Abstract. Aim: In patients with advanced estrogen-dependent type I endometrial cancer (EC), pharmacological treatment with progestins or antiestrogens is recommended, but primary and secondary resistance are common. The aim of our study was to investigate single-agent and dual-agent therapeutic strategies in estrogen receptor-positive human EC cells. Material and Methods: Human EC cells Ishikawa and HEC1A were cultivated under estrogen-reduced conditions and exposed to 4-hydroxytamoxifen (OHT), fulvestrant, gefitinib, everolimus, and the AKT inhibitor perifosine. Effects of drugs were analyzed by proliferation and apoptosis assays. Additionally, we analyzed expression of aromatase, phosphatase and tensin homolog (PTEN), AKT and pAKT and G protein-coupled receptor 30 (GPR30). Results: Neither OHT nor fulvestrant inhibited cell growth, nor did they induce apoptosis. Gefitinib, everolimus and perifosine inhibited proliferation in all cell lines. Only perifosine induced apoptosis. In PTEN-positive HEC1A cells, combined treatment of gefitinib plus OHT showed increased antiproliferative effects. In Ishikawa cells, combined treatment of everolimus plus gefitinib had synergistic antiproliferative effects. The most effective single-agent treatment and the only drug that induced apoptosis was perifosine. Activation of AKT had no predictive value for the effects perifosine. Due to mutation of PTEN, activated AKT was highly expressed in Ishikawa cells and scarcely detectable in HEC1A cells. Conclusion: Under estrogen-reduced conditions, growth of ER-positive EC cells can be reduced by inhibitors of AKT, mTOR and the erbB pathway, whereas antiestrogens have no effects. In PTEN-positive HEC1A cells, the absence of estradiol probably restores OHT-induced ER-mediated repression of nuclear co-activators and increases susceptibility to inhibitors of the erbB pathway. In PTEN-negative Ishikawa cells, OHT in combination with any drug had no effects, but inhibition of the PI3K/AKT/mTOR pathway by everolimus in combination with gefitinib showed synergistic effects.

Endometrial cancer (EC) is the most common malignancy of the female genital tract in the developed world. The outcome for patients with advanced or recurrent disease remains poor. Since the majority of relapses develop in distant sites, the pharmacological treatment plays a major role in the management of recurrent disease. Chemotherapy provides moderate response rates but shows increased toxicity (1). The more frequent type I EC is associated with an endocrine milieu of estrogen predominance, where loss of phosphatase and tensin homolog (PTEN) tumor suppressor gene expression and dysfunction of DNA-mismatch repair genes are involved (2). Although type I EC strongly expresses estrogen receptors (ER), the selective ER modulator tamoxifen, which is very effective in the treatment of ER-positive breast cancer, shows only low response rates in patients with EC (3,4). In contrast, exposure to tamoxifen significantly increases the hazard ratio for the incidence of EC (5). It was demonstrated that other selective ER modulators such as arzoxifene and raloxifene and the selective estrogen receptor antagonist fulvestrant act differently in EC cells, due to different effects on ER-modulated gene expression, and these drugs showed promising preliminary results in vitro and in vivo (6). Increased understanding of the mechanisms of resistance to hormone therapy and chemotherapy in EC offers new therapeutically approaches for specific inhibitors targeting modulators of growth factor receptor cascades. Beside ER-dependent growth regulation, the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway and crosstalk mechanisms to mediators of the epidermal growth factor (EGF) receptor...
family represent key pathways controlling cell growth, proliferation and survival in EC. PTEN dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate and acts as an indirect inhibitor of AKT phosphorylation. Reduced PTEN expression leads to increased activation of AKT and therefore increased cell proliferation and inhibition of apoptosis (7). Loss of PTEN expression and AKT activation was shown to be associated with poor prognosis in patients with EC (8). In addition, estrogen-dependent activation of ERα can up-regulate the expression of the insulin-like growth factor receptor (IGF-R) and autocrine IGF-R-mediated growth mechanisms can activate the PI3K/AKT pathway independently of PTEN (2). Besides this, the PI3K/AKT pathway can also be activated by the transmembrane G protein-coupled ER, GPR30 (9). AKT activates mTOR and various analogs of rapamycin are currently under investigation in the treatment of different types of cancer.

The EGF-signaling pathways involve four known transmembrane tyrosine kinase receptors (EGF-R, erbB2/HER-2, erbB3 and erbB4) and activation by its ligands leads to receptor dimerization followed by intracellular activation of ras and its downstream substrates of the mitogen-activated protein kinase (MAPK) family. Overexpression of EGF-R and of HER-2 is associated with tumor metastasis and poor survival in patients with EC (10). Single-targeting of any growth factor receptor cascade can provide complex mechanisms for rapid resistance in tumor cells based on crosstalk on different levels in their signal transduction.

Vivacqua et al. showed 4-hydroxytamoxifen (OHT) induced growth stimulation through GPR30 by activation of the ERK-1/2 and PI3K/AKT pathways in ERα-positive EC cells Ishikawa and HEC1A (11). Trastuzumab, which is highly effective in erbB2/HER-2 overexpressing breast cancer, induced apoptosis in Ishikawa and HEC1A cells only in the absence of estradiol (12, 13). However, different cell culture models to investigate the mechanisms of signal-transduction of growthfactor receptor signaling representing type I and II EC with detailed description of cellular expression profiles have been introduced (13-15). The aim of our study was to investigate single-agent and dual-agent therapeutic strategies in Ishikawa and HEC1A cells under estrogen-reduced conditions targeting the described pathways.

Materials and Methods

Cell lines and culture conditions. The human endometrial cancer cell lines HEC1A and Ishikawa were purchased from the American Type Culture Collection (ATCC, Manassas, V A, USA). Cells were cultured at 37˚C in a humidified atmosphere of 5% CO2. The medium used was minimal medium with Earl’s salts (MEM-Earll), 0.22% (w/v) NaHCO3, phenol red as an indicator and 2% (v/v) L-glutamine together with the experimental drugs or vehicle. Treatment of the cells started at day 0. For apoptosis assays and cell cycle analysis, cells were plated in 6-well plates in normal culture medium, washed with PBS after 24 h and cultured in MEM-Earll’s medium supplemented with 2% L-glutamine without FCS and phenol red for a further 24 h. Then cells were cultured for 96 h in MEM-Earll’s medium without phenol red, 10% (v/v) charcoal-stripped FCS (Allgaug BioTech Service, Goerisried, Germany), 0.22% (w/v) NaHCO3 and 2% L-glutamine (Sigma-Aldrich, Deisenhofen, Germany). Treatment of the cells started at day 0. For apoptosis assays and cell cycle analysis, cells were plated in 6-well plates in normal culture medium, washed with PBS after 24 h and cultured in MEM-Earll’s medium supplemented with 2% L-glutamine without FCS and phenol red for a further 24 h. Then cells were cultured for 96 h in MEM-Earll’s medium without phenol red, 10% (v/v) charcoal-stripped FCS, 0.22% (w/v) NaHCO3 and 2% (v/v) L-glutamine together with the experimental drugs or vehicle.

Proliferation assays. Cells were placed in a 96-well microplate (HEC1A: 500 cells/well; Ishikawa: 300 cells/well) in 100 μl normal culture medium without phenol red per well. After 24 h 100 μl of medium with the appropriate concentration of OHT (2.5 μM and 5 μM; Sigma-Aldrich), gefitinib (ZD1839/Iressa; 4.5 μM and 9 μM; Sigma-Aldrich), perifosine (20-50 μM; Zentaris, Frankfurt, Germany), gefitinib (ZD1839/Iressa; 4.5 μM and 9 μM; AstraZeneca), fulvestrant (ICI 182780; 10 - 250 nM; AstraZeneca) or everolimus (RAD001; 2-200 nM; Novartis, Basel, Switzerland) or vehicle were added and replaced with fresh medium containing the drugs in the same concentrations after 24 h, 48 h and 72 h. After 96 h, 20 μl of AlamarBlue™ indicator dye (AbD Serotec, Düsseldorf, Germany) were added to each well and microplates were incubated for an additional 4 h in an incubator at 37˚C. Absorbance at 570 nm and as a reference at 600 nm was then measured in each well in a microplate reader (BioTek, Bad Friedrichshall, Germany). Growing cells caused a chemical reduction of AlamarBlue™, and absorption was measured at 570 nm and 600 nm. The difference in absorbance is thus relative to the extent of growth.

Figure 1. A: Immunoblotting analysis of AKT, phosphorylated AKT, PTEN and GPR30 in HEC1A and Ishikawa cells. B: Immunoblotting of aromatase in HEC1A and Ishikawa cells, with placental cells as control. Actin was used as loading control. Immunoblots were repeated at least three times with similar results, a single representative blot is shown.
Apoptosis assays. To quantify apoptosis, a procedure based on detecting advanced DNA degradation was used (16). Analysis was performed on FACSCalibur equipment using Cellquest software (Becton Dickinson, Heidelberg, Germany). In each experiment, 1×10^5 cells were counted. To analyze apoptosis induced by the experimental drugs, cells were harvested after 96 h and prepared as described for further analysis (16). To verify our results we also used the APO LOGIX™ - JC-1 Mitochondrial Membrane Potential Detection Kit (Bachem, Weil am Rhein, Germany). The obtained results of the APO LOGIX™-JC-1 test of early apoptosis events yielded comparable percentages of apoptotic cells to those with delayed using the advanced DNA degradation detected by the method described above. In this paper, we present the results of cells assayed for advanced DNA degradation.

Preparation of cellular extract and immunoblotting. Cells were washed twice with PBS and then lysed on ice using a lysis buffer (Cellytic™ MT Cell Lysis Reagent; Sigma-Aldrich). To avoid degradation and dephosphorylation of proteins, a protease-inhibitor (Sigma-Aldrich) and a phosphatase-inhibitor cocktail was added.

Figure 2. Quantification of proliferation by AlamarBlue® assay in HEC1A cells after 96 h exposure to fulvestrant (250 nM), 4-hydroxytamoxifen (OHT; 5 μM), gefitinib (4.5 μM), everolimus (20 nM) and perifosine (50 μM) and respective combinations. Columns represent means±S.E. obtained from three independent experiments in three different passages of the cell line. The values represent the percentage of growing cells compared to the normalized (100%) controls (control vs. gefitinib: p<0.001; control vs. everolimus: p<0.001; control vs. perifosine: p<0.001; gefitinib plus OHT vs. gefitinib: p<0.05; everolimus plus OHT vs. everolimus: n.s.; perifosine plus OHT vs. perifosine: n.s.; everolimus plus gefitinib vs. gefitinib: n.s.; everolimus plus gefitinib vs. everolimus: p<0.05; n.s., not significant).

Figure 3. Quantification of proliferation by AlamarBlue® assay in Ishikawa cells after 96 h exposure to fulvestrant (250 nM), 4-hydroxytamoxifen (OHT; 5 μM), gefitinib (4.5 μM), everolimus (20 nM) and perifosine (50 μM) and respective combinations. Columns represent means±S.E. obtained from three independent experiments in three different passages of the cell line. The values represent the percentage of growing cells compared to the normalized (100%) controls (control vs. gefitinib: p<0.001; control vs. everolimus: p<0.001; control vs. perifosine: p<0.001; gefitinib plus OHT vs. gefitinib: n.s.; everolimus plus OHT vs. everolimus: n.s.; perifosine plus OHT vs. perifosine: n.s.; everolimus plus gefitinib vs. gefitinib: p<0.05; everolimus plus gefitinib vs. everolimus: p<0.01; n.s., not significant).
Protein content was quantified by Bradford Assay (Bio-Rad, Munich, Germany). Equal amounts of lysate were mixed with 2 X Laemmli sample buffer and electrophoresed using SDS/PAGE (7.5% acrylamide/N,N,N,N'-bis-methylene-acrylamide; AppliChem GmbH, Darmstadt, Germany). After electrophoretic separation proteins were electroblotted on to nitrocellulose membranes (Hybond-ECL nitrocellulose membrane; GE Healthcare, Munich, Germany). The nitrocellulose membranes were blocked in 5% instant skimmed-milk powder, spray-dried (Töpfer GmbH, Dietmannsried, Germany) in TBST buffer (137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20 and 25 mM Tris/Cl, pH 7.4) for 1 h at room temperature, washed with TBST, and then incubated overnight at 4˚C with polyclonal rabbit Akt antibody, monoclonal rabbit Phospho-Akt (Ser473) antibody, monoclonal rabbit PTEN antibody (all Cell Signaling Technology, Danvers, MA, USA), polyclonal rabbit aromatase antibody (Abcam, Cambridge, UK), GPR30 (GeneTex, Irvine, CA, USA) or polyclonal rabbit actin antibody (Sigma-Aldrich) at a 1:1000 or a 1:2000 dilution in a 5% bovine serum albumin TBST solution overnight. After washing with TBST, nitrocellulose membranes were incubated with peroxidase-conjugated polyclonal anti-rabbit IgG (1:10000 in TBST; GE Healthcare, Munich, Germany) for 1 h at room temperature. Visualization was achieved by using an enhanced chemiluminescence (ECL) detection system (Millipore, Billerica, MA, USA) and radiographic film (Kodak BioMax MR film; Kodak, Rochester, NY, USA).

Reverse transcription-PCR for GPR30. Total RNA from HEC1A and Ishikawa cells was isolated using RNeasy™ Mini Kit (Qiagen, Hilden, Germany), following manufacturer’s instructions. Total RNA (1 μg) was reversed transcribed using Superscript II (Promega, Mannheim, Germany), following company instructions. cDNA was amplified by PCR using Taq DNA polymerase (Roche, Mannheim, Germany) consisting of 30 cycles with specific primers (sense: 5’-AGA TGT ACA GAA CTG AAA TTC and antisense: 5’-ATT TAC CAA GAG ATC GAG CAA-3’). PCR products were analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide for UV light transilluminator visualization and following analysis.

Statistical analysis. All experiments were repeated three times with different passages of the respective cell lines. Unless otherwise indicated, data were tested for significant differences by one-way analysis of variance followed by Newman-Keuls Multiple Comparison Test. Analysis of data was carried out with GraphPad Prism 5 software (Graphpad Software, La Jolla, USA).

Results

Expression of AKT, pAKT, PTEN, aromatase and GPR30. To compare the results of our proliferation and apoptosis assays with previous reports we analyzed the expression of PTEN, basal and activated AKT, and GPR30 by Western blotting in our cell lines. According to previous data, Ishikawa and HEC1A cells express high basal levels of AKT, whereas pAKT was only elevated in Ishikawa cells and rarely detectable in HEC1A cells. Here, PTEN expression was detected in HEC1A cells, but Ishikawa cells showed complete loss of PTEN expression. Expression of aromatase was also not detected in our cell lines. Expression of GPR30 was detected by PCR analysis in both cell lines (data not shown) and confirmed on protein level by Western blotting. Representative results of Western blotting for both cell lines are shown in Figure 1.

Proliferation assays. Antiproliferative effects of OHT, and fulvestrant were analyzed as single-agent treatment and in combination with gefitinib, everolimus or perifosine. Effects

Figure 4. Flow-cytometric analysis of advanced DNA degradation and quantification of apoptosis in Ishikawa cells after 96 h exposure to perifosine (50 μM). Histograms represent one of at least three experiments. Cells with advanced DNA degradation are quantified in the subdiploid fraction.
on proliferation were compared to untreated control cells (100%). Fulvestrant (10-250 nM) had no antiproliferative effect on either cell lines (Figures 2 and 3) and also did not affect the antiproliferative effect of gefitinib, everolimus or perifosine compared to respective single-agent treatment (data not shown). OHT as single agent (2.5 μM and 5 μM) had no effect on proliferation in either cell line (Figure 2 and Figure 3). In HEC1A cells, the combination of OHT (5 μM) and gefitinib (4.5 μM) had a significantly greater antiproliferative effect compared to gefitinib alone (Figure 2). In Ishikawa cells, the effect of combined gefitinib and OHT treatment was comparable to that of gefitinib alone, but this effect was not statistically significant (Figure 3). The antiproliferative effect of everolimus reached a maximum at a concentration of 20 nM in both cell lines. Perifosine (10 and 50 μM) showed a dose-dependent antiproliferative effect in Ishikawa cells (10 μM: 52.97±2.71%, p<0.001; 50 μM: 18.63±0.26%, p<0.001), and, to a lesser extent, also in HEC1A cells (10 μM: 43.5%±1.01%, p<0.05; 50 μM: 26.97%±10.79%, p<0.01) (Figures 2 and 3). The effect of the combination of gefitinib (4.5 μM) and everolimus (20 nM) was significantly additive in Ishikawa cells (Figure 3) but of only marginal significance in HEC1A cells (Figure 2). All other dual agent combinations had no significantly additive effect compared to the respective single-agent treatments.

Effects on apoptosis. Fulvestrant (10-250 nM), OHT (2.5 μM and 5 μM), gefitinib (4.5 μM and 9 μM), everolimus (2-200 nM) did not induce apoptosis in either of our cell lines (data not shown). The only agent that induced dose-dependent apoptosis in both cell lines was perifosine at a minimum concentration of 20 μM in HEC1A cells and 40 μM in Ishikawa cells (representative histograms are shown in Figure 4). Results of apoptosis assays with perifosine are shown in Figures 5 and 6. The addition of any of the other drugs to perifosine did not significantly affect apoptosis induced by perifosine alone in all cell lines (data not shown). No other combination of any two drugs showed significant effects on apoptosis in Ishikawa and HEC1A cells compared to untreated control cells.

Discussion

This study presents the results of experiments of single- and dual-agent therapeutically strategies in ER-positive human EC cells under estrogen reduced conditions using medium with stripped FCS and without phenolred. Although it has been reported that the membrane-associated ER GPR30 is down-regulated in EC cells without exogenous estrogens (17), we were able to detect GPR30 at the RNA and the protein level in our cells. However, since we found no expression of aromatase, it is unlikely that autocrine estrogen-mediated growth control via GPR30 played a major role in our experiments. Fulvestrant, which acts as pure antagonist by blocking ER dimerization (18, 19), showed no effects, alone or in combination. This suggests estrogen-independent cell growth for these cells. Tamoxifen acts as a non-selective agonist and is equally effective at regulating
genes with ERα and ERβ, whereas its agonistic or antagonistic effects are regulated by nuclear co-activators and co-inhibitors (20). It was shown that OHT at high concentrations, of 10-25 μM, induced apoptosis in HEC1A and Ishikawa cells in medium containing estradiol (12, 13). In our experiments, treatment with OHT showed no effects, probably diminished by cross-talk mechanisms with the EGF-R/MAPK and the AKT/mTOR pathways. Beyond the effects on the AKT/mTOR pathway, PTEN exerts its antitumoral effects by a blockade of Shc phosphorylation resulting in the inhibition of ERK-1/2 MAPK, the downstream pathways of the erbB-receptor family, leading to reduced proliferation and induction of apoptosis (21). One major difference between Ishikawa and HEC1A cells is PTEN mutation found in Ishikawa cells. Interestingly, under estrogen-reduced conditions, there was no alteration of total AKT expression but reduced activation of AKT was described in both cell lines (17). The AKT inhibitor perifosine induces the extrinsic pathway of apoptosis by activation of caspase-8 and death receptor 5 (22). These effects are associated with a decrease of the plasma membrane localization of AKT, inhibition of AKT phosphorylation, and reduction of the levels of total AKT (22, 23). Engel et al. demonstrated dose- and time-dependent growth inhibition and inhibition of AKT phosphorylation by perifosine in Ishikawa and HEC1A cells in medium containing estradiol (24). In comparison to their findings, much higher concentrations of perifosine were needed in our study to reduce cell growth of these cells under estrogen-reduced conditions, indicating that these effects are affected by the presence of estrogens. However, in our experiments, AKT inhibition by perifosine resulted in comparable rates of apoptosis in Ishikawa and HEC1A cells, suggesting no predictive value of activated AKT levels for the effects of perifosine. Inhibition of AKT affects the ERK-1/2 MAPK and the more downstream mTOR pathway. Our findings are in accordance with those of Treeck et al. showing antiproliferative effects of everolimus but no induction of apoptosis in HEC1A cells and no additive effects of dual agent with OHT (12). In our experiments, everolimus in combination with gefitinib had only a minor additive effect on HEC1A cells. These data suggest that in cells with wild-type PTEN expression inhibition of the mTOR pathway does not increase susceptibility to OHT and only marginally increase sensitivity to inhibitors of the ERK-1/2 MAPK pathway. Basal mTOR expression was described in Ishikawa and HEC1A cells, but activated mTOR was shown to be increased only in PTEN mutated Ishikawa cells (25, 26). In contrast to HEC1A cells, in Ishikawa cells we found additive effects of everolimus in combination with gefitinib but not with OHT, suggesting ER-independent and predominantly ERK-1/2 MAPK- and AKT/mTOR-mediated growth control in these cells.

It was shown that gefitinib blocked the autophosphorylation of EGF-R and reduced MAPK ERK-1/2 activity in EC cells (14). Pfeiler et al. showed that the MEK-inhibitor U0126, which inhibits ERK-1/2 signaling, induced apoptosis in HEC1A cells but not in Ishikawa cells (13). The antiproliferative effects of gefitinib were increased by re-expression of PTEN in Ishikawa cells (27). Interestingly, in a panel of 19 different human EC cells the dual HER-2 and EGF-R kinase inhibitor lapatinib showed antiproliferative effects independently of PTEN expression, and the antiproliferative effects were not associated with inhibition of AKT activity (28).

In conclusion, Ishikawa and HEC1A cells model ER-positive EC and show comparable expression profiles of growth factor receptors. One major difference between these cell lines is PTEN mutation in Ishikawa cells. Gefitinib did not induce apoptosis under estradiol-reduced conditions in our experiments and in the presence of estradiol as shown by Pfeiler et al. (13). But gefitinib had significantly antiproliferative effects in both of our cell lines and these effects were significantly increased when gefitinib was given in combination with OHT in HEC1A cells. Our data demonstrate that in PTEN-positive and ER-positive EC cells, the combination of OHT and gefitinib inhibits erbB- and ER-mediated growth regulation in a synergistic manner in the absence of estrogens. Tamoxifen competes with estradiol in binding to ERα and ERβ, whereas tamoxifen acts as an agonist for both receptors, but shows in part opposite regulatory gene expression to estradiol on ERβ (20). Fulvestrant has no agonistic effects on ER and failed to inhibit proliferation, both alone and in combination. In PTEN-negative and ER-positive Ishikawa cells, we suggest circumvention of ER dependence by activation of the AKT/mTOR pathway. In these cells, the combined inhibition of erbB- and mTOR-mediated signal transduction by gefitinib plus everolimus showed synergistic growth inhibitory effects. This study provides further information for strategies in targeted therapies of EC. It is important to determine the predictive value of mediators of the erbB and PI3K/AKT/mTOR signal transduction pathways prior to clinical evaluation of any targeted therapy, not only of the basal expression level, but also at the activated level. In particular, although single-agent treatment with everolimus showed comparable effects in HEC1A and Ishikawa cells in our experiments, pmTOR seems to be a predictive marker for response to analogs of rapamycin (25, 26), at least for combination treatment strategies, whereas activation of AKT seems to have no predictive value for treatment with perifosine.

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