Published in final edited form as:

Clin Cancer Res. 2011 June 15; 17(12): 4042–4051. doi:10.1158/1078-0432.CCR-10-3387.

Foretinib (GSK1363089), an orally available multi-kinase inhibitor of c-Met and VEGFR-2, blocks proliferation, induces anoikis, and impairs ovarian cancer metastasis

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Abstract

Purpose—Currently, there are no approved targeted therapies for the treatment of ovarian cancer, despite the fact that it is the most lethal gynecological malignancy. One proposed target is c-Met, which has been shown to be an important prognostic indicator in a number of malignancies, including ovarian cancer. The objective of this study was to determine if an orally available multi-kinase inhibitor of c-Met and VEGFR-2 (foretinib, GSK1363089) blocks ovarian cancer growth.

Experimental Design—The effect of foretinib was tested in a genetic mouse model of endometrioid ovarian cancer, several ovarian cancer cell lines, and an organotypic 3D model of the human omentum.

Results—In the genetic mouse model, treatment with foretinib prevented the progression of primary tumors to invasive adenocarcinoma. Invasion through the basement membrane was completely blocked in treated mice, while in control mice invasive tumors entirely replaced the normal ovary. In two xenograft mouse models using human ovarian cancer cell lines the inhibitor reduced overall tumor burden (86% inhibition, $P < 0.0001$) and metastasis (67% inhibition, $P < 0.0001$). The mechanism of inhibition by foretinib involved (a) inhibition of c-Met activation and downstream signaling, (b) reduction of ovarian cancer cell adhesion, (c) a block in migration and invasion, (d) reduced proliferation mediated by a G₂/M cell cycle arrest, and (e) induction of anoikis.

Conclusions—This study shows that foretinib blocks tumorigenesis and reduces invasive tumor growth in different models of ovarian cancer by affecting several critical tumor functions. We believe that it provides a rationale for the further clinical development of foretinib for the treatment of ovarian cancer.

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Conflict of interest: Glaxo Smith Kline (GSK) provided the drug and funding for the xenograft experiments (Fig. 2). GSK did not interfere with the planning, execution, interpretation of the results, or with the composition of the manuscript.

Keywords

c-Met; HGF/SF; ovarian cancer; inhibitor; invasion; genetic mouse model

Translational Relevance

Since liposomal doxorubicin in 1999, no new drugs have been approved for the treatment of ovarian cancer. We and others have shown that c-Met is a valuable therapeutic target in ovarian cancer, however, few clinically viable therapeutics exist to inhibit c-Met function. Foretinib is a multi-kinase inhibitor of c-Met and VEGFR-2 which is undergoing clinical testing in Phase II studies for different cancer types. This study reports on pre-clinical studies aimed at evaluating foretinib's effectiveness in ovarian cancer and understanding its mechanism of action. In a genetic model of ovarian cancer as well as in two ovarian cancer xenograft models, foretinib successfully inhibits tumor growth and metastasis. The inhibitor induces anoikis and blocks many functions important for ovarian cancer metastasis including, c-Met signaling, adhesion, invasion, and proliferation. Based on an understanding of the drug's mechanism as well as its effectiveness in multiple *in vivo* models we believe that foretinib should be considered for clinical trials in ovarian cancer.

Introduction

Ovarian cancer is the most lethal gynecological malignancy because it is often diagnosed at a late stage, after tumor cells are widely metastasized within the peritoneal cavity. Despite aggressive treatment, which includes surgical cytoreduction followed by combination chemotherapy with paclitaxel and carboplatin, more than two thirds of all patients succumb to the disease (1,2). Clearly, novel treatments targeting key cancer functions are needed in order to improve survival of patients with this deadly disease.

Deregulation of the c-Met/HGF/SF signaling axis has been identified as a contributing factor to tumorigenesis and tumor progression in numerous cancers (3). We and others have shown that c-Met is overexpressed in ovarian cancer, and that this is associated with an adverse prognosis (4-8). Recently, we demonstrated that blocking c-Met expression, using adenovirus mediated delivery of a c-Met siRNA, inhibited adhesion, peritoneal dissemination, and tumor growth in ovarian cancer xenografts (7). In addition, inhibition of c-Met using an inhibitor reduced ovarian cancer growth in a xenograft model of ovarian cancer (9). However, using adenoviruses in patients is problematic and xenograft models have a low predictive value for future success in the clinic (10).

Foretinib is an orally available, small molecule inhibitor (11) designed to target the receptor tyrosine kinases c-Met and vascular endothelial growth factor receptor-2 (VEGFR-2) both of which have been implicated in the development, progression, and spread of cancer. Phase II studies published as abstracts in papillary renal cell (12) and gastrointestinal carcinoma (13) indicated that foretinib is well tolerated and exhibits anti-tumor activity. A recently published phase I study determined the maximally tolerated dose and showed that foretinib inhibited c-Met phosphorylation and decreased proliferation in tumors biopsied after treatment (14,15). Given the important role of c-Met in epithelial ovarian cancer, the lack of effective treatments for patients with ovarian cancer, and the availability of a multi-kinase inhibitor already in clinical testing which allows for convenient oral administration, we set out to understand its mechanism(s) of action in ovarian cancer.

Our results show that foretinib is an efficient inhibitor of HGF/SF/c-Met signaling, negatively affecting several key tumor functions: In a genetic mouse model of ovarian cancer the inhibitor blocked invasion of cancer cells through the basement membrane and in two xenograft mouse models it reduced tumor burden through inhibition of angiogenesis and induction of apoptosis. Exposure of ovarian cancer cell lines to foretinib *in vitro*, reduced cellular adhesion in a 3D model, reduced cellular proliferation through a G₂/M cell cycle arrest, and induced caspase-dependent anoikis. These data suggest that foretinib, should be considered for clinical testing in patients with ovarian cancer.

Materials and Methods

Reagents

Foretinib and pazopanib were a gift from Dr. Tona Gilmer at GlaxoSmithKline (Research Triangle, NC). Anti-phospho-c-Met (Tyr^{1230/1234/1235} and Tyr¹⁰⁰³) antibody was from BioSource (Camarillo, CA). Total c-Met (C-28) was from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against p44/42 MAPK, phospho-p44/42 MAPK, Akt, phospho-Akt (Ser⁴⁷³), cdc25C, total caspase-3, cleaved caspase-3, actin rabbit antibodies, cyclin B1, p21 Waf1/Cip1, VEGFR-2 mouse antibodies were obtained from Cell Signaling (Beverly, MA). Anti-PARP mouse monoclonal antibody was purchased from BIOMOL (Plymouth Meeting, PA). c-Met was inhibited using a mixture of 4 siRNA's with the following target sequences; 1: GAAACUGUAUGCUGGAUGA; 2: GAACAGAAUCACUGACAUAA; 3: CCAGAGACAUGUAUGAUAA; 4: GAAGAUCAGUUUCCUAAUU (siGENOME SMARTPOOL, Dharmacon, Lafayette, CO).

Cells lines

The human ovarian cancer cell lines, CaOV3, CaOV-4, SKOV-3, OVCAR-5 and MCF-7 were purchased from American Type Culture Collection (Rockville, MD). OVMZ-6 cells were provided by Dr. Volker Möbus (Hospital Frankfurt-Höchst, Germany), SKOV3ip1 and HEY cells were from Dr. Gordon Mills (MD Anderson Cancer Center, Houston, TX). Cell lines were authenticated by STR DNA fingerprinting using the AmpF[®]STR Identifier kit (Applied Biosystems). The STR profiles were compared to known ATCC fingerprints, to the Cell Line Integrated Molecular Authentication database (CLIMA), and to the MD Anderson fingerprint database.

Proliferation assay and cell cycle analysis

Cells were treated with foretinib for 24h, fixed and resuspended in Propidium Iodide (PI)/RNase staining buffer. Cells were analyzed with a FACS Calibur (Becton Dickinson, San Jose, CA). The percentage of cells in the G₂/M and the sub-G₀-G₁ population (apoptotic cells) was determined using FlowJo software. Control stimulations were performed with leucine-zipper Fas ligand (LzFasL). Proliferation was measured using a fluorescence dye intercalating into DNA (7).

Quantitative RT-PCR for p21

2µg of total RNA was reverse transcribed and quantitative RT-PCR was performed using SYBR Green. Primers: p21 forward: AAGACCATGTGGACCTGTCACTGT; p21 reverse: AGGGCTTCCTCTTGGAGAAGATCA.

Western blot

For the detection of cell cycle proteins, cells were seeded for 24 hours in normal growth media prior to administration of foretinib. For caspase-3, only non-adherent cells (except for

the untreated control cells) were collected. Cells were lysed using RIPA buffer and 15µg lysate separated by SDS-PAGE.

Branching morphogenesis

The assay was performed as previously described (16). Briefly, 2,000 cancer cells were seeded in 100µl of a 1:1 mix of growth-factor reduced Matrigel and serum free DMEM. The matrigel plug was allowed to gel for 30min at 37°C. 100µl of media supplemented with 40ng/ml of HGF/SF, 1µM inhibitor or vehicle was placed on top.

Trypan blue and Hoechst staining

CaOV3 cells were pretreated for 1h with 40µM of the pan-caspase inhibitor zVAD-fmk followed by 10µM foretinib or vehicle (DMSO). Non-adherent cells were collected and stained with trypan blue or fixed with formaldehyde and stained using Hoechst 33342 stain.

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

CaOV3 cells pretreated with 40µM of zVAD-fmk for 1h prior to stimulation with foretinib (10µM) were collected and stained with MitoShift dye (Trevigen, Gaithersburg, MD) and analyzed by flow cytometry (17).

Primary cells, 3D model, adhesion, migration, and invasion assay

Primary human peritoneal mesothelial cells (HPMC) and the 3D omental culture were assembled as described (18). Adhesion, invasion, and migration assays were performed as described (19).

Xenograft models

SKOV3ip1 or HeyA8 cells (1×10^6) were injected intraperitoneal (i.p.) into 6 week old female athymic nude mice. Tumors were allowed to initiate growth for 4 or 11 days post injection for HeyA8 and SKOV3ip1, respectively, and then treatment with the indicated doses of foretinib or the vehicle (1% hydroxypropyl methylcellulose, 0.2% sodium lauryl sulfate) began. Treatment lasted for 16 or 21 days for HeyA8 and SKOV3ip1, respectively, and was given 6 days/week p.o. Mice were sacrificed and tumor burden was analyzed by excision of the tumors to determine total tumor weight as well as the number of metastasis.

LSL-Kras^{G12D}/+Pten^{loxP/loxP} ovarian cancer mouse model (20,21)

Tumors were initiated by injection of AdCre virus in the right ovarian bursa. The left ovary was not injected and served as an internal control. Oral treatment with foretinib, administered at 30mg/kg/day, 6x/week, began 4 weeks after injection of the virus and continued for 3 weeks. Mice were then sacrificed and the primary tumor was excised and embedded in paraffin for staining.

Immunohistochemistry

Slides were stained with H&E, or incubated with antibodies against Ki-67, CD31, and cleaved caspase-3. Detection was performed with the Vectastain ABC kit. Negative controls were prepared by omitting the primary antibody. Staining was evaluated by two gynecologic pathologists (AM, TK). Stromal invasion was classified according to criteria applied for human cancers as either advanced invasive adenocarcinoma or non-invasive surface hyperplasia.

Statistical analysis

For the xenograft and *in vitro* experiments, statistical differences between treated and control groups were determined using an unpaired, two-sided Student's *t*-test. For the genetic mouse model, two-sided Fisher's exact test was used to determine significance between control and treated groups and a two-sided Wilcoxon-Mann-Whitney test was used to determine statistical difference in cytology.

Results

Foretinib prevents invasion in a genetic mouse model of ovarian cancer

c-Met is expressed in human ovarian tumors (7) and in most ovarian cancer cell lines (Supplementary Fig. S1A). We initially confirmed that foretinib indeed blocks c-Met activation and the canonical downstream c-Met signaling; namely Erk/MAPK and PI3K/AKT (Supplementary Fig. S1B). Foretinib also inhibited HGF/SF induced branching morphogenesis (Supplementary Fig. S1C). Knockdown of c-Met using siRNA mimicked the effect of foretinib on c-Met signaling and branching morphogenesis (Supplementary Fig. S1D,E). Since foretinib is a multi-kinase c-Met/VEGFR-2 inhibitor (11) we also examined expression of VEGFR-2 but found it to be undetectable in the cell lines used in this study (Supplementary Fig. S1F). Having shown that foretinib inhibits c-Met signaling *in vitro*, we sought to test whether the compound was able to affect early tumorigenesis in a genetic mouse model of ovarian cancer. Immune competent mice carrying Cre-inducible oncogenic K-ras in combination with deletion of Pten in the ovaries develop invasive endometrioid ovarian cancer about 7 weeks after injection with adeno Cre virus (20). Mice were treated six times a week by oral gavage with the inhibitor or vehicle starting 4 weeks after tumor induction. At 7 weeks after induction, 7 out of 11 control treated mice had a high grade invasive adenocarcinoma which completely replaced the normal ovary (Fig. 1A-C). Staining for cytokeratin 19 confirmed the epithelial origin of the tumor cells (Fig. 1C). In contrast, in 9 out of 10 mice that received the c-Met inhibitor the ovarian tumors did not invade through the basement membrane into the normal ovarian stroma ($P=0.027$) (Fig. 1A-C and Table 1). The treated mice showed non-invasive surface hyperplasia of low nuclear grade (H&E, Ki-67). To determine whether foretinib affected the mouse tumor cells *in vitro*, we treated a cell line we recently established (21) from an ovarian cancer formed in the *LSL-K-ras^{G12D}/+Pten^{loxP/loxP}* mice with foretinib. The inhibitor blocked migration and invasion (Fig. 1D) as well as proliferation in soft agar and branching morphogenesis (Supplementary Fig. S2, A and B) while the VEGFR family inhibitor, pazopanib, had no effect. Finally, treatment of the K-ras/Pten cell line with foretinib induced apoptosis as evidenced by an increase in sub-G₀/G₁ cells (Supplementary Fig. S2C). Collectively, these data shows that treatment with foretinib significantly suppressed early ovarian cancer growth and invasion in a genetic mouse tumor model.

Foretinib reduces tumor burden in a xenograft model of metastatic ovarian cancer

To study the *in vivo* efficacy of foretinib in an additional model, female athymic nude mice with established intraperitoneal SKOV3ip1 tumors received either the inhibitor orally or vehicle alone. Foretinib reduced the number of metastatic tumor nodules (30mg/kg: 67% inhibition, $P<0.0001$) and tumor weight (30mg/kg: 86% inhibition, $P<0.0001$) in a dose dependent fashion (Fig. 2A). Similar effects were also seen in a second xenograft model using HeyA8 cells in reduction of tumor weight (71% inhibition, $P<0.0001$) (Fig. 2B). Western analysis on tumors extracted from several mice showed reduced c-Met phosphorylation upon treatment with foretinib (Supplementary Fig. S3A). Treated tumors consistently revealed significant areas of necrosis, impaired angiogenesis with a significant reduction ($P<0.005$) in CD31 positive endothelial cells, a reduced proliferation rate (Ki-67 staining), and an increase in caspase-3 a marker of tumor cell apoptosis (Fig. 2C).

In order to study the early steps (adhesion/invasion) of tumor cell metastasis to the peritoneum/omentum (2,19), we recently established a 3D culture model assembled from primary human mesothelial cells and fibroblasts extracted from human omentum (18) (Fig. 2D). Treatment with foretinib significantly abrogated SKOV3ip1 attachment to the 3D culture (Fig. 2D) and adhesion to various ECMs or primary human mesothelial (Supplementary Fig. S3B). Similarly to the mouse K-ras/Pten cell line, addition of foretinib to SKOV3ip1 cells (Supplementary Fig. S3C) nearly completely blocked migration and invasion while pazopanib had no significant effect suggesting that it is the inhibition of c-Met, not of VEGFR-2, which is mediating these effects.

Foretinib inhibits cell proliferation via a G₂/M cell cycle arrest

Because we observed a profound reduction proliferation in foretinib tumors treated as well as impaired *in vitro* proliferation (Supplementary Fig. S4A), we asked whether foretinib inhibits cell cycle progression or affects apoptosis. In both SKOV3ip1 and CaOV3 cells, foretinib profoundly increased the percentage of cells in the G₂/M phase (Fig. 3A) while decreasing the percentage of cells in the G₀/G₁ phase. Moreover, in CaOV3 cells a significant number of cells underwent cell death, as indicated by the appearance of sub-G₀/G₁ cells (Fig. 3A (see"#")), Supplementary Table S1). A similar decrease in the number of cells in the G₁ and S phase was observed with both cell lines (Supplementary Table S1), indicating that treatment with foretinib leads to a G₂/M cell cycle arrest. Next, we studied the effect of foretinib on the expression of proteins that regulate the G₂/M transition. Cdc25C is a phosphatase which functions in the nucleus to drive cell cycle progression by activating the Cdc2-cyclin B mitotic kinase complex, thereby permitting cell entry into mitosis (22). In SKOV3ip1 and CaOV3 cells, foretinib treatment resulted in a decrease of both Cdc25C and cyclin B1 protein expression over time (Fig. 3B). Both mRNA and protein expression of the G₂/M checkpoint regulator p21 (23,24) was significantly increased (Fig. 3C) following inhibitor treatment, while a p53 luciferase reporter construct transfected into SKOV3ip1 cells was not induced by foretinib, suggesting p53 independent transcriptional regulation of p21 (Supplementary Fig. S4B). These data show that foretinib inhibits the proliferation of ovarian cancer cells through a G₂/M cell cycle arrest.

Foretinib induces cell death in a two stage process

The increase of cleaved caspase-3 in foretinib treated xenograft tumors (Fig. 2C) and the increase in cells in the sub-G₁ phase (Fig. 3A, Supplementary Table S1), suggests that foretinib induces apoptosis. This was confirmed by detecting increased PARP cleavage upon treatment with foretinib in two cell lines (Fig. 4A). To determine whether the apoptosis was caspase dependent, CaOV3 cells were pretreated for 1 hour with the oligo-caspase inhibitor, zVAD-fmk, followed by treatment with the inhibitor. Foretinib induced a time-dependent increase in apoptotic cells, as evidenced by cells containing sub-G₁ nuclei (Fig. 4A). The caspase inhibitor substantially inhibited apoptosis induced by foretinib. Interestingly, we noticed that CaOV3 cells began to round up and detach as early as 8 hours after the addition of foretinib (data not shown), when apoptosis was not detectable. The detachment of cells induced by foretinib could not be inhibited by zVAD-fmk, indicating that this process is caspase independent. One of the earliest events in the apoptosis pathway (17) is the collapse of the mitochondrial transmembrane potential ($\Delta\Psi_m$). Twelve hours after addition of foretinib only a small number of cells lost their $\Delta\Psi_m$, as determined by staining with Mitotracker, but this number increased significantly 24 hours after treatment. The loss of $\Delta\Psi_m$ could be blocked by zVAD-fmk, demonstrating that it is caspase dependent (Fig. 4B).

These data imply that soon after foretinib treatment, cells detach and only secondarily undergo caspase-mediated apoptosis. To test this hypothesis, we determined the $\Delta\Psi_m$ in the attached and detached cells after treatment with foretinib (Fig. 4C). Sixteen hours after

addition of foretinib the number of attached cells with reduced $\Delta\Psi_m$ had not decreased, suggesting that cell detachment is not the result of apoptosis. While most detached cells had reduced $\Delta\Psi_m$, about a third of the cells still had intact mitochondrial membrane potential. This number decreased over the next 8 hours, indicating that it is the detached cells that undergo apoptosis. Consistently, the detached cells showed signs of nuclear fragmentation (Fig. 5A) and a low level of activation of caspase-3 (Fig. 5B) both of which could be blocked by zVAD-fmk. In order to determine the order of events, we quantified the number of dead and alive detached cells at different times after addition of foretinib (Fig. 5C). Eight hours after the addition of foretinib about half of the detached cells were dead, a number that was not reduced by treatment with zVAD-fmk. Over the next 40 hours the number of dead cells increased. This increase could be inhibited by zVAD-fmk, indicating that it was caused by a caspase-dependent process. Based on this data, we conclude that foretinib induces cell death of ovarian cancer cells through a two step mechanism; cells first detach and then subsequently undergo caspase-dependent apoptosis, similar to the process of anoikis (suspension induced apoptosis).

Discussion

This study reports on the efficacy and mechanism of action of the small molecule multi-kinase inhibitor foretinib in preclinical models of ovarian cancer metastasis. Our data suggest four principal mechanisms how foretinib inhibits ovarian cancer growth and metastasis. In ovarian cancer cell lines, the inhibitor: (i) blocked activation of c-Met signaling; (ii) reduced proliferation mediated by a G₂/M cell cycle arrest; (iii) induced cell death through a two-step mechanism in which cells detach followed by a caspase-dependent form of anoikis; and (iv) reduced proliferation, adhesion, migration and invasion during early tumor development. In mouse models of ovarian cancer metastasis, foretinib reduced tumor burden and metastasis mediated by reduced angiogenesis, proliferation, and increased apoptosis. The multiple activities of foretinib are consistent with the numerous effects that have been attributed to c-Met and angiogenesis in the context of cancer (3).

Foretinib targets c-Met and VEGFR-2 with the highest affinity but also inhibits platelet-derived growth factor- β (PDGFR β), Tie-2, RON, c-Kit and FLT3 kinases *in vitro* although with lower affinity (11). In this study, we used three ovarian cancer cell lines which do not express VEGFR-2. Treatment with the anti-angiogenic inhibitor pazopanib, which targets the VEGFR family (-1,-2, and -3) along with PDGFR- α/β and c-Kit, did not inhibit migration and invasion at a dose more than 300-fold greater than the reported IC₅₀ (25). Therefore, the *in vitro* effects observed with foretinib treatment are less likely to be due to the inhibition of VEGFR-2. Nonetheless, a potent reduction in microvessel density was seen in tumors from treated mice when compared to those of control mice and was probably mediated by inhibition of VEGFR-2 on mouse endothelial cells. Many studies have demonstrated the important role of angiogenesis in ovarian cancer and there are currently several agents (bevacizumab, sunitinib, sorafenib) in late phase clinical testing (26,27). Given the very efficient inhibition of c-Met mediated function(s), additional inhibition of angiogenesis would likely only add to foretinib's anti-tumor efficiency and relevance for ovarian cancer treatment.

Our data suggest that anoikis induction contributes to the potent antitumor effects of the inhibitor. Characterization of the cell death observed *in vitro* revealed that the cells first detach from the extracellular matrix and then undergo anoikis. Consistent with a mechanism of anoikis, cell death occurred in two distinct stages; detachment followed by caspase mediated apoptosis. Furthermore, inhibition of caspase activation with zVAD-fmk did not prevent cell detachment but blocked apoptosis. Upon adhesion to extracellular matrices, tumor cells activate HGF/SF c-Met signaling (28,29). We recently demonstrated that

knockdown of c-Met via siRNA inhibits adhesion to various ECM's (7), suggesting that inhibition of c-Met will affect tumor cell survival. Consistent with our findings, the activation of c-Met signaling has been shown to play a role in cell survival by conferring resistance to anoikis through coordinate activation of ERK/MAPK and PI3K/AKT pathways (30,31). Even with inhibition of c-Met signaling it is probable that cancer cells are capable of activating these signaling pathways via alternate mechanisms that will ultimately lead to resistance against the c-Met inhibitor. It would be of interest to determine whether combining a c-Met inhibitor with a PI3K pathway inhibitor would be more effective in inducing cell death than the c-Met inhibitor alone.

One of the mechanisms identified for the inhibition of metastasis by foretinib is through the induction of a G₂/M cell cycle arrest. In eukaryotic cells, entry into mitosis is controlled by the activation of the cyclin B/Cdc2 protein kinase, resulting in the degradation of cyclin B. The cyclin B/Cdc2 complex is activated by dephosphorylation of key residues by the Cdc25 family of phosphatases. We have shown by FACS analysis that treatment with foretinib leads to the accumulation of cells in G₂/M phase and to the downregulation of both cyclin B1 and Cdc25C expression. In addition, protein and mRNA expression of the cyclin-dependent kinase inhibitor, p21, was upregulated after exposure to foretinib, providing an explanation for the observed cell cycle inhibition. Our results are supported by the recently published results of a phase I study on foretinib which reported decreased Ki-67 staining and increased TUNEL staining in patient biopsies (14).

The results of the *in vitro* and xenograft experiments demonstrate that foretinib inhibits ovarian cancer growth in multiple ways, suggesting that several key ovarian cancer functions may be targeted by the compound. However, these experiments cannot substitute for one involving immune competent mice with relevant genetic lesions. In the K-ras/Pten genetic mouse model (20,21), endometrioid ovarian cancer develops at correct anatomical locations (primary cancer in the ovary, metastases in the peritoneum) with non-clonal, diverse tumors mimicking the presentation of ovarian cancer in patients. Although this genetic model produces endometrioid ovarian cancer rather than the more common serous papillary subtype, both subtypes are reported to have high c-Met overexpression at relatively equal frequency (32). Our data demonstrates, for the first time, the efficacy of a small molecule inhibitor in preventing ovarian cancer progression in a genetic mouse tumor model. Foretinib almost completely prevented cancer cells from breaking through the basement membrane raising the intriguing possibility that c-Met inhibition might be further developed for cancer prevention.

Because ovarian cancer is rare, the opportunity to test new compounds in clinical trials is limited. Preclinical testing of a compound in several *in vitro* (cell lines, 3D models) and *in vivo* models (genetic mouse models, xenograft) as well as understanding its mechanism of action might help to evaluate pre-clinically if a compound should be further developed for clinical testing. We believe that the preclinical results presented here suggest that foretinib may be particularly effective for the treatment of patients with advanced ovarian cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We greatly appreciate Gail Isenberg's work editing the manuscript. The leucine-zipper Fas ligand was provided by Dr. H. Walczak.

This work was supported by the Ovarian Cancer Research Fund (Liz Tilberis Scholars Program) and NIH grant R01 CA111882 (to E.L.).

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Fig. 1. Treatment with foretinib inhibits progression to an invasive adenocarcinoma in a genetic mouse model of endometrioid ovarian cancer

A, H&E staining of a normal ovary (left), an ovarian tumor from a control mouse (middle), and an ovary from a mouse which received treatment with foretinib (right), magnification 10x. Tumors were initiated by injection of AdCre virus in the right ovarian bursa of LSL-K-ras^{G12D}/+Pten^{loxP/loxP} mice. The left ovary was not injected and served as an internal control. Oral treatment with foretinib was administered 6x/week and began 4 weeks after injection of the virus, continuing for 3 weeks. The primary tumor was excised and stained with H&E. (“fo.”, follicle; “Inv. Ca”, invasive cancer; “Hyperplasia”, non-invasive surface hyperplasia; “BM”, basement membrane). The basement membrane (BM) is outlined with a dashed line. B, H&E staining showing representative ovarian tumors from 2 control and 2 treated mice. Magnification 100x, (inset 200x). The untreated mice showed an invasive cancer transforming the entire ovary while the treated mice showed non-invasive surface hyperplasia but no cancer. C, CK-19 staining detecting epithelial tumor cells and Ki-67 staining to evaluate proliferation, magnification 100x. D, migration and invasion with and without foretinib or pazopanib in K-ras/Pten mouse ovarian cancer cells. (“n.s.”, not significant; “***”, $P<0.0001$).

Fig. 2. Inhibition of tumor growth and metastasis in an ovarian cancer xenograft models

A, Mice injected i.p. with SKOV3ip1 cells underwent treatment 11 days post-injection with vehicle (control) or the indicated doses of foretinib p.o. 6 days/week for 21 days. The number of metastases and tumor weight was measured. Bar indicates the average for each group ("n.s.", not significant; "*", $P < 0.05$; "**", $P < 0.005$; "***", $P < 0.0001$). B, Overall tumor weight of mice with HeyA8 i.p. tumors treated with either vehicle or 30mg/kg foretinib. HeyA8 cells were injected intraperitoneally and underwent treatment 4 days post-injection with vehicle (control) or foretinib p.o. 6 days/week for 16 days. The tumor weight was measured. Bar indicates average for each group. ("***", $P < 0.0001$). C, immunohistochemistry of treated and control SKOV3ip1 tumors. H&E, CD31, Ki-67, active caspase-3, and negative control (upper). Magnification 200x, scale bar 100μM. ("‡", necrosis) ("***", $P < 0.005$) Quantification of staining (below). D, adhesion assay to a 3D omental culture (schematic, left). Fluorescently-labeled SKOV3ip1 cells were pre-treated with foretinib (10μM) and plated on the 3D culture. After 2 hours, the number of adherent cells was quantified by measuring fluorescence intensity. The experiment was repeated 3 times with different preparations of primary cells ("***", $P < 0.001$).

Fig. 3. Foretinib inhibits ovarian cancer proliferation mediated by G₂/M arrest

A, Flow cytometric analysis of PI staining of cancer cells after 48h in the presence of DMSO vehicle (control, left) or foretinib 10μM (right). Percentage of cells in G₂/M phase is shown. "#", indicates sub-G₀/G₁ phase. B, immunoblotting of G₂/M cell cycle regulators in SKOV3ip1 (left) and CaOV3 cells (right). Cells were treated with DMSO, 1 or 10μM of foretinib for 24 and 48h and membranes probed for Cdc25C and Cyclin B1. C, expression of p21. SKOV3ip1 cells were treated with 10μM foretinib or solvent control. Western blot (top) on lysates for p21. Quantitative RT-PCR (bottom) of p21 transcript. Mock is DMSO treatment. 1 μM nocodazole (Pos. Ctrl) was used as a positive control. ("***" for P<0.001).

Fig. 4. Foretinib induces ovarian cancer cell detachment and causes cell death

A, cells were pretreated with DMSO control or 40 μ M zVAD-fmk for 1h and incubated with 10 μ M foretinib or with 100ng/ml leucine-zipper Fas ligand (LzFasL). Attached and detached cells were pooled and analyzed for DNA fragmentation. Western blot analysis (inset) of PARP and β -actin of lysates obtained from cells treated with 10 μ M foretinib (F) for 48h. B, CaOV3 cells were pretreated with DMSO or 40 μ M zVAD-fmk and incubated with 10 μ M foretinib (F). Both adherent and detached cells were pooled and stained with Mitoshift to determine loss of mitochondrial transmembrane potential ($\Delta\Psi_m$). C, CaOV3 cells were treated with 10 μ M foretinib and the attached or detached cells were stained with Mitoshift to quantify the loss of $\Delta\Psi_m$.

Fig. 5. Foretinib induces anoikis through a two step process

A, Hoechst 33342 staining of detached cells. CaOV3 cells were pretreated with DMSO or 40 μ M zVAD-fmk for 1 h and incubated with 10 μ M foretinib for 48hr. B, detached CaOV3 cells were collected and lysates immunoblotted with total caspase-3 mAb. C, detached CaOV3 cells were collected, stained with Trypan blue, and counted for viability.

Table 1
Results of foretinib treatment in the LSL-K-ras^{G12D/+}Pten^{loxP/loxP} mouse model of endometrioid ovarian cancer

Control or foretinib treated tumors were categorized as either invasive adenocarcinoma or non-invasive surface hyperplasia. Tumor cell cytology was classified as either high or low grade. For tumor classification, significance was determined using a two-sided Fisher's exact test. For tumor cell cytology, significance was determined using a two-sided Wilcoxon-Mann-Whitney test. A p-value < 0.05 was considered significant.

	Control	Foretinib	P-value
Invasive adenocarcinoma	7/11	1/10	0.027
Non-invasive surface hyperplasia	4/11	9/10	
Grade (Tumor cytology)	Low 4/11	Low 9/10	0.038
	High 7/11	High 1/10	