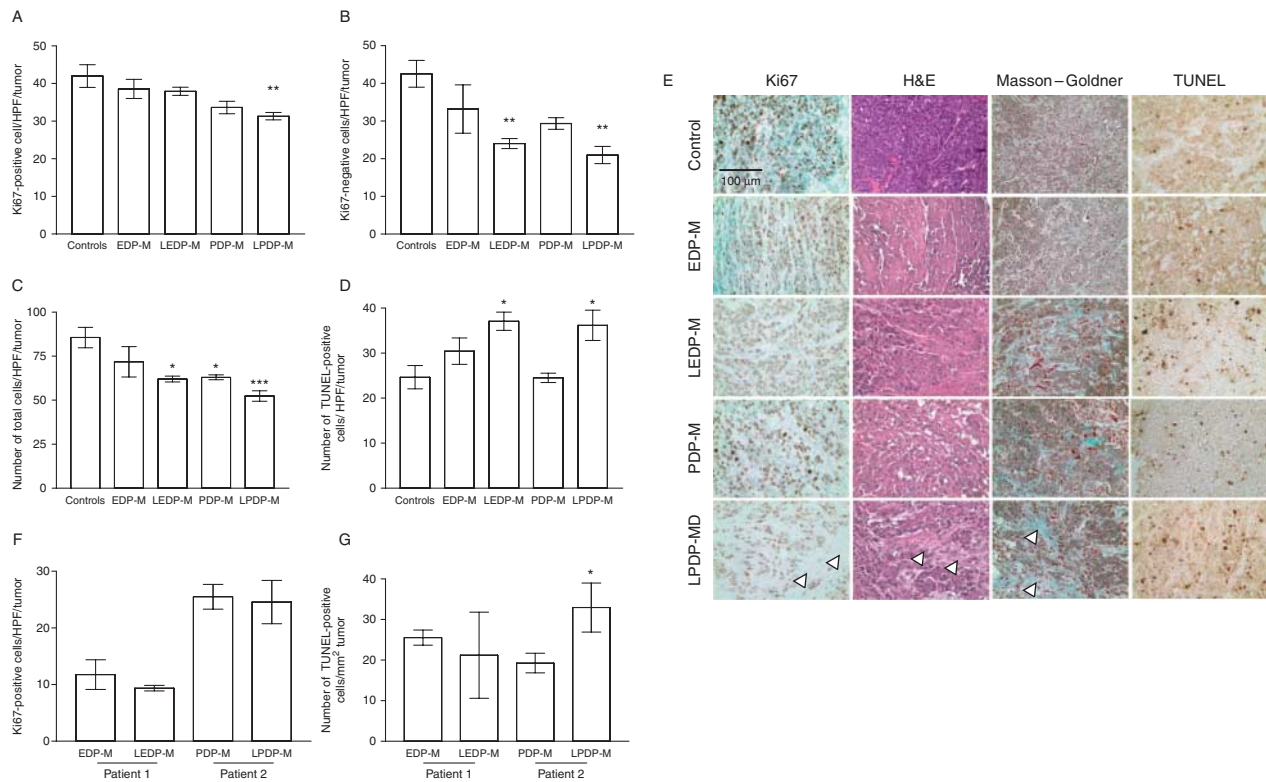


Figure 2 Treatment-dependent induction of caspase-3/7 (A) as well as inhibition of cell viability (B) and cell proliferation (C) upon treatment with different concentrations of EDP-M and PDP-M *in vitro*. Stars represent statistical significance of PDP-M over EDP-M.

**Figure 3**

Ki67 and TUNEL tumor analysis results obtained for NCIh295 tumors in the short-term therapeutic experiment. Quantification of the number of Ki67-positive cells (A), Ki67-negative cells (B), total cells (C), and TUNEL-positive cells (D), as well as representative pictures obtained from Ki67, H&E, Masson–Goldner, and TUNEL bright-field microscopy (E). Quantification of

the number of proliferating cells (F) and apoptotic cells (G) after subjecting patient ACC tumor xenografts to different treatments. Statistical significance vs controls (A, B, C and D) and (G) PDP-M group is denoted with stars (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

$P = 0.09$ vs EDP-M) and LPDP-M (36.2 ± 3.4 ; $P = 0.02$ vs controls and $P = 0.006$ vs PDP-M) was followed by a significant increase in the number of apoptotic cells in comparison with controls (24.7 ± 2.6). A comparable increase in apoptosis was not detectable in the PDP-M-treated (24.5 ± 1 ; $P = 0.96$ vs controls) and EDP-M-treated (30.5 ± 2.9 ; $P = 0.17$ vs controls) NCIh295 tumors (Fig. 3D).

Moreover, antitumoral effects were confirmed histologically in a semi-quantitative analysis of H&E- and MG trichrome-stained tumors: the number of hematoxylin-stained nuclei appeared to be decreased, cell borders were blurred, and a higher percentage of connective tissue was apparent mainly in the liposomal treatment groups (as indicated by arrows for LPDP-M in bright-field pictures in Fig. 3E). Each tumor was histologically analyzed and categorized with regard to differences in tumor density and tumor cell-free tissue, defined as blurred areas with reduced nuclear staining in H&E staining and green-stained connective tissue in MG staining (Table 1). LEDP-M- and LPDP-M-treated NCIh295 xenografts

exhibited a reduction in tumor density in comparison with controls and EDP-M- and PDP-M-treated NCIh295 xenografts. Tumor cell-free tissue exhibited a tendency to increase after LEDP-M, PDP-M, and LPDP-M treatments compared with controls and EDP-M-treated xenografts.

In addition to the experiments on NCIh295 xenografts, Ki67 (Fig. 3F) and TUNEL (Fig. 3G) analyses were also carried out on two different ACC-derived patient xenografts. Owing to the limitation of available fresh tumor material, only the relevant treatment groups were compared: mice carrying tumor derived from patient 1 were treated either with EDP-M or with LEDP-M, while mice carrying ACC xenograft derived from patient 2 were treated either with PDP-M or with LPDP-M. Considering a comparison of only the directly related treatment groups without controls, in patient xenografts (patient 1: EDP-M, 11.8 ± 2.1 vs LEDP-M, 9.4 ± 0.4 ; $P = 0.4$ and patient 2: PDP-M, 25.2 ± 1.6 vs LPDP-M, 24.5 ± 3.1 ; $P = 0.8$), we detected no significant differences in the number of Ki67-positive cells, but significantly more apoptotic cells after treatment with

Table 1 Semi-quantitative analysis results obtained for hematoxylin/eosin (H&E) and Masson–Goldner (MG) staining of NCIh295 tumors for all treatment groups ($n=6/7$ tumors per group). Each tumor was analyzed with regard to differences in tumor density and tumor cell-free tissue

	Masson–Goldner/H&E					
	Tumor density			Tumor cell-free tissue		
	Low	Medium	High	Low	Medium	High
Control ($n=6$)	0/0	1/2	5/4	2/2	4/4	0/0
EDP-M ($n=6$)	2/2	1/3	3/1	4/1	2/5	0/0
LEDP-M ($n=6$)	1/2	5/4	0/0	0/0	5/2	1/4
PDP-M ($n=7$)	2/2	3/4	2/1	0/0	6/4	1/3
LPDP-M ($n=7$)	2/2	5/5	0/0	0/0	5/3	2/4

LPDP-M than after treatment with PDP-M (patient 2: 32.9 ± 6 vs 19.3 ± 2.4 ; $P=0.05$), while LEDP-M treatment did not lead to significant differences compared with EDP-M treatment (patient 1: 21.2 ± 10.6 vs 25.6 ± 1.9 ; $P=0.8$).

Investigation of adverse effects after short-term therapeutic treatment

As leukopenia is a common side effect induced by the clinical EDP-M protocol, we investigated blood samples collected from controls and EDP-M-, PDP-M-, LEDP-M-, and LPDP-M-treated NCIh295 tumor-bearing mice at the end of the short-term experiment. While parameters such as erythrocytes and hemoglobin did not exhibit any significant changes after the different treatments protocols in comparison with those in untreated controls (Fig. 4A and B), we detected a significant decrease in the number of leukocytes (G/l) in mice subjected to the etoposide-based treatment schemes (EDP-M: 1.9 ± 0.2 ; $P < 0.01$ and LEDP-M: 2.1 ± 0.3 ; $P < 0.01$; vs controls: 6.0 ± 1.3 ; Fig. 4C). Blood samples collected from the PDP-M-treated (3.3 ± 0.6) and LPDP-M-treated (3.2 ± 0.2) mice also exhibited a tendency for leukocyte reduction, but for these groups, no statistically significant decrease vs controls was detectable.

As other targets of common side effects from the utilized drugs, we further examined hearts and kidneys collected from the treated animals. Following one therapeutic cycle, we did not detect severe morphological changes in the organs of mice belonging to any of the treatment arms. However, while the kidneys of the LEDP-M- and LPDP-M-treated mice appeared to be completely unaffected in comparison with those of controls, H&E staining of the kidney tissues of the EDP-M- and PDP-M-treated mice exhibited a tendency for reduction in nuclear staining intensity and more diffuse cell borders (Fig. 4D, E, F, G and H). Only a single kidney from the EDP-M group

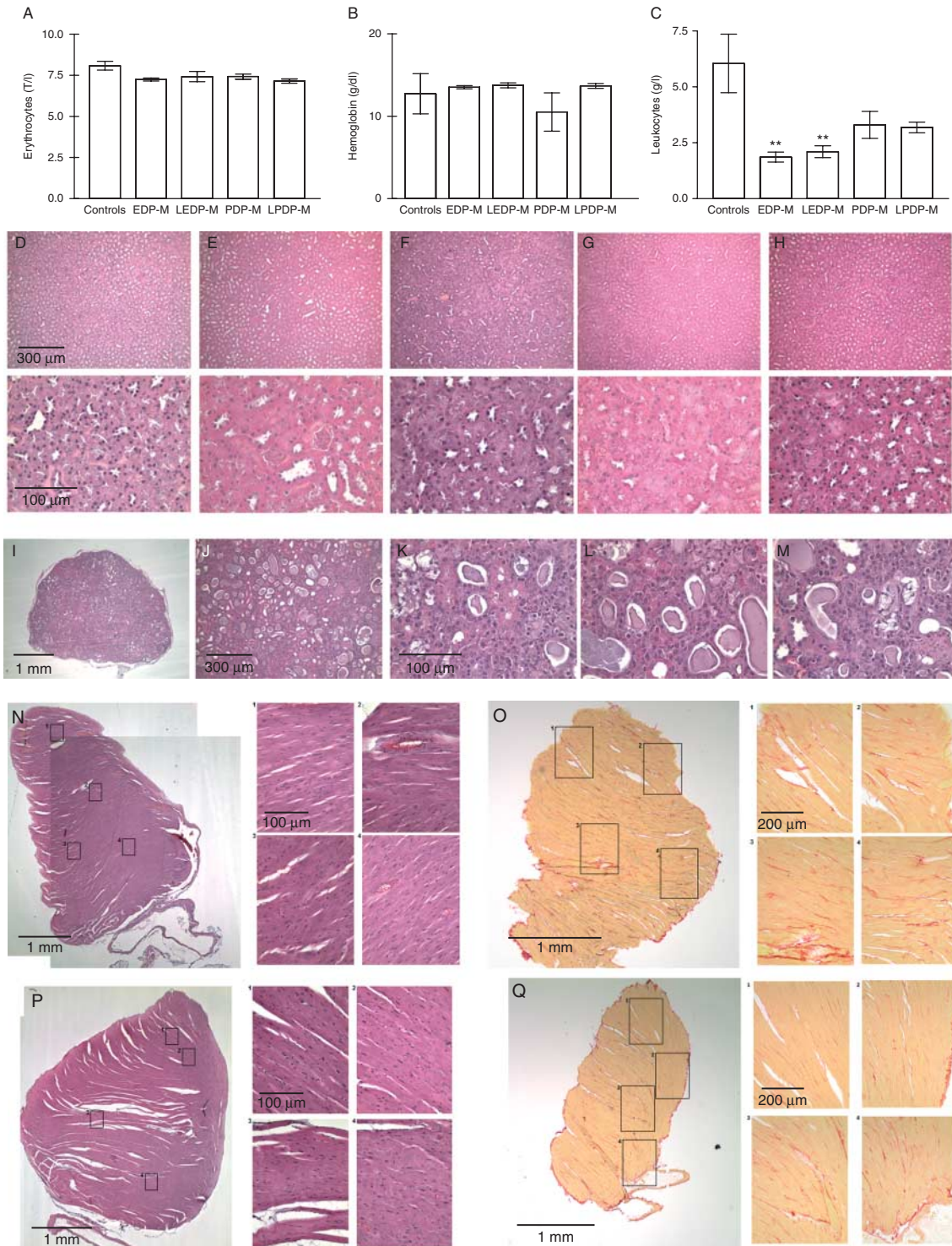
displayed more severe pathological changes (Fig. 4I, J, K, L and M). Comparison of hearts collected from the control animals with those collected from the EDP-M-treated mice, where predominantly effects were expected to be caused by free doxorubicin, did not reveal any changes in the analyzed tissues neither by H&E staining nor by picosirius-red staining (Fig. 4N, O, P and Q).

Investigation of long-term therapeutic efficacy in NCI-H295R xenografts

The primary endpoint of the long-term experiment was the investigation of NCIh295 tumor development by measuring the tumor sizes (expressed as length \times width (cm^2)) during treatment with EDP-M, PDP-M, LEDP-M, or LPDP-M (Fig. 5). During this experiment, the mice were subjected to two therapeutic cycles with each agent following the established treatment protocols. There was a therapy-free interval of 10 days between each cycle. This experiment demonstrated a clear therapeutic superiority of LEDP-M. Besides significant differences between controls (day 35, 0.3 ± 0.08 and day 53, 0.96 ± 0.35) and LEDP-M-treated tumors (day 35, 0.11 ± 0.02 , $P=0.02$ and day 53, 0.1 ± 0.03 , $P=0.03$), we detected from day 35 onwards (0.17 ± 0.02 ; $P=0.04$) up to day 53 when the experiment was concluded (0.3 ± 0.04 ; $P=0.0006$) highly significant reduced tumor sizes in LEDP-M-treated tumors in comparison with EDP-M-treated tumors.

Discussion

Herein, we report on the attempt to establish novel therapeutic protocols for the treatment of ACC patients through the modification of existing polychemotherapies with nanotechnologically advanced cytostatic drugs. The starting point for this preclinical study was our recent observation of active internalization of liposomes by

**Figure 4**

Influence of one therapeutic cycle on different blood parameters (A, B and C). Stars denote significant differences compared with controls. Pathological examination of kidneys obtained from different treatment groups: control (D), EDP-M (E), LEDP-M (F), PDP-M (G), and LPDP-M (H).

Pictures of a pathologically affected kidney from the EDP-M treatment group at different magnifications (I, J, K, L and M). Pictures of murine hearts from control (N and O), and EDP-M (P and Q) groups analyzed by H&E and picosirius-red staining.

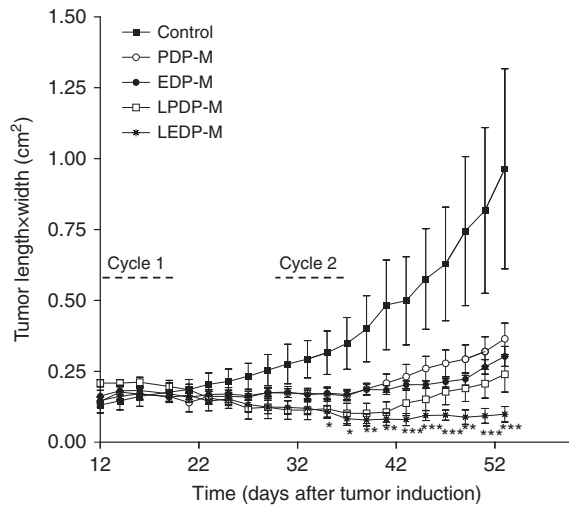


Figure 5
Effects on the tumor size of NCIh295 xenografts after two therapeutic cycles with different treatment modalities. Stars denote significant differences compared with the EDP-M group.

NCIh295 and SW-13 cells (Hantel et al. 2012), which indicated that liposomally encapsulated drugs could represent an interesting treatment option specifically advantageous for treating tumors of adrenocortical origin. As two components of the clinically utilized EDP-M scheme (Fassnacht et al. 2012), doxorubicin and cisplatin, are available as sterically stabilized liposomal formulations, we replaced these free agents with their liposomal variants. Although successful liposomal encapsulation has also been described for etoposide (Sengupta et al. 2000, Sistla et al. 2009), it is not yet available as a commercial preparation. The experimental results presented herein confirmed the results of our previous work and theoretical assumptions: short-term therapeutic treatment with LEDP-M led to a significant decrease in the number of Ki67-negative cells and total number of tumor cells in the investigated NCIh295 tumors, while no therapeutic effect was evident in animals treated with the classical EDP-M scheme. Strongest antitumoral impact against proliferating and non-proliferating cells was observed in the novel LPDP-M treatment arm. Paclitaxel as a single agent has previously been shown to reduce cell proliferation, viability, and hormone secretion in NCIh295 tumor cells *in vitro* (Fallo et al. 1996, 1998), and our *in vitro* experiments also support the superior antitumoral effects of the paclitaxel-based PDP-M scheme when compared with the EDP-M scheme. By contrast, weekly paclitaxel administration together with sorafenib as

second/third-line therapy in a current phase II study in ACC patients has not proved to be successful (Berruti et al. 2011). Along the same line, the therapeutic efficacy of free PDP-M *in vivo* was only mild (short-term experiment) or not significantly different from that of the classical EDP-M scheme (long-term experiment). By contrast, our data indicate a pronounced effect of the novel LPDP-M scheme after one therapeutic cycle.

Further histological examination with TUNEL staining as well as H&E and MG staining confirmed antitumoral effects primarily following treatment with the liposomal regimen LEDP-M or LPDP-M in NCIh295 tumors. This enhanced antitumor efficacy in comparison with the free drugs can be explained by the previously described general advantages mediated by sterically stabilized liposomes including slower drug release rates, increased plasma half-life, and passive drug accumulation of liposomes (Immordino et al. 2006, Gabizon et al. 2011). Furthermore, the active uptake of liposomes by ACC cells could have further contributed to this finding (Hantel et al. 2012). Moreover, we cannot exclude the possibility that due to its lipophilic nature, mitotane could bind to the administered liposomes, further resulting in higher intratumoral mitotane levels. Thus, it would be interesting to investigate putative alterations in mitotane tumor delivery within these treatment schemes in future studies. However, based on our current knowledge, we suggest the previously discussed effects as the main mediators for enhanced therapeutic efficacy.

Although both liposomal regimens led to a significant induction in the number of apoptotic cells compared with untreated controls, after one therapeutic cycle only LPDP-M regimen further resulted in significant differences compared with the corresponding free treatment scheme PDP-M. No such differences were detectable between EDP-M and LEDP-M regimens in the short-term setting. Interestingly, we were also able to confirm this pronounced effect of LPDP-M over PDP-M regarding the induction of apoptosis in patient-derived tumor xenografts. One explanation for the superior antitumoral effect of LPDP-M could be a specific pharmacokinetic interference between nab-paclitaxel and liposomal doxorubicin. For non-modified paclitaxel, an inhibitory effect has been described specifically on liposomal doxorubicin, resulting in further prolongation of plasma half-life and enhanced therapeutic efficacy (Gabizon et al. 2011). We cannot exclude the possibility that this observation might also be relevant for the albumin-bound form of paclitaxel. On the other hand, nab-paclitaxel could simply be more effective at mediating short-term apoptotic effects than

etoposide. The fact that LPDP-M was not superior to LEDP-M in the long-term therapeutic experiment supports the assumption for only short-term effects and potential development of secondary resistance of tumor cells. Another explanation for the unexpected lack of long-term efficacy of LPDP-M could be the delayed administration of liposomal cisplatin 48 h after paclitaxel treatment. Following the investigation of sequence and schedule dependency of paclitaxel-based polychemotherapies, a 48-h interval between paclitaxel and cisplatin administration has been recommended (Milross *et al.* 1995). However, as these findings account for the free substances, it is likely that the optimal time point for the administration of a combination of nab-paclitaxel and liposomal cisplatin might differ as pharmacokinetic parameters change with encapsulation and modification with albumin respectively. Although tumors in the LEDP-M arm were subjected to two complete treatment cycles on 1 day, therapeutic efficacy in the LPDP-M group could be averaged over days 6–8 and thus might not have had the same intensity. On the other hand, the same time schedule led to the highest antitumoral effects in the short-term experiment with the same delay in administration. Thus, we do not assume a general disadvantage of the 48-h treatment interval for the detected differences in the long-term therapeutic efficacy. To evaluate the full potential of nab-paclitaxel in this combinational regimen, additional preclinical experiments would be advantageous to specify the ideal time points of administration.

As the classical EDP-M protocol is associated with severe toxicity, we further initiated an investigation of putative therapy-related toxicities. Leukopenia is one of the dose-limiting side effects of the classical Berruti (EDP-M) protocol (Berruti *et al.* 2005) and further commonly observed serious adverse effects associated with doxorubicin or cisplatin include cardiotoxicity and nephrotoxicity (Fassnacht *et al.* 2012). Liposomal encapsulation of cisplatin (Boulikas 2004, Devarajan *et al.* 2004) and doxorubicin (Harrington *et al.* 2002, Huwyler *et al.* 2008) has been shown to quench these drug-specific side effects. In the present study, blood sampling revealed significantly reduced numbers of leukocytes compared with control animals following treatment with the etoposide-based scheme, but no differences between the EDP-M- and LEDP-M-treated animals. Mice in the PDP-M and LPDP-M groups exhibited slightly improved blood counts, indicating that the replacement of etoposide with paclitaxel could hold advantages. Histological examination regarding cardiotoxicity and nephrotoxicity revealed overall no severe pathological changes after one therapeutic cycle.

However, kidneys collected from the EDP-M- and PDP-M-treated mice seemed to be slightly affected compared with those collected from the control, LEDP-M, and LPDP-M groups, and a single kidney from the EDP-M group displayed more severe pathological changes. Following these morphological analyses, it was found that liposome-based regimens, despite their higher antitumor efficacy, were at least as well tolerated as and resulted in better tolerability than regimens based on free cytotoxic agents. However, this should be evaluated in more detail upon multiple treatment courses and include additional techniques such as real-time PCR.

In summary, modifications of the classical treatment schemes with advanced drugs such as liposomal and albumin-bound chemotherapeutic agents hold promise for the treatment of ACC patients. In addition to the development of novel substances, newly tested combinations of commercially already available drugs could lead to a faster improvement of the clinical situation, as these substances can be easily introduced into future clinical trials. To further improve the therapeutic efficacy of the novel treatment regimens, the replacement of etoposide and paclitaxel with liposomal preparations could present an interesting future perspective. Although currently no commercial liposomal preparation for etoposide is available, a vascular-targeting cationic liposomal preparation for paclitaxel has recently been established and is under clinical testing (Strieth *et al.* 2008, Eichhorn *et al.* 2010). Altogether our experiments indicate that the tested liposomal variants could represent interesting treatment options for ACC patients and should be investigated in more detail to allow a safe and effective translation into clinical studies.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-13-0439>.

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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