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MDR1 inhibition increases sensitivity to doxorubicin and etoposide in adrenocortical cancer

S G Creemers¹, P M van Koetsveld¹, W W De Herder¹, F Dogan¹, G J H Franssen², R A Feelders¹ and L J Hofland¹

¹Department of Internal Medicine, Division of Endocrinology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, the Netherlands

²Department of Surgery, Erasmus MC, University Medical Center Rotterdam, Rotterdam, the Netherlands

Correspondence should be addressed to L J Hofland: l.hofland@erasmusmc.nl

Abstract

Chemotherapy for adrenocortical carcinoma (ACC) has limited efficacy and is accompanied by severe toxicity. This lack of effectiveness has been associated with high tumoral levels of the multidrug resistance (MDR) pump P-glycoprotein (P-gp), encoded by the *MDR1* gene. In this study, effects of P-gp inhibition on the sensitivity of ACC cells to cytotoxic drugs were evaluated. *MDR1* mRNA and P-gp expression were determined in human adrenal tissues and cell lines. H295R, HAC15 and SW13 cells were treated with mitotane, doxorubicin, etoposide, cisplatin and streptozotocin, with or without the P-gp inhibitors verapamil and tariquidar. Cell growth and surviving fraction of colonies were assessed. *MDR1* mRNA and P-gp protein expression were lower in ACCs than in adrenocortical adenomas ($P < 0.0001$; $P < 0.01$, respectively). *MDR1* and P-gp expression were positively correlated in ACC ($P < 0.0001$, $\rho = 0.723$). Mitotane, doxorubicin, cisplatin and etoposide dose dependently inhibited cell growth in H295R, HAC15 and SW13. Tariquidar, and in H295R also verapamil, increased the response of HAC15 and H295R to doxorubicin (6.3- and 7.5-fold EC_{50} decrease in H295R, respectively; all $P < 0.0001$). Sensitivity to etoposide was increased in H295R and HAC15 by verapamil and tariquidar (all $P < 0.0001$). Findings were confirmed when assessing colony formation. We show that cytotoxic drugs, except streptozotocin, used for ACC treatment, inhibit ACC cell growth and colony formation at clinically achievable concentrations. P-gp inhibition increases sensitivity to doxorubicin and etoposide, suggesting that MDR1 is involved in sensitivity to these drugs and could be a potential target for cytotoxic treatment improvement in ACC.

Key Words

- ▶ adrenal cortex
- ▶ carcinoma
- ▶ chemotherapy
- ▶ oncology
- ▶ gene regulation

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Introduction

Adrenocortical carcinoma (ACC) is a highly malignant disease with limited treatment options (Creemers *et al.* 2016a). The only curative treatment is tumor-directed surgery (R0 resection) in case of local disease. The high recurrence rates (30–50%) after surgery, especially in high-risk patients, provide the rationale for adjuvant treatment with mitotane as standard of care (Bellantone *et al.* 1997,

Hahner & Fassnacht 2005, Allolio & Fassnacht 2006, Fassnacht *et al.* 2009, Grubbs *et al.* 2010, Libe *et al.* 2015, Margonis *et al.* 2016). In case of progression of advanced disease, mitotane can be combined with cytotoxic drugs. In the first randomized, controlled trial in ACC, the FIRM-act trial, mitotane in combination with etoposide, doxorubicin and cisplatin (M-EDP) resulted in a longer

median progression-free survival compared to patients receiving mitotane and streptozotocin (Fassnacht *et al.* 2012). However, the median overall survival for this regimen was still only 14.8 months, but is now considered the treatment of choice (Fassnacht *et al.* 2012). These data suggest that the resistance to chemotherapy is still a major impediment of successful systemic treatment of ACC patients. The limited efficacy of chemotherapy in ACC has been associated with the presence of high levels of P-glycoprotein (P-gp) in normal and tumoral adrenal tissue, an enzyme which actively pumps a variety of substrates like chemotherapy out of the cell (Flynn *et al.* 1992, Sharom 2008). P-gp is also expressed in several other types of cancer, such as colon, kidney, liver and pancreas tumors (Goldstein *et al.* 1989). Data on the role of mitotane as P-gp inhibitor are equivocal. Mitotane has been shown to inhibit P-gp – encoded by the multidrug resistance protein 1 (*MDR1*) gene – activity *in vitro*, suggesting that this could reverse the chemoresistant character of ACC (Bates *et al.* 1991, Gagliano *et al.* 2014). In contrast, Theile and colleagues demonstrated the induction of not only P-gp, but also MRP2 (*MDR1*-related protein 2) and breast cancer resistance protein (BCRP), under mitotane treatment *in vitro* (Theile *et al.* 2015). A phase II trial in 35 ACC patients investigating mitotane as a P-gp antagonist in combination with doxorubicin, etoposide and vincristine showed no *in vivo* P-gp inhibition by mitotane (Abraham *et al.* 2002). The response rate to the chemotherapeutic drugs in combination with mitotane was 22%, a percentage difficult to interpret due to the lack of comparison in this study with mitotane or chemotherapy monotherapy (Abraham *et al.* 2002).

Several P-gp inhibitors have been described in literature. Verapamil is a first-generation P-gp inhibitor and substrate (competitive inhibitor), first described in 1981 (Tsuruo *et al.* 1981, Sharom 2008). Although verapamil was able to enhance drug accumulation *in vitro*, its clinical application is somewhat limited due to the challenging balance between the required doses to inhibit P-gp and potential verapamil-associated toxicity (Benson *et al.* 1985, Raderer & Scheithauer 1993). Tariquidar (XR9576), a non-transporter P-gp inhibitor, belongs to the third-generation P-gp inhibitors, described as most selective and potent inhibitors obtained by design (Palmeira *et al.* 2012). Tariquidar inhibits the basal ATPase activity associated with P-gp (Martin *et al.* 1999) and has shown to inhibit P-gp activity in ACC patients *in vivo* (Menefee *et al.* 2008). Tariquidar also inhibits BCRP,

another frequently studied ATP-binding cassette (ABC) protein associated with decreased accumulation of anticancer drugs (Kannan *et al.* 2011). The specificity depends upon the concentration and the relative density and capacity of P-gp vs BCRP. To the best of our knowledge, the effect of tariquidar on the efficacy of cytotoxic drugs has not yet been tested *in vitro* for treatment of ACC.

The aim of the present study is to further explore the potential value of inhibiting P-gp activity. We therefore assessed *MDR1* mRNA and P-gp expression in normal adrenals (NAs), adrenocortical adenomas (ACAs) and ACCs. Thereby, we evaluated the effects of the currently clinically used cytotoxic drugs, in combination with verapamil, and tariquidar, on the growth of human adrenocortical cancer cells.

Materials and methods

Adrenocortical tissues

Adrenocortical tissues were obtained between June 1994 and August 2016 at the Department of Surgery, Erasmus MC, Rotterdam. Directly after resection, adrenal tissues were embedded in Tissue-Tek and stored at -80°C . Diagnosis was confirmed using the Weiss score or Van Slooten index (van Slooten *et al.* 1985, Lau & Weiss 2009), dependent on the year of diagnosis. Normal adrenals were collected during nephrectomy and confirmed by the pathologist as being normal. Patient and tumor characteristics were obtained from electronic patient records. The study was approved by the Medical Ethics Committee of the Erasmus University Medical Center, and informed consent was obtained from all patients.

Real-time quantitative PCR

MDR1 mRNA expression was measured in cell lines and adrenocortical tissues. RNA isolation, cDNA synthesis and RT-PCR were performed as previously described, but using other primers (Supplementary Table 1, see section on supplementary data given at the end of this article; Sigma-Aldrich) (Creemers *et al.* 2016b). Three housekeeping genes were used to normalize mRNA levels using the Vandesompele method: hypoxanthine-guanine phosphoribosyl transferase 1 (*HPRT1*; Sigma-Aldrich), Beta-actin (*B-actin*; Thermo Fisher Scientific) and glucuronidase beta (*GUSB*; Thermo Fisher Scientific) (Vandesompele *et al.* 2002).

P-gp immunohistochemistry

Two tissue microarrays (TMA) comprising specimens from NAs, ACAs and ACCs were constructed, as previously described using the automated TMA constructor (ATA-27 Beecher Instruments, Sun Prairie, WI, USA) available at Erasmus MC, Department of Pathology (Papathomas *et al.* 2014). For an optimal representation, an expert pathologist identified three areas per specimen in hematoxylin and eosin-stained slides. Cores with a diameter of 1 mm were extracted from 'donor' blocks and brought into the 'recipient' paraffin block at predefined coordinates. In addition, formalin-fixed paraffin-embedded whole sections were used from three cases not included in the TMA. Five micrometer sections were cut and used for immunohistochemistry (IHC). Sections were deparaffinized, rehydrated and heated for 20 min in TRIS-EDTA buffer (pH 9.0). Endogenous peroxidase was blocked for 15 min in hydrogen peroxide/PBS 3% solution in darkness. Afterward, sections were washed and incubated with the rabbit monoclonal anti-P-gp antibody (dilution 1:500; ab170904; Abcam), overnight at 4°C. After several wash steps, sections were incubated for 30 min with the second antibody horseradish peroxidase rabbit/mouse, where after bound antibody was visualized with freshly prepared DAB+ Chromogen 100 µL (both Dako Detection System). Slides were counterstained with hematoxylin and coverslipped. Sections were scored blind for tissue type by two investigators (S G C, L J H) independently of each other using a semi-quantitative well-established immunoreactivity score (IRS), calculated by the product of the percentage positive cells (4, >80%; 3, >51–80%; 2, >10%; 1, 0) and intensity of staining (3, strong; 2, moderate; 1, mild; 0, no staining) (Remmele & Stegner 1987).

Cell lines and culture conditions

Three available human ACC cell lines were used: H295R, HAC-15 and SW13 obtained from the American Type Culture Collection, ECACC (Salisbury, Wiltshire, UK) and from Dr W Rainey (as a kind gift), respectively. Short tandem repeat profiling using a Powerplex Kit (Promega) of NCI-H295R and SW13 gave results consistent with the ATCC database. HAC15 showed a genetic profile identical to H295R, which is consistent with a previous report by Rainey *et al.* (Wang & Rainey 2012). Culture conditions were described in detail previously (van Koetsveld *et al.* 2006). Mitotane, etoposide and verapamil stock solutions were diluted in absolute ethanol (EtOH),

whereas doxorubicin, cisplatin and streptozotocin were diluted in H₂O. Tariquidar was diluted in 100% DMSO. All compounds were obtained from Sigma-Aldrich, except tariquidar, which was obtained from Tocris Biosciences (Abingdon, United Kingdom). To assess the optimal concentration of verapamil and tariquidar for the combination experiments with cytotoxic drugs, a dose-response curve was obtained for verapamil and tariquidar on cell growth in all three cell lines. After trypsinization, cells were plated at a concentration of 100,000, 75,000 and 20,000 cells per well for H295R, HAC15 and SW13, respectively, in order to obtain 80% confluency at the end of the experiment. The doubling times of H295R, HAC15 and SW13 are 30, 49 and 22 h, respectively (data not shown). The next day, incubations were started in quadruplicate and control cells were vehicle treated. Medium and compounds were refreshed after 3 days. All cell culture experiments were carried out at least twice in quadruplicate.

Cell proliferation assay

Dose-dependent effects of mitotane, doxorubicin, cisplatin, etoposide and streptozotocin, with or without the P-gp inhibitors, were assessed on cell amount after 7 days of treatment. After 7 days of cell culture experiments, media were removed and plates were stored at –20°C until DNA measurement. Measurement of total DNA was performed with the bisbenzimidazole fluorescent dye (Hoechst 33258, Sigma-Aldrich) as previously described (Hofland *et al.* 1990).

Colony forming assay

The colony forming assay (CFA) is considered the gold standard for measuring the effects of cytotoxic agents on cancer cells *in vitro*. We determined the effects of cytotoxic drugs with and without verapamil or tariquidar on colony formation in H295R, HAC15 and SW13. Effects on surviving fraction and colony size were assessed at cell line-specific concentrations of cytotoxic drugs that appeared to be effective in cell growth inhibition at monolayer culture (EC₅₀ values after 7 days). Concentrations were adjusted when too strong or no effects were observed on colony formation. Final concentrations were 5, 10 and 25 µM for mitotane and 0.01, 0.01 and 0.05 µM for doxorubicin in H295R, HAC15 and SW13, respectively. For etoposide, 0.1 µM was used in all cell lines, whereas a concentration of 1 µM was used for cisplatin. For verapamil and tariquidar, 10 µM and 1 µM were used, respectively. Plates were

coated with 1 mL poly-L-lysine (10 µg/mL), where after 3000, 1500 or 500 cells were plated in a six-well plates for H295R, HAC15 and SW13, respectively. After 1 day, drug treatment was initiated. Media were removed after 3 days and refreshed without treatment. After 2 weeks, the cells were washed and fixed. Cells were colored with hematoxylin, and amounts and sizes of colonies were measured using MultiImage TM Light Cabinet (Alpha Innotech). Surviving fraction was calculated as previously described (Franken *et al.* 2006).

Statistical analysis

For statistical analysis, Graphpad Prism 6.0 (Graphpad Software) and SPSS Statistics 21 (SPSS 21.0; SPSS Inc.) were used. Kruskal–Wallis, followed by Dunn's multiple comparisons test, or the Mann Whitney *U* test when applicable was used to assess the differences in *MDR1* mRNA and P-gp expression levels between different adrenal tissues. Correlations were tested using Spearman's rank coefficient. Non-linear regression curve fitting program was used to calculate the EC₅₀ values on cell growth in the ACC cell lines. For analysis of the combined effect of P-gp inhibitors and cytotoxic drugs on cell growth and colony formation, verapamil or tariquidar monotherapy was set as control. One-way ANOVA with Tukey's multiple comparisons test was used to assess the differences in colony formation between monotherapy and combination of cytotoxic drugs with P-gp inhibitors.

Values of $P < 0.05$ were considered significant. Data are presented as mean $\pm$ S.E.M., unless specified otherwise.

Results

Patient characteristics

Ten NAs, obtained during nephrectomy, 16 ACAs and 42 ACCs were included. *MDR1* mRNA expression was assessed in 58 adrenal specimens (8 NAs, 13 ACAs and 37 ACCs), while P-gp protein expression, as determined by IHC, was analyzed in 59 tissues (7 NAs, 14 ACAs and 38 ACCs). Four of the 42 ACC were recurrent cases, and two patients received mitotane before surgery. There were no patients, both primary and recurrent cases, who received chemotherapy before surgery. Patient and tumor characteristics are listed in Table 1.

MDR1 mRNA and protein expression in human adrenocortical carcinoma cell lines and tissues

MDR1 mRNA expression was higher in H295R cells compared to HAC15 ($P < 0.05$; Fig. 1A). *MDR1* mRNA expression could not be detected in SW13 cells. *MDR1* mRNA expression was lower in ACCs compared to ACAs and NAs, with a 7.7-fold lower median expression level in ACC than in ACA ($P < 0.0001$; $P < 0.01$, respectively; Fig. 1B).

P-gp expression, as determined by IHC, was high in almost all adrenal tissues, with a mean IRS of

Table 1 Patient and tumor characteristics of patients included in this study.

	Adrenocortical carcinomas ($n = 42$)	Adrenocortical adenomas ($n = 16$)
Mean age at diagnosis (years, median, IQR)	52 (42–65)	45 (38–56)
Mean follow-up (months, median, IQR)	18.5 (8.0–89)	31 (13–55)
Male (%)	20 (48%)	3 (19%)
Mean tumor size (cm, median, IQR)	14 (8.0–19)	2.70 (1.6–4.8)
Secretion		
Androgens	9 (21%)	1 (6%)
Glucocorticoids	20 (48%)	6 (38%)
Mineralocorticoids	2 (5%)	5 (31%)
Precursors	5 (12%)	0 (0%)
Estradiol	4 (10%)	0 (0%)
Non-secreting	15 (36%)	4 (25%)
Weiss score (median, IQR)	6.0 (5.0–7.0), $n = 35$	0.00 (0.0–0.8)
VanSlooten index (median, IQR)	21 (18–25), $n = 36$	1.6 (1.6–1.6)
ENSAT		
I	2 (5%)	14 (88%)
II	18 (47%)	2 (12%)
III	3 (8%)	0 (0%)
IV	15 (40%)	0 (0%)

Van Slooten index and Weiss score were not available for all patients, dependent on the year of diagnosis.
ENSAT, European Network for the Study of Adrenal Tumors; IQR, interquartile range.

Figure 1

MDR1 mRNA expression in adrenal cell lines (A) and adrenal tissues (B) and P-glycoprotein expression in human adrenocortical tissues (C). Correlation between *MDR1* mRNA expression and P-glycoprotein expression in ACCs (D). Representative example of P-gp immunohistochemical staining in adrenocortical carcinoma as determined by immunohistochemistry (E). Sections were blinded and independently evaluated by two investigators. Microscopic magnification 40 $\times$, 200 $\times$, and 400 $\times$. Lines represent medians. ρ represents Spearman's rank correlation coefficient. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; IRS, immunoreactivity score; NA, normal adrenals; ND, not detectable.

9.8 (range 0–12; Fig. 1C). Staining was primarily located in the cell membrane (Fig. 1E). P-gp expression was significantly lower in ACCs compared to ACAs ($P < 0.01$; Fig. 1C), which seems to be primarily driven by a subgroup of ACC patients with a P-gp expression below the median. *MDR1* mRNA expression was positively correlated with P-gp expression not only in the whole group of patients ($P < 0.0001$, $\rho = 0.723$) but also in the group of ACCs separately ($P < 0.0001$, $\rho = 0.6978$; Fig. 1D). No correlations were found between *MDR1* mRNA or P-gp expression and the Van Slooten index or the Weiss score. Expression levels were also comparable between different ENSAT stages. *MDR1* mRNA expression was negatively correlated with the maximum tumor diameter

in ACCs ($P = 0.002$, $\rho = -0.461$). Furthermore, P-gp expression was significantly higher in cortisol-producing ACC than in other ACC ($P = 0.048$, $n = 38$).

Effects of cytotoxic drugs on human adrenocortical carcinoma cell lines

H295R, HAC15 and SW13 demonstrated a dose-dependent inhibitory response to mitotane (EC_{50} range: 2.63–12.3 μ M), doxorubicin (EC_{50} range: 0.0110–0.0608 μ M), etoposide (EC_{50} range: 0.175–0.708 μ M) and cisplatin (EC_{50} range: 0.579–1.14 μ M), but not to streptozotocin (Fig. 2 and Table 2).

Figure 2

Dose-response curves of mitotane, doxorubicin, etoposide, cisplatin and streptozotocin on total DNA measurement, as a measure of cell amount, in H295R (left panel), HAC15 (middle panel) and SW13 (right panel) after 7 days of treatment. Values represent mean $\pm$ s.e.m. and are shown as percentage of control.

Table 2 EC₅₀ values of mitotane, doxorubicin, cisplatin and etoposide on cell growth in human adrenocortical cell lines with or without the P-gp inhibitors verapamil or tariquidar.

		MIT	DOX	ETO	CIS
H295R	Monotherapy	2.96 (2.47–3.56)	0.0575 (0.0415–0.0795)	0.674 (0.565–0.803)	0.579 (0.460–0.728)
	+ Verapamil	2.70 (2.11–3.44)	0.00765 (0.00596–0.00982)****	0.181 (0.124–0.265)****	0.676 (0.556–0.822)
	+ Tariquidar	2.45 (1.64–3.66)*	0.00914 (0.00582–0.0144)****	0.0732 (0.0596–0.0900)****	1.48 (1.12–1.95)****
HAC15	Monotherapy	2.63 (3.03–4.13)	0.0608 (0.0464–0.0796)	0.708 (0.576–0.870)	1.10 (0.907–1.322)
	+ Verapamil	3.45 (2.56–4.65)*	0.0435 (0.0293–0.0647)	0.194 (0.146–0.259)****	0.855 (0.668–1.10)*
	+ Tariquidar	2.65 (1.77–3.96)	0.0152 (0.0103–0.0225)****	0.116 (0.0836–0.161)****	3.73 (3.17–4.39)****
SW13	Monotherapy	12.3 (9.09–16.7)	0.0110 (0.00694–0.0175)	0.175 (0.112–0.275)	1.14 (0.897–1.44)
	+ Verapamil	9.57 (5.85–15.6)	0.0130 (0.00873–0.0194)	0.116 (0.0768–0.174)**	0.731 (0.564–0.949)****
	+ Tariquidar	9.25 (6.53–13.1)*	0.0109 (0.00720–0.0165)	0.169 (0.0926–0.308)	3.51 (1.96–6.31)****

EC₅₀ values are presented in micromolar (μM, 95% CI). Controls represent vehicle control for monotherapy of cytotoxic drugs, and treatment with verapamil (10 μM) or tariquidar (1 μM) for combination therapy. *P* values compare EC₅₀ value of combination therapy with P-gp inhibitors verapamil or tariquidar to monotherapy of either mitotane (MIT), doxorubicin (DOX), etoposide (ETO), or cisplatin (CIS). EC₅₀ values depicted in bold are significantly lower compared to monotherapy.

P* < 0.05, *P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

Effects of the P-gp inhibitors verapamil and tariquidar on the response of human adrenocortical carcinoma cell lines to cytotoxic drugs

First, we examined the possible effects of verapamil and tariquidar alone on cell growth in H295R, HAC15 and SW13. To avoid cytotoxicity, a concentration of 10 μM verapamil and 1 μM tariquidar was chosen for combination experiments with cytotoxic drugs. Verapamil and tariquidar in these concentrations only minimally affected cell growth in HAC15 with respectively 11 and 9.7% (*P* < 0.001), whereas no effects were observed in H295R and SW13 on cell growth. Subsequently, we analyzed whether both P-gp inhibitors could sensitize ACC cells reflected by a decrease in the EC₅₀ value of the cytotoxic drugs currently used for treatment of ACC (Fig. 3 and Table 2).

When tariquidar was added to different doses of mitotane, sensitivity to mitotane only modestly increased in H295R and SW13, with 1.2- and 1.3-fold lower EC₅₀ values, respectively (both *P* < 0.05 vs mitotane monotherapy). Verapamil, however, decreased the response to mitotane in HAC15, but not in H295R and SW13 (*P* < 0.05 vs mitotane monotherapy). Verapamil and tariquidar both increased the response of H295R cells to doxorubicin, as shown by a 7.5- and 6.3-fold decrease in EC₅₀ value compared to doxorubicin monotherapy, respectively (both *P* < 0.0001 vs doxorubicin monotherapy). In HAC15, sensitivity to doxorubicin only increased when tariquidar was used as shown by a 4-fold decrease in EC₅₀ (*P* < 0.0001 vs doxorubicin monotherapy). Sensitivity to doxorubicin did not change in SW13 cells with addition of P-gp inhibitors. Sensitivity to etoposide increased when the compound was combined with

verapamil or tariquidar, as indicated by a 3.7- and 9.2-fold significant decrease in EC₅₀ value in H295R and a 3.6 and 6.1 times decrease in HAC15, respectively. In both H295R and HAC15 cells, tariquidar more strongly sensitized cells to etoposide compared to verapamil (both *P* < 0.01 vs etoposide monotherapy). In SW13, effects of etoposide were only slightly potentiated by verapamil (2.0-fold decrease in EC₅₀; *P* < 0.0001). In all three ACC cell lines, tariquidar counteracted the effect of cisplatin on cell growth (range increase EC₅₀ 2.6–3.4 fold; all *P* < 0.0001 vs cisplatin monotherapy). In HAC15 and SW13, verapamil increased sensitivity to cisplatin (*P* < 0.05, *P* < 0.0001, respectively).

Effects of cytotoxic drugs, with and without P-gp inhibitors, on colony formation in human ACC cell lines

The effects of the compounds with and without P-gp inhibitors on colony formation appeared to primarily be cytotoxic, as stated by an effect on the fraction of colonies rather than an effect on colony size. Effects on the size of colonies are presented in Supplementary Fig. 1. In H295R, mitotane in combination with tariquidar resulted in a less strong effect on surviving fraction of colonies compared to mitotane alone (*P* < 0.01 vs mitotane monotherapy). In HAC15, verapamil counteracted whereas tariquidar potentiated the effect of mitotane (Fig. 4B; *P* < 0.0001, *P* < 0.05, respectively). Both verapamil and tariquidar strongly potentiated the effects of doxorubicin in H295R and HAC15 (all *P* < 0.0001 vs doxorubicin monotherapy). P-gp inhibition did not alter cytotoxic effects of mitotane and doxorubicin in SW13 cells.

Figure 3

Effects of mitotane (MIT), doxorubicin (DOX), etoposide (ETO) and cisplatin (CIS), with or without the P-gp inhibitors verapamil (10 μM) or tariquidar (1 μM) on cell growth in three human ACC cell lines H295R (left panel), HAC15 (middle panel) and SW13 (right panel) after 7 days of treatment. Gray solid lines represent the effect of monotherapy. Dotted lines represent the combination of verapamil (black dotted lines) or tariquidar (gray dotted lines) with cytotoxic therapy. Data are presented as percentage of vehicle treated control. When combination of cytotoxic drugs with verapamil (+V) or tariquidar (+T) were examined, the control was set as verapamil (10 μM) or tariquidar (1 μM) monotherapy, respectively. Values represent mean ± S.E.M. and are shown as percentage of control.

In H295R, the effect of etoposide on surviving fraction of colonies was enhanced when combined with verapamil or tariquidar (Fig. 4G; both $P < 0.05$ vs etoposide monotherapy), whereas this effect in HAC15 was only observed in combination with tariquidar ($P < 0.01$ vs etoposide monotherapy). In SW13, verapamil potentiated the effect of etoposide (Fig. 4I; $P < 0.01$). In H295R, the effect of cisplatin was slightly counteracted by tariquidar ($P < 0.001$ vs cisplatin monotherapy), whereas no

effects were seen on sensitivity to cisplatin in HAC15 and SW13.

Discussion

In this study, we show that P-gp is highly expressed in normal and neoplastic adrenal tissues, with lower levels in ACC compared to ACA and NA. Previously, it has been shown that verapamil might increase sensitivity to

Figure 4

Effects of cytotoxic drugs with or without the P-gp inhibitors verapamil (V) or tariquidar (T) on the surviving fraction of colonies in H295R (left panel), HAC15 (middle panel) and SW13 (right panel). Data are presented as percentage change compared to vehicle treated control. When combination of cytotoxic drugs with verapamil (+V) or tariquidar (+T) were examined, the control was set as verapamil (10 μ M) or tariquidar (1 μ M) monotherapy, respectively. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 vs vehicle treated control or as stated by the lines. CIS, cisplatin; DOX, doxorubicin; ETO, etoposide; MIT, mitotane. Values represent mean $\pm$ S.E.M.

doxorubicin in ACC (Gagliano *et al.* 2014). This is the first study investigating the effects of verapamil and the clinically more promising P-gp inhibitor tariquidar on a panel of the most important clinically used chemotherapeutic drugs for ACC. We demonstrate that inhibition of P-gp in human ACC cells *in vitro* can increase sensitivity to specific cytotoxic drugs, used to treat ACC *in vivo*.

Chemotherapeutic drugs have demonstrated disappointing results in ACC patients so far. Targeting P-gp expression would potentially increase tumor cell

exposure to cytotoxic agents and thereby improve the efficacy of chemotherapy. In this study, we confirm the previously reported high P-gp expression in the majority of ACC cases (Flynn *et al.* 1992, Haak *et al.* 1993, Backlin *et al.* 1994). *MDR1* mRNA expression and P-gp expression are positively correlated in ACCs and lower in ACAs. The lower expression in ACCs compared to ACAs might potentially be the result of involvement of the *MDR1* gene in the pathogenesis of ACC, as P-gp silencing is also an early event in colorectal carcinogenesis

(Andersen *et al.* 2013). Dedifferentiation of ACCs could be another explanation of lower *MDR1* mRNA and P-gp expression, although no correlation was found with histopathology. ACCs appeared to have a more variable P-gp expression compared to ACAs and NAs, and the hypothesis is that ACC patients with high P-gp expression might be more resistant to chemotherapeutic drugs, and these patients may particularly benefit from simultaneous treatment with P-gp inhibitors. Most importantly, the majority of patients seem to have P-gp expression comparable with NAs, whose expression is thought to be high relative to other normal tissues (Thiebaut *et al.* 1987, Cordon-Cardo *et al.* 1990, Sugawara 1990). Furthermore, these ACC cases have *MDR1* mRNA expression comparable with the expression in the cell lines that have been used as a model.

Previously, the antitumoral effects of the M-EDP regimen were already demonstrated in NCI-H295R cells *in vitro* and in xenografts (Hantel *et al.* 2014). In the present study, we report the *in vitro* effects, in pharmacological concentrations, of currently used cytotoxic drugs in ACC separately. Surprisingly, streptozotocin did not inhibit the growth of ACC cells *in vitro*. The first rationale for streptozotocin in the treatment of ACC was based on increased concentration of streptozotocin in the mice adrenal cortex after i.v. injection (Tjalve *et al.* 1976). In the FIRM-act trial, however, streptozotocin in combination with mitotane appeared to be less effective compared to the M-EDP regimen (Fassnacht *et al.* 2012), which turns out to be concordant with our *in vitro* results. We demonstrate that the other drugs used to treat ACC (mitotane, cisplatin, etoposide and doxorubicin) effectively inhibited cell growth in ACC cell lines, all with EC₅₀ values within the range of achievable plasma concentrations after administration in humans (Baudin *et al.* 2001, Urien & Lokiec 2004, Barpe *et al.* 2010, Tazawa *et al.* 2016). An important consideration is that plasma concentrations do not necessarily represent intratumoral concentrations.

The findings on cell growth inhibition in ACC cell lines were confirmed by performing a CFA, which predominantly revealed comparable results. There is a clear difference between both assays; the CFA provides information on the long-term effects (2 weeks without treatment) of a short-term (3 days) treatment with cytotoxic drugs on colony growth, whereas the cell growth assay studies the direct effect of cytotoxic drugs after 7 days of treatment. This difference could explain inequalities in effect of compounds. In H295R and HAC15, especially an increased response to doxorubicin

and etoposide was found under P-gp inhibition both on cell growth and on the surviving fraction of colonies. Increased sensitivity can be explained by the fact that doxorubicin and etoposide are substrates of P-gp (Sharom 2008). In a study investigating the effects of mitotane on drug elimination pathways, it was suggested that mitotane retained its antineoplastic efficacy even in tumors overexpressing drug transporters, due to lack of substrate characteristics (Theile *et al.* 2015). Another explanation of lack of increased sensitivity to mitotane could be potential P-gp inhibition, although the role of mitotane in P-gp inhibition is debatable (Bates *et al.* 1991, Gagliano *et al.* 2014, Theile *et al.* 2015). In line with this finding, only subtle differences in mitotane sensitivity were observed under P-gp inhibition on cell growth in ACC cells in our study. P-gp inhibition also only minimally affected the cytotoxic effects of mitotane on colony formation in H295R and SW13. In HAC15, however, verapamil counteracted the effect of mitotane on colony formation, corresponding with the effect in monolayer culture on cell growth. The minimal or even absence of enhanced cisplatin effects after co-treatment with verapamil in H295R, HAC15 and SW13 could be explained by the fact that cisplatin is not a substrate of P-gp, but of several *MDR1*-related proteins (MRPs) (Jedlitschky *et al.* 2006). Decreased sensitivity to cisplatin was particularly observed with respect to cell growth when cisplatin was combined with tariquidar and was confirmed in H295R in the CFA. This attenuated sensitivity could potentially be explained by a compensatory increase in drug efflux by other transporters. Compensatory upregulation of other transporters has been demonstrated in mutant animals with deficiency of a specific drug efflux transporter (Johnson *et al.* 2006). This in turn would indicate that it could be required to inhibit multiple drug transporters to reach optimal efficacy, which would also be reasonable in case there is expression of other ABC transporters in ACC as well. This could be subject to further investigation in ACC. In SW13, effects of P-gp inhibitors in general were much less pronounced, which is concordant with the undetectable *MDR1* mRNA expression in SW13 cells, in contrast to H295R and HAC15.

In preclinical studies, the question always arises whether the obtained findings can be translated to clinical practice. The present study uses ACC cell lines. Considering the limited availability of ACC cell line models, a subclone of H295R was also used (HAC15), aiming to obtain a better representation of the heterogeneity of ACC. Further research could focus on primary cultures. Finally, tariquidar in combination with mitotane, doxorubicin

and etoposide could be tested in ACC patients with disease progression under the conventional M-EDP regimen. Tariquidar is well tolerated and a peak serum concentration of 2.3 μM can be reached (Wagner *et al.* 2009, Bauer *et al.* 2013). In the present study, a lower concentration (1 μM) was used and appeared to significantly increase sensitivity to doxorubicin and etoposide. Tariquidar *in vivo* already demonstrated to induce inhibition of P-gp in patients with ACC as determined by increased accumulation of $^{99\text{m}}\text{Tc}$ -sestamibi in ACC lesions and blockade of rhodamine efflux in CD56+ mononuclear cells (Menefee *et al.* 2008). Where verapamil is often used as a positive control for P-gp inhibition in preclinical studies, tariquidar and other third-generation P-gp inhibitors are currently evaluated in several clinical trials in other types of cancer. The fact that this preclinical study combines currently clinically used chemotherapeutic drugs with a P-gp inhibitor that is already investigated in patients with ACC could lead to a faster introduction in clinical trials aiming to improve sensitivity to chemotherapy in ACC. A recent review summarized the efficacy of clinical trials using third-generation P-gp inhibitors until now, revealing however disappointing results in general (Chung *et al.* 2016). Several challenges have been encountered such as the limited fraction of tumors expressing the *MDR1* gene. P-gp is for instance expressed in less than 50% of breast cancers (Trock *et al.* 1997). No preselection based on P-gp expression was made in previous clinical trials. In ACCs, however, we show that P-gp is expressed in 95% of cases, of which 78% of all cases with an IRS of at least six. This observation provides a rationale for targeting this transporter in selected patients, which is associated with the limitation that P-gp inhibition might not be effective in a subgroup of ACC patients. An important consideration, however, is that P-gp expression can be upregulated following chemotherapy (Zhou *et al.* 1995, Chevillard *et al.* 1996), so baseline P-gp expression levels may not be predictive for clinical outcome of combined treatment with chemotherapy and P-gp inhibition. The large variability of P-gp expression observed in the present ACC panel could not be caused by upregulation, since no patients were pretreated with chemotherapy, and only two patients received mitotane before surgery. Thereby, we have to acknowledge that P-gp is only one of the multitudes of mechanisms that can lead to drug resistance, and P-gp is part of a family of ABC transporters (Sharom 2008).

In conclusion, we show that cytotoxic drugs, except streptozotocin, used for treatment of ACC inhibit cell growth in ACC cell lines. P-glycoprotein is expressed in

ACC, expression level is in the majority of cases comparable with the high expression in NAs, and inhibition of P-gp increases sensitivity to particularly doxorubicin and etoposide. This study thereby marks the relevance of carefully selecting compounds as well as patients when designing clinical trials with P-gp inhibitors in patients with ACC. These findings suggest that the *MDR1* gene plays a role in sensitivity of ACC cells to these cytotoxic drugs and could be a potential target for improvement of efficacy of cytotoxic drugs in ACCs.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-18-0500>.

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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