Combinatorial Therapy Using Dovitinib and ICI182.780 (Fulvestrant) Blocks Tumoral Activity of Endometrial Cancer Cells

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Abstract

Mutations in fibroblast growth factor receptor 2 (FGFR2) have been recently described as a molecular-specific feature in endometrioid carcinomas and the presence of activated FGFR2 mutations is associated with poor prognosis. For that reason, inhibition of FGFR2 could be a therapeutic target in the treatment of endometrioid carcinomas. In this work, we investigated the antitumoral activity of dovitinib (a multiple kinase inhibitor) in human endometrial cancer cell (ECC) lines. We found that dovitinib caused cell growth arrest, loss of clonogenic growth, and cell-cycle arrest in FGFR2-mutated ECCs in in vitro and in vivo experiments. Next, we investigated the mechanistic basis of dovitinib effects. We could determine that dovitinib modified expression levels of well-known key cell-cycle regulatory proteins that induce cellular senescence. To further investigate the role of dovitinib, we analyzed its effect on estrogen receptor α (ER-α) expression. Surprisingly, we discovered that dovitinib enhances ER-α expression in FGFR2-mutant ECCs. Because blocking one signaling pathway is often not sufficient to cause total tumor regression and the effectiveness of individual inhibitors is often short-lived, we examined the impact of targeting FGFR2 with dovitinib in combination with a selective ER antagonist, fulvestrant (ICI182.780). Combination of dovitinib plus ICI182.780 resulted in a significantly higher inhibition of cell growth than dovitinib treatment alone. These findings suggest that combinatory therapies using dovitinib plus ICI182.780 treatment can be truly effective in patients with endometrial carcinomas carrying FGFR2 mutations. Mol Cancer Ther; 13(4); 776–87. ©2014 AACR.

Introduction

Endometrial carcinoma is one of the most commonly diagnosed malignant tumors of the female genital tract in industrialized countries. The majority of women presenting endometrial cancers are surgically cured with a hysterectomy; however, about ~15% of patients show persistent or recurrent tumors that are refractory to current chemotherapies (1). There are 2 main clinic-pathological variants of endometrial carcinomas: type I tumors or endometrioid carcinomas (about 80%) are usually low-grade and estrogen-related carcinomas. Type I endometrioid carcinomas are developed in pre- or perimenopausal women and are associated with obesity, hyperlipidemia, anovulation, and infertility (2). In contrast, type II tumors (about 10%) are nonendometrioid carcinomas that arise from endometrial polyps or precancerous lesions present in atrophic endometrium. Type II tumors are not associated with estrogen stimulation and hyperplasia, readily invade the myometrium and vascular spaces, and are associated with poor prognosis (3). Classification of endometrial carcinomas in these 2 types is probably too rigid, as tumors showing combined or mixed features are not infrequent in pathology practice. Type I and type II endometrioid carcinomas display different mutations. Endometrioid carcinomas are characterized by a high frequency of inactivating mutations in PTEN (26%–80%; ref. 4), activating KRAS mutations (10%–30%; ref. 5), gain-of-function CTNNB1 (β-catenin) mutations (28%–35%; ref. 6), and microsatellite instability (20%–30%; ref. 7). Recently, some investigators have suggested that alterations in fibroblast growth factor receptor 2 (FGFR2) could be added to the molecular-specific features of endometrioid endometrial carcinomas with a frequency of 6.5% to 12% (8–11). Moreover, Pollock and colleagues have recently reported that presence of FGFR2 mutations is significantly associated with shorter disease-free survival (12). The discovery of activating FGFR2 mutations in...
endometrial carcinomas raises the possibility of using anti-FGFR molecularly target therapies in patients with advanced or recurrent endometrial carcinomas. Indeed, recent studies have identified FGFRs as promising targets for anticancer therapy. The efficiency of FGFR inhibitors has been investigated in a variety of malignancies, including bladder cancer and advanced melanoma (13).

FGFRs signal through FGFRs and regulate essential developmental pathways in the early embryo and its effects extend many physiologic roles in the adult organism. FGFRs are expressed on many different cell types, including endometrial epithelial cell, and regulate key cell behaviors, such as proliferation, differentiation, and survival, which makes FGF signaling susceptible to subversion by cancer cells. FGFRs compromise a large group of heparin-binding growth factors that include 22 ligands that exert their function through 4 high-affinity tyrosine kinase receptors (FGFR1, FGFR2, FGFR3, and FGFR4; ref. 14). Binding of FGFRs to the extracellular domains of FGFRs results in dimerization and conformational shift in receptor structure, leading to activation of the intracellular kinase domain. Activation of FGFR triggers multiple antiapoptotic pathways, including peritoneal lymphocytes γ, phosphoinositide 3-kinase (PI3K)-AKT, mitogen-activated protein kinase (MAPK), and STATs. This pro-survival effect has also been linked to resistance to chemotherapy (11, 15). However, one of the predominant signaling pathway–activated downstream of FGFR is RAS–MAPK. Following FGFR activation, MAPK signaling induces cell proliferation, through induction of CYCLIN D1. Together, these data confirm that cell survival is a major readout of FGF signaling, and multiple pathways can result in similar outcomes.

Dovitinib (TKI258; formerly CHIR-258) is a potent small-molecule inhibitor of multiple receptor tyrosine kinases, showing biochemical IC50 values <20 mmol/L for VEGFR-1 to -3, PDGFR-β, FGFR-1 to -3, and C-KIT (16). According to previous studies, dovitinib exhibits potent tumorigrowth inhibition in a broad range of preclinical animal models. For example, dovitinib specifically inhibited proliferation of primary cells and cell lines with FGFR1 fusion genes associated with the 8p11 myeloproliferative syndrome (17). In addition, dovitinib induces apoptosis in FGFR-expression hepatocellular carcinoma cells and in mammary cells via inhibition of PI3K/AKT signaling pathway (18). Recently, dovitinib has been tested in endometrial epithelial cells. The in vitro data support the antitumorogenic effect of dovitinib, especially in ECC lines with activating FGFR2, although dovitinib may also have other direct antitumoral activity because of its multitarget specificity with other kinases, such as C-KIT or PDGFR-β. The in vivo results also suggest that dovitinib also inhibits angiogenesis, thus dovitinib may be a promising therapeutic agent to treat patients with advanced and/or metastatic endometrial cancer (19). Dovitinib is being tested in clinical trials for the treatment of both solid tumors and hematologic malignancies (20), such renal cell carcinoma (NCT01223027), advanced breast cancer (NCT00958971), relapsed multiple myeloma (NCT01058434), and urothelial cancer (NCT00790426). A phase II, single-arm nonrandomized multicenter study is currently ongoing to evaluate the efficacy of oral dovitinib (500 mg for 5 days/week) as second-line therapy in patients with either FGFR2-mutated or wild-type advanced or metastatic endometrial cancer (EUDRACT number 2011-000266-35).

A significant proportion of endometrial carcinomas express estrogen receptor (ER) and progesterone receptor (PR), and positivity for these receptors is associated with better outcome (21). Receptor-positive and well-differentiated tumors represent a subset of responding tumors in hormonal therapy trials. It has been demonstrated that ER modulators, such as the selective ER antagonist fulvestrant (ICI182.780), act differently in endometrial carcinoma cells, because of distinct effects on ER-modulated gene expression. ICI182.780 has showed promising preliminary results in patients with advanced or recurrent endometrial cancer (22).

In this report, we first explored the molecular mechanisms of antineoplastic activity of dovitinib on endometrial cancer cells (ECC). We found that dovitinib treatment resulted in increased senescence-associated β-galactosidase (SA-β-Gal) accumulation and increased expression of senescence-associated markers such as p21 and p14 ARF, suggesting that it induces tumoral senescence. Immunohistochemical analysis of tumors revealed that dovitinib responsiveness correlated with a marked increase of ER-α expression. We found that only in endometrial cells that are sensible to dovitinib, a combined exposure to dovitinib and ICI182.780 resulted in a synergic antitumoral activity.

Materials and Methods

Cell lines, culture conditions, and transfection

The Ishikawa 3-H-12, a well-differentiated human endometrial carcinoma cell line with FGFR2 and ER-α wild-type expression, and MFE-296, moderately differentiated human endometrial carcinoma cell line with FGFR2-mutated and ER-α wild-type expression were purchased in 2011 from the American Type Culture Collection (ATCC). AN3CA and HEC-1-A (HEC) cells were a gift from Dr. Reventos (Hospital Vall d’Hebron, Barcelona) and were validated, in 2013, by sequencing methods for FGFR2-activating mutations and Western blot method for ER-α expression status. Endometrial carcinoma cells were grown in Dulbecco’s modified Eagle medium (Sigma) supplemented with 10% FBS (Invitrogen, Inc.), 1 mmol/L HEPES (Sigma), 1 mmol/L sodium pyruvate (Sigma), 2 mmol/L L-glutamine (Sigma), and 1% of penicillin/streptomycin (Sigma) at 37°C with saturating humidity and 5% CO2. All experiments were conducted with low passage cells from recently resuscitated frozen stocks. Cell lines obtained from ATCC and European Collection of Animal Cell Cultures were subjected to comprehensive quality control and authentication procedures, including short tandem repeat profiling for human cell lines, species verification by DNA barcoding,
verification of morphology, and testing for fungi, mycoplasma, and other bacteria.

**Chemical reagents**

Dovitinib (TKI258; formerly CHIR-258) was obtained from Novartis. A 50 mmol/L stock was prepared in dimethylsulphoxide (DMSO) and stored at −20°C in 5 μL aliquots until further use. MTT, DMSO, 5-bromo-deoxyuridine (BrdUrd), bis-benzimide fluorescent dye (Hoechst 33258), and antibodies to p-pRB (#9307), p21 (#1484), p14 ARF (#P2610), p53 (#P5813), p16 (#4828), p-erk (#9101S), and tubulin (#T5168) were from Sigma (St Louis). Anti–ER-α (sc-543) and cyclin D1 (sc-20044) were from Santa Cruz Biotechnology Inc. Anti-BrdUrd was purchased from Dako (Denmark). p-Akt (ser 473) and cleaved caspase-9 antibodies were from Cell Signaling. p-Stat3 (#04-1059) was purchased from Cell Signaling Technology. p53 and p16 antibodies were from Dako (Denmark). Anti-BrdUrd was purchased from Dako (Denmark). DMSO to dissolve the blue formazan crystals produced by the mitochondrial succinate dehydrogenase of the living cells. Drug resistance was represented as the percentage of live cells surviving after drug treatment relative to control cells. Absorbance was measured using a spectrophotometer (Bio-Rad) at a dual wavelength of 595 and 620 nm. Apoptotic cells were identified by nuclear staining with bis-benzimide fluorescent dye (Hoechst 33258), after the indicated treatments, to a final concentration of 0.5 mg/mL to each M24 well. Cells were counted under epifluorescence microscope (Leica Microsystems).

**Cell viability assays and assessment of apoptosis**

The general mitochondrial activity of endometrial carcinoma cell lines was determined by assaying reduction of MTT to formazan. Endometrial carcinoma cells were plated on 96-well plates at 1.5 × 10³ cells per well. After the indicated treatments, cells were incubated for 30 minutes with 0.5 mg/mL of MTT reagent and lysed with DMSO to dissolve the blue formazan crystals produced by the mitochondrial succinate dehydrogenase of the living cells. Cell viability proportionate to optical density was measured using a colorimetric assay of mitochondria activity. Drug resistance was represented as the percentage of live cells surviving after drug treatment relative to control cells. Absorbance was measured using a spectrophotometer (Bio-Rad) at a dual wavelength of 595 and 620 nm. Apoptotic cells were identified by nuclear staining with bis-benzimide fluorescent dye (Hoechst 33258), after the indicated treatments, to a final concentration of 0.5 mg/mL to each M24 well. Cells were counted under epifluorescence microscope (Leica Microsystems).

**Clonogenic formation assay**

Cell colonies were evaluated after 15 days in culture as previously described (23).

**Xenografting and dovitinib and ICI182.780 administration**

Immunodeficient female SCID mice (age 12 weeks; weight 20–25 g) were maintained in specific pathogen free (SPF) conditions. Animals were subcutaneously injected with HEC-1A and AN3CA cells (1.5 × 10⁶) suspended in 100 μL PBS + Matrigel (1:1) in the flank. Tumors were allowed to grow for 15 days. Mice were then stratified into treatment groups with 2 tumors per mouse on the basis of their weight and tumor volume at the start of the experiment, such that the starting weight and tumor volume in each group were uniform. Mice were treated via oral gavage with (i) vehicle or dovitinib 20 mg/kg. In the second set of experiments, xenografted mice were treated orally with vehicle or dovitinib 20 mg/kg and/or subcutaneous injection of ICI at 5 mg/kg 3 days/week for 2 consecutive weeks. Tumors were measured every 2 days with calipers. Tumor size was calculated using the formula: tumor weight (TW, mg) = (D × d²)/2 = mm³.

**Histology and immunohistochemical analysis**

Mice were euthanized by cervical dislocation after 2 weeks of dovitinib treatment and/or ICI182.780 injections. Tumor samples were collected and formaline-fixed overnight at 4°C. Tumors were paraffin embedded for further histologic analysis. Paraffin blocks were sectioned at 3 μm, dried for 1 hour at 65°C before pretreatment procedure of deparaffinization, rehydration, and epitope retrieval in the pretreatment module, PT-LINK (DAKO) at 95°C for 20 minutes in 50× Tris/EDTA buffer, pH 9. Before staining the sections, endogenous peroxidase was blocked. Representative images were taken with Leica DMD108 microscope.

**Bromodeoxyuridine incorporation**

For the determination of DNA and after the indicated treatments, cells were incubated with 3 ng/mL of BrdUrd (Sigma) for 20 minutes and then fixed with 4% paraformaldehyde. After DNA denaturing with 2 mol/L HCl for 30 minutes and neutralization with 0.1 mol/L Na₂B₄O₇ (pH 8.5) for 2 minutes, cells were blocked in PBS solution containing 5% horse serum, 5% FBS, 0.2% glycine, and 0.1% Triton X-100 for 1 hour. Subsequently, cells were subjected to indirect immunofluorescence with a mouse anti-BrdUrd monoclonal antibody (Dako), and fluorescein isothiocyanate-conjugated anti-mouse secondary antibody (Molecular Probes). Nuclei were counterstained with 5 mg/mL Hoechst 33258 and cells were visualized under an epifluorescence microscope (Leica Microsystems).

**Western blot analysis**

Western blot analysis was performed as previously described (24).

**Real-time quantitative PCR**

For RNA extraction, we used RNeasy Mini Kit with DNase I (Qiagen) to keep the same level of quality among all the RNA samples. Expression of mRNA was measured using TaqMan Gene Expression Assays on demand (Applied Biosystems) as described in ref. 25. The following assays were used: p16 (Hs02902543_m1), p14 ARF (Hs00924091_m1), and GAPDH (Hs99999905_m1) as a control. Quantitative reverse transcriptase PCR (RT-PCR) analysis was performed in triplicate with the ABI Prism 7900 Sequence Detector System (Applied Biosystems). The PCR cyclin conditions were the standard 95°C for 10 minutes for 1 cycle, 95°C for 15 seconds, and 60°C for 1 minute for 40 cycles.

**SA-β-Gal activity**

β-Gal activity was performed as previously described (26).
Detection of changes in cell-cycle profiles

Changes in cell-cycle profile after drug treatments were determined by propidium iodide staining and flow cytometry. Following treatment, approximately 1 × 10^6 cells were fixed in 70% ethanol for at least 1 hour on ice. The cells were then resuspended in 2 mL of cell-cycle buffer (20 μg/mL propidium iodide, in PBS containing 0.1% Triton X-100 and 50 μg/mL RNAse A) for 1 hour at 37°C. Propidium iodide fluorescence emission was measured using a FACSCanto II (BD Biosciences), and cell-cycle distribution was analyzed with ModFIT LT software (Verity Software House).

Statistical analysis

Linear models were used to evaluate the main effect of each compound on cell survival but also their interaction and, therefore, an analysis of variance was performed to assess statistical significance as described in refs. 27 and 28. All analyses were obtained with R statistical package and a 0.05 threshold was used as significance level.

Results

Dovitinib treatment reduces cell-cycle progression and proliferation on ECC lines in vitro and in vivo

To begin with, we explored the viability of ECC lines after dovitinib treatment by a MTT toxicity assay. For this purpose, we used 2 ECC lines expressing wild-type FGFR2: Ishikawa and HEC-1A-1A (HEC-1A); and 2 ECC lines bearing FGFR2 mutations: AN3CA and MFE-296. All 4 ECCs were exposed to different concentrations (0–2.5 μmol/L) of dovitinib for periods of 48 or 72 hours. Treatment of cell lines harboring FGFR2 mutations with dovitinib 0.5 μmol/L for 72 hours resulted in a significant decrease in cell viability of AN3CA (37.80%) and MFE-296 (53.14%). Conversely, in cell lines expressing wild-type FGFR2 the effects of dovitinib were nearly indiscernible (Supplementary Fig. S1A). Because we observed similar response to dovitinib in the 2 wild-type FGFR2 expression cell lines, we chose HEC-1A cells to further analyze dovitinib effects.

Some reports have demonstrated that dovitinib has antiproliferative effects on different cancer cell lines (9, 10, 19). To investigate whether dovitinib was able to reduce cellular proliferation in FGFR2 mutant cells, we treated HEC-1A, AN3CA, and MFE-296 cells with 0.5 μmol/L of dovitinib during 72 hours. Treatment of AN3CA and MFE-296 cells with dovitinib resulted in an important decrease of BrdUrd-positive cells (Supplementary Fig. S1B) and G2–M arrest with a reduction on G1/G0 and S phases (Fig. 1A), supporting that dovitinib induces cell-cycle arrest. Dovitinib also caused a significant reduction in number of clonogenic capability of ECC lines (Fig. 1B).

HEC-1A and AN3CA cells are capable of generating subcutaneous tumors when xenografted in immunocompromised mice (SCID). To assess antitumoral activity of dovitinib in vivo, we injected these 2 cell lines subcutaneously into SCID mice, and tumors were allowed to grow for 15 days. At this point, tumor-bearing mice were treated orally with vehicle or dovitinib at dose of 20 mg/kg/day for 2 weeks. All animals tolerated the treatment well without observable signs of toxicity. No gross pathologic abnormalities were noted at necropsy. Tumor growth was not significantly modified in FGFR2 wild-type cell line HEC-1A; however, in mice grafted with the FGFR2 AN3CA mutant cell line, dovitinib treatment not only resulted in a significant growth inhibition, but also in a considerable tumor regression (Fig. 1C). At the end of the treatment, we dissected tumors from treated and untreated mice and we analyzed by immunohistochemistry (IHC) cyclin D1 expression. As shown in Fig. 1D, dovitinib treatment significantly reduces cyclin D1 expression in AN3CA xenografts. Conversely, the same treatment was ineffective in HEC-1A tumor xenografts. Taken together, these results indicate that dovitinib reduced tumoral growth of xenografted FGFR2-mutated cell line.

Dovitinib blocks signaling pathways and induces cellular senescence in FGFR2-activated endometrial cells

To elucidate the underlying mechanisms by which dovitinib induces cell-cycle arrest in endometrial cancer cells, we examined the effects of dovitinib on signal transduction pathways related with regulation of cell cycle. As shown in Fig. 2A, dovitinib caused a decrease in STAT3, ERK, and AKT phosphorylation in a dose-dependent manner. The phosphorylation of STAT3 at residue 705 is associated with cell survival and proliferation. Consequently, the downstream effectors driven by STAT3, such as cyclin D1 or Rb, were also repressed by dovitinib in the 2 sensitive endometrial cell lines, supporting the observation that dovitinib induced cell-cycle arrest.

Deregulation of apoptotic cell death is also a critical event in development and progression of cancer. Once ascertained that dovitinib treatment reduced both proliferation and anchorage-independent cell growth, its potential role in regulation of apoptosis was investigated. After 72 hours of treatment, we quantified the nuclei potential role in regulation of apoptosis was investigated. After 72 hours of treatment, we quantified the nuclei displaying picnotic morphology by Hoechst staining. As Supplementary Fig. S2 shows, dovitinib does not cause any apoptotic effect in any cell lines even at the highest dose. Moreover, levels of caspase-9 were analyzed, and as Fig. 2A shows, dovitinib poorly increased cleavage of caspase-9, supporting the observation that dovitinib does not induce apoptosis in endometrial epithelial cell.

Having established that dovitinib treatment reduces proliferation of FGFR2 mutant cell lines, we wanted to examine the differential expression levels of key cell-cycle regulatory proteins. Usually, senescent cells show large changes in gene expression, including changes in well-known cell-cycle inhibitors or activators. Two of the main cell-cycle inhibitors often expressed by senescent cell are the cyclin-dependent kinase inhibitors (CDKIs) p21 and p16 (29). These CDKIs are components of a tumor-suppressor
pathway governed by the p53 and the retinoblastoma (pRB) protein, respectively. Both pathways can establish and maintain the growth arrest typical of senescence (30). At this stage, we wanted to analyze the expression levels of different senescence markers. FGFR2 mutant cells treated with dovitinib (0.5 μmol/L) during 72 hours showed a significant increase of p21, p14 ARF, and p53 protein levels (Fig. 2B) and a robust increase of transcriptional levels of p14 ARF and p16 (Fig. 2C). To demonstrate that dovitinib was also triggering senescence in vivo, we stained sections xenograft with p16 antibody. As Fig. 2D shows, dovitinib treatment induces an increase of p16 expression in FGFR2-mutant cell line. Besides CDKIs expression, one of the most used cellular markers of cellular senescence is SA-β-Gal. As shown in Fig. 2E, dovitinib treatment caused an important increase in positive stained β-Gal activity cells. We confirmed these outcomes by assessing SA-β-Gal activity over sections of AN3CA xenografts (Fig. 2F). These results suggest that dovitinib treatment reduces cellular proliferation by inducing cellular senescence.

**Dovitinib treatment increase ERα expression in FGFR2-activated endometrial cells**

It has been described that in hormone-related cancers, members of FG family can play key roles in acquisition of hormone independence (31). Moreover, in endometrial tumorigenesis, FGFR2-activated mutations occur in type I ECC, which are estrogen-related carcinomas. For that reason, we wanted to analyze ERα expression in cells treated with dovitinib. Unexpectedly, FGFR2-mutated cell lines treated with dovitinib at different doses (0.1 and 0.5 μmol/L) during 72 hours, showed a marked increase of ERα expression, although cyclin D1 expression and PI3K/AKT and MAPK pathways were downregulated (Fig. 3A). Next, we wanted to confirm that ERα was upregulated in xenografted cells treated with dovitinib in vivo. For that reason, we stained xenograft sections with ERα antibody and as we found in in vitro analysis, AN3CA cells, but not HEC-1-A, showed positive ERα immunostaining in the nucleus after 15 days of dovitinib treatment (Fig. 3B).

**The combined treatment with dovitinib and ICI182.780 results in a synergic decrease of cellular proliferation in FGFR2-mutated cells in in vitro and in vivo assays**

Motivated by the results described above, we hypothesized that ERα inhibition could enhance growth inhibitor effects of dovitinib. To assess this point, we treated HEC-1A (ERα+/FGFR2wt), Ishikawa (ERα+/FGFR2wt), AN3CA (ERα+/FGFR2mut), and MFE-296 (ERα+/FGFR2mut) cells with different doses of dovitinib (0.1–0.5 nmol/L) plus the selective ER inhibitor ICI182.780 (100 nmol/L). The drugs were used alone or in combination. Using a MTT assay we observed that combination of inhibitors caused a clear decrease in cell viability in FGFR2-mutated ECC lines (Fig. 4A). Moreover, we used linear models to study the effect of each compound and their interaction on cell viability. Statistical analysis concluded that dovitinib and ICI182.780 showed a synergic combined effect, which reached statistical significance for FGFR2-mutated cell lines (Supplementary Table S1 and Fig. S3). Similarly, we also observed a synergic effect on clonogenic ability of FGFR2-mutant cell lines (Fig. 4B). In addition, we wanted to analyze whether dovitinib and ICI182.780 cotreatment was able to cause a further inhibition of the main molecular pathways target by dovitinib alone. Combination of both drugs had a synergic effect in expression levels of downstream effectors such as p-STAT3, p-AKT, p-ERK, cyclin D1; strengthen the idea that dovitinib and ICI182.780 have synergic effects in FGFR2 mutant ECC in vivo (Fig. 4C). It should be remark that although Ishikawa cells has normal expression of ERα, ICI182.780 treatment does not affect its proliferation rate as well as signal transduction pathways related with cell-cycle regulation. It is important to note that although ERα levels at 72 hours on ICI182.780 treatment condition are quite similar to control levels, ICI182.780 treatment reduced the steady-state levels of the ER at 24 and 48 hours (Supplementary Fig. S4). To explain this phenomenon, some authors have described that pure anti-estrogen ICI182.780 reduces levels of ER by diminishing the half-life of the protein, and this circumstance can lead to an increase of receptor turnover after some hours of ICI182.780 treatment (32, 33).

Next, we evaluated the synergic antitumoral effects of dovitinib and ICI182.780 combination in vivo. For this purpose AN3CA were xenografted into SCID mice. Dovitinib was dosed daily and ICI182.780 subcutaneously 3 times/week, during 2 weeks. As Fig. 5A and B display, and consistent with the above results, dovitinib treatment inhibited growth of xenografts. Moreover, we could observe a slightly decrease (although not statistically significant) in tumor growth in ICI182.780 alone condition. This result is not surprising because ERα is required to drive mitogenic effects of a large variety of growth

Figure 1. Dovitinib decreases in vitro and in vivo growth of endometrial carcinoma cell lines. A, ECCs were treated with dovitinib at 0.5 μmol/L or vehicle for 72 hours; after which propidium iodide staining was done in order to check the DNA content. B, MFE-296, AN3CA, and Hec-1-A cells were cultured for 14 days, with 0.1–0.5 μmol/L of dovitinib or without treatment. Dovitinib was added to the media during the first 3 days. After 14 days, colonies were stained with MTT, fixed and counted. The graphs on the left show the number of colonies obtained for each condition. C, representative pictures of SCID mice injected with the indicated cell lines. HEC-1A (n = 6) and AN3CA (n = 7) tumor bearing mice were treated orally every day with dovitinib 20 mg/kg or vehicle during 15 days. Tumors were collected and prepared for immunostaining. Left, representative images of tumors. Right, graph of tumor growth over time of SCID mice xenografted with HEC-1A and AN3CA cell lines. D, immunostaining against cyclin D1 of HEC-1-A and AN3CA-xenografted tumors. All the experiments were performed at least 3 times on 3 independent days. Representative images and quantifications are shown. Errors bars indicate ± SD. Asterisks denote significant differences between cell lines. **, P ≤ 0.05 (Mann–Whitney test).
Discussion

Patients with recurrent endometrial cancer have a poor prognosis, with median survival time of approximately 12 months and an overall survival rate less than 8% (36, 37). The usual treatment for advanced diseases is cytotoxic chemotherapy and hormonal therapy (38) and, although several therapeutic agents are currently under investigation, there are no approved molecular target therapies for this disease (38). Recent advances in the understanding of molecular and genetic causes of endometriod carcinomas have led to the development of targeted therapies that inhibit pathways involved in cell growth and proliferation. Endometrial tumors are characterized by heterogeneity of genetic alterations, including defects in FGFR2 expression. Indeed, several studies have identified FGFR2 mutations in a frequency of 6.5% to 12% (8–11). However, some authors consider that these frequencies may be an underestimation because studies of frequency mutation of FGFR2 in endometrial carcinomas have only included patients with primary cancers (19). These mutations primarily occur in the extracellular or kinase domains and lead to ligand hyper-sensitivity receptor dimerization or constitutive kinase activation. Such activating mutations can confer oncogenic stimulus to tumor cells and their presence can serve to identify potential therapeutic targets and predict clinical response to pathway inhibition. Furthermore, it has become clearer that the response to target inhibition is also affected in a context-dependent manner wherein these mutations occur. We have previously demonstrated that FGFR2 expression in endometrial carcinomas is significantly associated with increased ER and PR (11). ER- and PR-positive expression in endometrial carcinomas have better outcomes and hypothetically are likely to respond better to hormonal therapy. Despite this theoretical advantage, the response rate to hormonal agents in ER-positive patients is disappointing low (16%; ref. 39). In this work, we sought to evaluate the therapeutic potential of targeting activated FGFR2 by investigating the biologic consequences of blocking FGFR2 using dovitinib, in endometrial cancer cells possessing activating mutations in FGFR2.

Dovitinib displays a charming potential as antineoplastic agent for endometrial cancer (8, 10, 11). Dovitinib has been shown to be an effective anti-neoplastic agent for different type of tumors such as breast cancer, renal cell carcinoma, and hepatocellular carcinoma among factors including IGF-1/Insulin or EGF in endometrium and other tissues (34, 35). Combination of both treatments resulted in an important decrease of tumor growth when compared with either agent alone. To confirm ER-α expression inhibition by ICI182.780 treatment, mice uteri were analyzed (Supplementary Fig. S5). Finally, we analyzed the expression of cyclin D1 and ER-α in xenograft tumors by IFIC. As shown in Fig. 5B, dovitinib treatment alone enhanced ER-α expression, although cyclin D1 expression is significantly reduced (as described before). Nevertheless, cotreatment with dovitinib and ICI182.780 significantly inhibited ER-α as well as cyclin D1 expression on AN3CA tumors.
Figure 4. Combined treatment of dovitinib (dov) and ICI182.780 results in synergic effects in vitro. A, AN3CA, MFE-296, Ishikawa, and HEC-1A were treated with vehicle, dovitinib, and/ or ICI at indicated doses for 72 hours; cell viability was assessed by MTT assay. B, HEC-1A, AN3CA, Ishikawa, and MFE-296 were subjected to Western blot analysis for the expression of ER-α, cyclin D1, phospho-Stat3, phospho-ERK, and phospho-AKT. Tubulin is shown as a loading control. All the experiments were performed at least 3 times on 3 independent days. Representative images and quantifications are shown.
However, in case of ECC, a recent study reported that dovitinib inhibits proliferation inducing cell-cycle arrest and a significant increase of apoptosis in \textit{in vitro} and \textit{in vivo} assays (19). Here, we confirm that ECC cell lines with activated FGFR2 pathway were sensitive to dovitinib, whereas in \textit{wild-type} cell lines little or no effect was seen. Next, we have focused our investigations on the study of pathways and mechanisms involved in cell-cycle arrest and inhibited proliferation triggered by dovitinib. We found that dovitinib cause a strong inhibition of ERK and PI3K pathways, associated with an inhibition of p-STAT3 and CYCLIN D1, which are proteins activated as a result of signaling pathways downstream FGFR2. Because dovitinib treatment caused an inability to progress through the cell cycle in FGFR2 mutant cell lines, we examined the expression of several hallmarks of cellular senescence. We have demonstrated that dovitinib altered the p53 and p16-pRB tumor suppressor pathways, which basically establish and maintain the senescence growth arrest. Taking together all these results, our findings suggest that dovitinib could have an antitumor activity in FGF-pathway–amplified tumors inducing cellular senescence. Induction of cellular senescence probably suppresses tumor progression because cancer development requires cell proliferation (43); for that reason, induction of senescence possesses a formidable, although not insurmountable, barrier to tumor progression.

One of the key findings of this work is the increase of ER-\(\alpha\) expression after dovitinib treatment that specifically affects FGFR2-activated cell lines. Such increase could be explained by a modest modulation of different FGF family members that could modify response to hormones and its receptors in some hormone-related cancers or through the inhibition of other kinases shared in the FGFR2-activated AN3CA and MFE-296 cell lines. To this regard, the role of FGFR2 activation or modulation (or other FGF family proteins) in hormonal response changes is still poorly understood and controversial; however, some authors have postulated that several FGF family members could

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\caption{Combined treatment of dovitinib and ICI182.780 results in synergic effects \textit{in vivo}. A, AN3CA tumor-bearing mice were treated orally every day with dovitinib 20 mg/kg and/or subcutaneously with ICI182.780 5 mg/kg 3 times weeks, during 15 days. Tumors were collected and prepared for immunostaining. Top, tumor weight measurements. \*, \(P < 0.1\); **, \(P < 0.05\) (Mann-Whitney test). Bottom, representative images of tumors and uteri of treated mice. B, immunostaining against ER-\(\alpha\) and cyclin D1 on AN3CA-xenografted tumors.}
\end{figure}
play key roles in the acquisition of hormone independence (44, 45).

The most important consequence of ER-α expression increase in FGFR2 mutant cell lines is the opportunity for combinatorial therapy using dovitinib and selective ER inhibitors such as ICI182.780. Our results provide the first evidence that dovitinib treatment sensitizes FGFR2 mutant cells to pure steroidal selective ER modulators such as ICI182.780. In breast cancer, some authors have reported in vitro and in vivo assays using combinational therapy using ICI182.780 with chemotherapeutic agents such as Doxorubicin (46); Paclitaxel (47), Vinorelbine (48), and Doxotaxel (49), although the results are quite promising, the number of studies is limited and combinational treatment using modern hormone therapy with chemotherapeutic agents has not been examined toughly, especially in endometrial carcinoma treatment. However, several experimental approaches using anticancer kinase inhibitors in tumor cells have revealed numerous feedback loops and have shown that blocking one signaling pathway can block tumor growth partially, but they are not sufficient to cause a complete tumor regression, thereby allowing resistant cells to emerge (50). An important goal of this work was to uncover a tyrosine kinase receptor that when inhibited would upregulate ER-α expression. The upregulation of ER-α expression after dovitinib treatment provides us new combinational therapy approaches for patient with endometrial carcinoma treatment because many times the effectiveness of individual inhibitors is short-lived and resistances emerges.

In summary, we provide the first demonstration that simultaneous blockade of mutant FGFR2 and ER-α activity results in robust synergistic antiproliferative effects. Prominently, our findings provide further support for targeting activating mutations of FGFR2 in endometrial carcinomas; and that FGFR2 mutational status is reasonably to predict those patients most likely to respond to combinational therapy. Recapitulating, our findings support the investigation of dovitinib and ICI182.780 as a novel combinational therapeutic approach for patients with endometrial tumors with well-defined molecular features.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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