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Induction of Mitotic Cell Death by Overriding G2/M Checkpoint in Endometrial Cancer Cells with Non-functional p53

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Abstract

Objective—Endometrial tumors with non-functional p53, such as serous uterine endometrial carcinomas, are aggressive malignancies with a poor outcome, yet they have an Achilles' heel: due to loss of p53 function, these tumors may be sensitive to treatments which abrogate the G2/M checkpoint. Our objective was to exploit this weakness to induce mitotic cell death using two strategies: (1) EGFR inhibitor gefitinib combined with paclitaxel to arrest cells at mitosis, or (2) BI2536, an inhibitor of polo-like kinase 1 (PLK1), to block PLK1 activity.

Methods—We examined the impact of combining gefitinib and paclitaxel or PLK1 inhibitor on expression of G2/M checkpoint controllers, cell viability, and cell cycle progression in endometrial cancer cells with mutant p53.

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

K.K.L. and X.M. conceptualized the hypotheses and designed the research strategy; X.M., L.L.L., E.A.K., S.Y., Z.X., D.Z., X.W., P.B., D.D., Y.Z., X.W., L.A., and M.A.M. performed the experiments; D.L. performed the statistical analysis; M.A.M. contributed new analytic tools; F.I. and E.A.K. analyzed the LSDCAS data; and K.K.L., X.M., and F.I. wrote the paper. All authors read and approved the final manuscript.

Results—In cells lacking normal p53 activity, each treatment activated CDC25C and inactivated Wee1, which in turn activated cdc2 and sent cells rapidly through the G2/M checkpoint and into mitosis. Live cell imaging demonstrated irreversible mitotic arrest and eventual cell death. Combinatorial therapy with paclitaxel and gefitinib was highly synergistic and resulted in a 10-fold reduction in the IC50 for paclitaxel, from 14 nM as a single agent to 1.3 nM in the presence of gefitinib. However, BI2536 alone at low concentrations (5 nM) was the most effective treatment and resulted in massive mitotic cell death. In a xenograft mouse model with p53-deficient cells, low dose BI2536 significantly inhibited tumor growth.

Conclusions—These findings reveal induction of mitotic cell death as a therapeutic strategy for endometrial tumors lacking functional p53.

Keywords

EGFR; cell cycle checkpoint; CDC25C; p38; PLK1; mitotic catastrophe

Background

Cancer of the uterine corpus is the most common gynecologic malignancy in the United States [1]. Endometrial cancer has a generally favorable prognosis when detected early; however, a subset of tumors, termed type II endometrial cancers, display a more aggressive clinical course similar to serous epithelial cancers of the ovary [2, 3]. These tumors are poorly differentiated, may be histologically classified as serous adenocarcinoma, and often demonstrate loss of function of the tumor suppressor gene p53.

A potential target for treatment of type II endometrial cancers is the EGFR tyrosine kinase enzyme. The small molecule EGFR inhibitor, gefitinib, is a low molecular weight (447 Da), synthetic anilinoquinazoline, which is an orally active, selective, reversible inhibitor of the EGFR tyrosine kinase domain [4, 5]. However, as a single agent, it has limited activity in gynecologic malignancies. The combination of targeted, non-cytotoxic agents with traditional cytotoxic chemotherapeutics has now become a common practice in colon and breast cancers due to the resulting synergistic action [6-8]. This strategy has not yet been employed for the treatment of gynecologic cancers and bears investigation.

Augmentation of cell growth inhibition, induction of apoptosis, and increased antitumor activity *in vitro* and *in vivo* has been observed when gefitinib is combined with cisplatin, carboplatin, oxaliplatin, paclitaxel, docetaxel, doxorubicin, etoposide, topotecan, or raltitrexed [9, 10]. In some cases, gefitinib in combination with cytotoxic agents promotes tumor regression in nude mice bearing prostate, lung, and colon cancer xenografts [10, 11]. Moreover, gefitinib inhibits the proliferation of ovarian, breast, and colon cancer cells and produces a synergistic enhancement of the inhibitory action of cytotoxic drugs [5, 9].

Paclitaxel is an attractive agent for combination therapy in type II endometrial cancer [12]. However, current therapeutic dose levels of paclitaxel in combination with doxorubicin and cisplatin result in a median survival of only 15.3 months with blood and neurological toxicity [13]. Thus, new combination treatments, such as paclitaxel combined with gefitinib, may provide improved responses and reduced toxicity.

Other agents that have been shown to be effective as mitotic regulators in cancer cells are the inhibitors of polo-like kinase 1 (PLK1) [13]. PLK1 is a member of the serine/threonine protein kinase family, cdc5/polo subfamily and it has been shown to regulate cyclin B1/cdc2 through phosphorylation and activation of CDC25C phosphatase. PLK1 is expressed only in dividing cells and it is required for mitotic entry, spindle assembly, chromosome segregation, and cytokinesis [14, 15]. Depletion of PLK1 results in mitotic catastrophe and

spindle disruption, and deregulation of expression of PLK1 is correlated with development of many malignancies [14, 15]. Because PLK1 is regarded as a good potential therapeutic target in cancer, a number of small-molecule inhibitors have been developed and have entered phase I trials [16-18].

In the present study, our objective was to explore the growth inhibitory effects of gefitinib, paclitaxel, the combination of paclitaxel and gefitinib, or the PLK1 inhibitor BI2536 in endometrial cancer cells with absent p53 (Hec50co) compared to cells with a gain-of-function p53 mutation [19]. The goal was to abrogate the G2/M checkpoint and induce widespread mitotic cell death. Studies in a xenograft model of endometrial cancer explored how induction of mitotic arrest affected tumor growth. The mechanistic analysis of molecular events in sensitive versus resistant cells highlights for the first time the phenotype of endometrial cells most likely to respond to therapeutic strategies that induce mitotic arrest.

Methods

Detailed methods can be found in *Supporting Methods*.

Ethics statement

Animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Experimental protocols were approved by the University of Iowa Institutional Animal Care and Use Committee (approval #1102038).

Cell lines and culture conditions

Hec50co endometrial cancer cells, a subline of Hec50 cells, were kindly provided by Dr. Erlio Gupide (New York University) and KLE cells were purchased from ATCC. Cells were cultured in DMEM (Sigma-Aldrich) with 10% fetal bovine serum (Gemini Bio-Products) and 2 mM L-glutamine (Invitrogen). For p53 knockdown in KLE cells, cells were transfected with p53 or scrambled control shRNA constructs per manufacturer's instructions (ORIGENE). Individual cell clones resistant to puromycin were expanded and screened for p53 expression by Western blotting.

Drugs and antibodies

All antibodies were purchased from Cell Signaling. Gefitinib (ZD1839, Iressa) and paclitaxel were suspended in dimethyl sulfoxide (DMSO). PLK1 inhibitor BI2536 (Selleck Chemicals) was resuspended in saline with 0.1 N HCl.

Cell cycle analysis by flow cytometry

Cell cycle analysis was performed as previously described [20].

Western blot analysis

Lysates were analyzed for protein expression/phosphorylation as described in *Supporting Methods*.

LSDCAS imaging

Large Scale Digital Cell Analysis System (LSDCAS) was used for live cell imaging for 5 days as described in *Supporting Methods* [21, 22]. Following image acquisition, images were streamed as a video using *casViewer* [22]. After identification of cell events, data were

modeled using an acyclic graph representation and graphs reporting the yield of each event of interest were built.

Mouse xenograft of human endometrial cancer

Experimental procedures for creation of tumor xenografts and injection of therapeutic agents were reported previously [23]. Briefly, 6-8 week old, Crl: NU/NU-nuBR female athymic mice [24] were injected subcutaneously with 2.5×10^6 Hec50co cells. Four weeks later, 30 mg/kg BI2536 was given through tail vein injection once a week and tumors measured weekly. At sacrifice, tumors from control animals as well as those tumors that grew in the presence of BI2536 were collected and cultured in vitro (see *Supporting Methods*).

Data analysis

The IC_{50} values were acquired using the SigmaPlot (SPSS Inc.) sigmoidal dose response equation with variable slope. The combination index (CI) was calculated using the following equation: $CI = (Am)_{50}/(As)_{50} + (Bm)_{50}/[5]_{50}$, in which $(Am)_{50}$ is the concentration of drug A necessary to attain a 50% inhibitory effect in the combination; $(As)_{50}$ is the IC_{50} concentration of drug A alone; $(Bm)_{50}$ is the concentration of drug B that will generate a 50% inhibitory effect in the combination; and $[5]_{50}$ is the IC_{50} concentration of drug B alone. A CI >1 indicates antagonism, <1 indicates synergy, and equal to 1 indicates an additive effect [23, 25]. For tumor growth analysis, statistical significance was assessed by analysis of variance (one way ANOVA), and a p value <0.05 was considered statistically significant.

Results

Paclitaxel IC_{50} is reduced by 10-fold in the presence of gefitinib

We first determined that the IC_{50} (the concentration of drug that reduces the cell number by 50% at 24 h) for paclitaxel is 14.7 nM in the Hec50co poorly differentiated type II endometrial cancer cells [26] that do not express functional p53 protein owing to a missense mutation and premature stop codon [27] (Figure 1A). By contrast, we were not able to achieve an IC_{50} for gefitinib in Hec50co cells, in accordance with the cytostatic properties of gefitinib as compared to the cytotoxic properties of paclitaxel. The IC_{50} for the combination treatment of paclitaxel with 10 μ M gefitinib was 1.3 nM, thus achieving a 10-fold reduction in IC_{50} for paclitaxel (Figure 1A) with a combination index of 0.25. These results indicate therapeutic synergy between paclitaxel and gefitinib in Hec50co cells.

Paclitaxel and gefitinib combination treatment overrides the G2/M checkpoint and produces a high percentage of abnormal mitotic cells

Flow cytometric measurements were performed to determine the effects of paclitaxel, gefitinib, and the combination treatment on cell cycle in Hec50co cells. The most interesting change was a significant 81.8% increase in the percentage of cells in G2/M phase with the combination treatment compared to 43.2% with paclitaxel alone and less than 10% for gefitinib alone or control (Figure 1B). These findings indicate that paclitaxel treatment in the presence of gefitinib results in a significant enhancement of the G2/M population. The contribution of apoptotic cell death, as indicated by the percent of cells in the sub-G1 phase, was slightly enhanced in cells treated with paclitaxel alone compared to controls; however, the sub-G1 phase did not increase when cells were treated with gefitinib and paclitaxel. This fact indicates that the significant reduction in cell number with the combination treatment cannot be attributed to an increase in programmed cell death.

Next, we determined the percentage of mitotic cells following the different drug treatments. In the control group and gefitinib groups, only about 5% of the cells were in mitosis, while

paclitaxel treatment resulted in about 15% of mitotic cells (Figure 1C). However, the addition of gefitinib to paclitaxel resulted in 60% of cells entering and remaining in mitosis, indicating that the majority of cells in the G2/M phase in Figure 1B were blocked in M phase. Note that while a small percentage of the cells in the control group were found to be in anaphase, none of the cells entered anaphase when treated with the combination of paclitaxel and gefitinib, which is likely due to disruption of spindle function by paclitaxel.

Mechanism of mitotic cell death induction by paclitaxel and gefitinib combination treatment

To understand the mechanism of synergistic cell death induction by gefitinib and paclitaxel combination treatment, we assayed the effects on protein expression and post-translational modification of critical cell cycle regulators at the G2/M transition. Total cdc2 levels remained unchanged (Figure 2A). The active form of CDC25C, a phosphatase that mediates dephosphorylation and thereby activation of cdc2 at Tyr15, was significantly increased in cells treated with the combination of paclitaxel and gefitinib as demonstrated by a slower migrating band compared to control or either drug alone. We also observed a reduction of the inhibitory form of CDC25C (Ser216), the more rapidly migrating band, with the combination treatment. In addition, a significant reduction in the phosphorylated form of p38 MAP kinase at Thr180/Tyr182, an increase in phosphorylation of PLK1 at Thr210 and Myt1 at Ser83, and a decrease in phosphorylation of Wee1 at Ser642 and total Wee1 were detected after the combination treatment but not by single agent treatment. Rather, individual drug treatment appeared to enhance the phosphorylation of p38, consistent with observations by others [28-31]. Paclitaxel and gefitinib combination treatment also promoted a significant increase in phosphorylation of the mitosis marker histone H3 at Ser10. Consistent with the activation of cdc2, treatment with paclitaxel and gefitinib inactivated stathmin-1 (STMN1), a microtubule destabilizer, via hyperphosphorylation. Collectively, these data demonstrate a molecular signature indicative of mitotic arrest in cells subjected to gefitinib and paclitaxel combination treatment.

As a confirmation of the role played by the p53 protein in treatment response, we examined the effect of gefitinib and paclitaxel on KLE cells that harbor the p53 gain-of-function mutation R175H linked to constitutive p38 MAPK activation [32]. KLE cells demonstrated significant resistance to treatment (Figure 2B). In particular, these cells displayed no significant induction of the active form of CDC25C after treatment with paclitaxel and gefitinib, no significant reduction in phosphorylated p38 MAPK, and no loss of cdc2 phosphorylation. KLE cells demonstrated constitutively elevated phospho-p38 regardless of treatment conditions. Also in contrast to Hec50co cells, paclitaxel alone or in combination with gefitinib only slightly increased histone H3 Ser10 phosphorylation and STMN1 hyperphosphorylation. Knockdown of p53 in KLE cells restored sensitivity to the paclitaxel and gefitinib combination as determined by a marked reduction in cell viability (Figure 2C, D), thus providing evidence for the critical role of the p53 gain-of-functional mutant in the resistance mechanism.

To determine the requirement for blockade of p38 MAPK in mitotic cell death, we assessed the cell cycle profile of Hec50co cells treated with a combination of the p38 MAPK inhibitor SB203580 and paclitaxel (Supplementary Figure S1). Combination treatment resulted in 61.7% of the cells arrested in the G2/M phase. By contrast, only 45.6% of cells were in G2/M after treatment with paclitaxel alone and no G2/M arrest was observed with SB203580 alone. These findings confirm that p38 MAPK is a critical controller of drug sensitivity in p53 inactive cells.

PLK1 inhibitor BI2536 induces mitotic arrest and cell death in p53 null Hec50co cells but not in KLE cells with gain-of-function p53 mutation

PLK1 is a critical regulator of various processes in mitosis [15, 33]. Therefore, we next determined whether direct inhibition of PLK1 alone is an attractive therapeutic strategy in p53-deficient endometrial cancer cells. Significant loss of viability and G2/M arrest was observed in Hec50co cells treated with 2.5-10 nM of the PLK1 inhibitor BI2536 (Figure 3A, $IC_{50} = 5$ nM). More than 84% of cells treated with 10 nM BI2536 accumulated in the G2/M phase compared to 15% of control cells (Figure 3A). As in the case of combining gefitinib with paclitaxel, most of the PLK1 inhibitor-treated cells were in mitosis (Figure 3B) with cdc2 dephosphorylation and CDC25C activation (Figure 3C). Conversely, KLE cells were relatively resistant to the PLK1 inhibitor treatment.

Live cell imaging of normal cell division, abnormal cell division and mitotic cell death

Kinetics of normal and abnormal cell division and mitotic cell death in Hec50co cells were determined in 5 day live cell imaging experiments using the Large Scale Digital Cell Analysis System (LSDCAS) (23) (Figure 4) and analyzing the events by applying an in-house developed program *cas Viewer* [21]. During the 5 days of image acquisition, gefitinib-treated cells grew exponentially at the same rate as the control sample, following first order kinetics (Figure 4A). Paclitaxel treatment alone slowed down cell division starting at about 40 h, though cell division remained exponential. Combination treatment with paclitaxel and gefitinib dramatically depressed normal division. Control, paclitaxel, and gefitinib alone treated cells did not display abnormal cell division, whereas cells treated with BI2536 lacked normal cell division (Figure 4A) and had the highest yield of abnormal cell division with an onset within the first 20 h (Figure 4B). Combined treatment with paclitaxel and gefitinib gave the second highest yield of abnormal division, with the kinetics of abnormal cell division following first order kinetics for the first 20 h then slowly plateauing between 40 to 120 h. All the abnormal cell division events in BI2536-treated cells resulted in cell death; cell death sharply increased at ~45 h, soon after all the cells in the sampled population abnormally divided (Figure 4B, C). Combination treatment with paclitaxel and gefitinib induced death with much slower kinetics and without death of all imaged cells (Figure 4C). Figure 4D reports the number of events analyzed during the 5 day imaging experiment with starting and ending numbers of sampled cells for each treatment.

LSDCAS composite still frames and video clips (Supplementary Figure S2 and Media Clips S1-5) visually demonstrate how the treatments affected cell proliferation and morphology. Further analysis demonstrates that cells treated with BI2536 were widely blocked in the first mitotic division after treatment. The cells remained rounded-up for a prolonged period, and most died while attempting division. Note that the rounding-up events mark the time that precedes cell division; imaged cells, growing as monolayer, detached from the growth surface, became rounder during spindle organization, and became translucent (see the arrow in Supplementary Figure S2G). Following a failed division, very few cells spread out, they demonstrated extensive nuclear fragmentation, and most died during interphase following the first failed division (Supplementary Figure S2H). Cells treated with paclitaxel and gefitinib underwent mitotic catastrophe-like events [34] with abnormal division and fusion and production of large, multinucleated cells (Supplementary Figure S2D, G). Furthermore, these cells displayed a long delay at the first division attempt and rounded-up for 5 to 12 h before completing the first division; about 13% of analyzed cells recovered and resumed division at a normal rate, with ~50% of multinucleated cells alive at the end of imaging acquisition (Supplementary Figure S2D).

Inhibition of tumor growth in Hec50co xenograft mice by BI2536

Based on our *in vitro* observation of significant mitotic cell death induction by the PLK1 inhibitor BI2536 at nM-range doses, we further tested the effect of low-dose BI2536 on tumor growth inhibition in a xenograft model of endometrial cancer using implanted Hec50co cells. Although BI2536 treatment by tail vein injection (30 mg/kg once a week for six weeks) did not cause complete tumor regression, significant tumor growth inhibition was observed (Figure 5A). Detailed analysis of the growth of each BI2536-treated tumor showed that responses differed between individual mice, with a dramatic inhibition of tumor growth in mice B, D, G and H, partial inhibition in A and E, but no evident inhibition in C, F, I and J (Supplementary Figure S3). The failure of BI2536 treatment in the latter group may be attributed to the increased tumor size at the beginning of the treatment (Supplementary Figure S3); however, the induction of resistance pathways in these cells may also have occurred. To indirectly examine whether the failure to respond in some treatments may have been due to drug bioavailability, we conducted molecular analysis of tumors excised 24 h after the final BI2536 treatment. We observed a significant increase in hyperphosphorylated STMN-1 and phosphorylated histone H3 in treated mice compared to controls (Figure 5B), suggestive of response to BI2536. Yet, for tumor I in the BI2536-treated group, the magnitude of the increase in hyperphosphorylated STMN-1 and histone H3 phosphorylation was less than that observed in the other individual tumors, indicating ineffective pathway blockade by BI2536 in these tumors. To explore the nature of sensitivity versus resistance in more detail, cell lines were derived from these individual tumors and challenged with BI2536 again *in vitro*. Interestingly, most of the cells derived from resistant tumors remained sensitive to BI2536, with IC₅₀ values similar to those observed in parental Hec50co cells (Figure 5C). We also observed the anticipated increase in phosphorylation of histone H3, hyperphosphorylated STMN-1, decreased cdc2 phosphorylation, and increased un-phosphorylated STMN-1 at Ser38 (Figure 5D). These data suggest that the absence of response to BI2536 for select xenograft tumors was due to suboptimal drug bioavailability to the tumor mass.

Discussion

Type II serous endometrial cancer is a lethal disease associated with high mortality rates. New, more effective therapeutic regimens are needed to treat these patients. Controlling cancer requires inhibiting proliferation and inducing cell death. Irreversible mitotic arrest leads to death in mitosis due to an inability to achieve cellular division. Also referred to as mitotic catastrophe, irreversible mitotic arrest can occur through multiple pathways depending on the nature of the inducer and the status of cell cycle checkpoints [35]. Induction of mitotic arrest by therapeutic manipulation is a new strategy for cancer treatment worthy of further exploration; however, a more complete understanding of which cells are most likely to respond and why is needed. We demonstrate that mitotic arrest can be accomplished in susceptible cells (those lacking functional p53) using either gefitinib or a specific blocker of p38 MAPK in the presence of an agent which disrupts the mitotic apparatus such as paclitaxel. Our data suggest a model in which, in response to gefitinib and paclitaxel combination, up-regulation of the active form of CDC25C enhances the transition of cells through G2 phase and into M phase by activating cyclin B1/cdc2. The normal p53 suppression of CDC25C is lost in p53 null Hec50co cells. Given that PLK1 is required for both the G2/M phase transition and mitosis, we hypothesize that the effects of BI2536 in this study are most likely due to inhibiting PLK1 activity in mitosis. However, we note that p53-deficient cells with BI2536 alone decreased p38 MAPK phosphorylation, thereby replicating the effects of gefitinib and paclitaxel. In fact, effects of PLK1 inhibitor BI2536 were more pronounced than those obtained with the gefitinib and paclitaxel combination. These cell

culture and tumor xenograft findings set the stage for using such strategies in clinical trials in endometrial tumors lacking functional p53.

We investigated the growth-limiting effects of paclitaxel, gefitinib, the combination of the two agents, and the PLK1 inhibitor BI2536 on cell cycle, mitotic arrest, and cell death. Our findings indicate that when Hec50co cells (a serous cell line which lacks p53) were treated with a combination of paclitaxel and gefitinib, the IC₅₀ for paclitaxel was reduced 10-fold and synergistic cell death resulted. Treatment with these two agents or with BI2536 alone caused a high percentage of cells to accumulate in mitosis. Live cell imaging demonstrated that the cells were blocked in mitosis and had undergone mitotic catastrophe based upon nuclear and cytoplasmic morphology [34, 36, 37]. In contrast, KLE cells (high grade endometrial cancer cells with a gain-of-function p53 mutation) did not undergo mitotic catastrophe, maintained constitutive p38 MAPK activation, and were resistant to treatment.

To investigate the mechanism by which mitotic arrest occurred, we assessed the expression of important controllers of the cell cycle at the G2/M transition. The G2/M checkpoint is very important for cancer cells with inactivated p53 because these cells do not have an intact G1/S checkpoint that can be activated in response to DNA damage. Cells with non-functional p53 rely on the G2/M checkpoint to initiate DNA repair; hence, loss of the G2/M checkpoint moves cells that are genomically unstable prematurely into M phase, leading to widespread cell death due to mitotic catastrophe [22, 38-40]. In the presence of paclitaxel combined with gefitinib (or a p38 MAPK inhibitor) or with the PLK1 inhibitor BI2536, the increase in the active form of the phosphatase CDC25C enhanced transition of cells through G2 and abrogated the G2/M checkpoint. CDC25C is also inhibited by p38 MAPK through the downstream kinase MK2 [41, 42]. Phosphorylation of p38 was inhibited by gefitinib and paclitaxel in combination, or by BI2536 alone, thereby relieving the inhibition of CDC25C and enhancing progression into mitosis. The increase in p38 MAPK phosphorylation when cells were treated with paclitaxel [28-30] or gefitinib [31] alone has been previously reported in the literature, though it is as yet unknown how the combination of gefitinib and paclitaxel signal to inhibit phosphorylation of p38. This is a critical question which deserves further investigation because the p38/MK2 signaling pathway is activated to maintain the G2/M checkpoint specifically in cells which lack functional p53. Inhibition of this pathway likely plays a significant role in the synergy achieved by the combination regimen.

The pronounced effect observed with PLK1 inhibitor BI2536 alone is likely attributed to inhibition of PLK1 activity in M phase. Experiments using xenograft models also demonstrate the effectiveness of this strategy. Interestingly, while some animals derived substantial clinical benefit from the PLK1 inhibitor, BI2536, others harbored tumors which were partially resistant. When the resistant cells were harvested from the tumors and grown *in vitro*, we observed sensitivity to PLK1 inhibition, suggesting that future studies that optimize drug delivery to larger tumors are necessary.

Taken together, these findings suggest that EGFR blockade using a tyrosine kinase inhibitor such as gefitinib in addition to chemotherapy with paclitaxel, could be an effective treatment. The strategy is particularly relevant for those cancers deficient in p53, a common finding in type II serous endometrial cancer. Given that the standard chemotherapy regimen for endometrial cancer patients is paclitaxel and carboplatin, future studies that examine how addition of carboplatin impacts the effects of paclitaxel and gefitinib combination on the G2/M checkpoint as well as on tumor growth in xenograft models in mice. It is also important to note that clinical studies in ovarian cancer demonstrated increased incidence of hematologic malignancies in patients treated with carboplatin, paclitaxel, and gefitinib [43], though studies in non-small cell lung cancer did not reveal similar adverse effects [44, 45]. Studies that examine how the timing and sequence of drug administration affect not only clinical

response but also incidence of adverse events are warranted. In addition to the combination of chemotherapy with gefitinib, PLK1 inhibitors, which our data demonstrate block p38 MAPK as well as disrupt the mitotic machinery, may have clinical utility as single agents in the same clinical setting. We propose that tumors with loss-of-function p53 mutations have a unique capacity to respond to therapies which target the G2/M checkpoint and the mitotic machinery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research Highlights

- Therapeutic synergy between gefitinib and paclitaxel is achieved by abrogating the G2/M cell cycle checkpoint in p53-deficient endometrial cancer cells.
- Endometrial cancer cells lacking functional p53, but not cells with gain-of-function p53 mutation, undergo mitotic arrest and cell death.
- PLK1 inhibition decreases growth of p53-null xenograft endometrial tumors, identifying a new therapeutic strategy for this molecular phenotype.

FIGURE 1.

Effect of paclitaxel alone or in combination with gefitinib on cell viability and cell cycle distribution in Hec50co cells. A. IC₅₀ was determined for Hec50co cells treated with paclitaxel alone (top left), or a combination of paclitaxel and gefitinib (bottom left) for 24 h. Paclitaxel IC₅₀ = 14.7 nM. Paclitaxel+gefitinib IC₅₀ = 1.3 nM. IgM = log of molarity. The combination index for paclitaxel+gefitinib = 0.25. B. Cell cycle distribution for Hec50co cells treated with vehicle (DMSO), 10 nM paclitaxel, 10 μM gefitinib, or a combination of 10 nM paclitaxel and 10 μM gefitinib. C. Representative nuclei in control cells or gefitinib and paclitaxel co-treated cells (top panel), and percentage of mitotic cells under different drug treatments (bottom panel: gefitinib 10 μM; paclitaxel 10 nM; paclitaxel and gefitinib 10 μM + 10 nM). Error bars = $\pm$ SEM.

FIGURE 2.

Combination treatment with paclitaxel and gefitinib induces variations in cell cycle regulatory proteins in Hec50co compared to KLE cells. A. Hec50co cells were treated for 24 h as follows: 1) control (vehicle, DMSO), 2) 10 μ M gefitinib, 3) 10 nM paclitaxel, and 4) 10 nM paclitaxel + 10 μ M gefitinib. Expression/phosphorylation of indicated proteins was assessed by Western blotting. B. KLE cells were treated and analyzed as in (A). C. Expression of p53 was examined in KLE cells, which harbor a p53 gain-of-function mutation, after stable expression of either a control or p53-specific shRNA. B-actin, loading control. D. Cell viability after treatment with either paclitaxel alone or in combination with 10 μ M gefitinib was assessed in KLE cells stably expressing either control shRNA (left panel) or p53 shRNA (right panel).

FIGURE 3.

PLK1 inhibitor BI2536 treatment induces accumulation at G2/M phase in Hec50co and KLE cells. A. Hec50co cells were treated with vehicle (DMSO, control), or 10 nM BI2536 for 24 h, followed by cell cycle analysis. B. Percentage of mitotic cells in control or 10 nM BI2536 treated Hec50co and KLE cells. C. Western blotting analysis of cell cycle regulatory proteins in Hec50co and KLE cells treated as in (A).

FIGURE 4. *casViewerLSDCAS* imaging data analysis. Yield of normal division (A), abnormal division (B), and cell death (C) in Hec50co cells treated with a combination of 2.5 nM paclitaxel and 10 μ M gefitinib, 5 nM BI2536, 2.5 nM paclitaxel, 10 μ M gefitinib, or DMSO (vehicle control). D. Number of cells sampled and the events (normal cell division, abnormal cell division, cell death) analyzed during the 5 days of live cell imaging.

FIGURE 5.

PLK1 inhibitor BI2536 inhibits tumor growth in Hec50co xenograft model of endometrial cancer. A. Effect of BI2536 on tumor growth. BI2536 (30 mg/kg), or vehicle control, were administered by tail vein injection once per week for 5 weeks. * indicates $p < 0.05$ vs. vehicle. B. STMN-1 hyper-phosphorylation and histone H3 phosphorylation (Ser10) were examined in tumor lysates from BI2536 treated mice 24 h following the final BI2536, or vehicle control, treatment. Letters denote individual tumors from control or BI2536 treated groups. C. WST-1 viability assay for the cancer cell lines derived from BI2536-treated mice re-exposed to BI2536 for 3 days *in vitro*. D. Levels of cell-cycle regulatory proteins in cell lines derived from BI2536-treated mice re-exposed to a dose of 10 mM of BI2536 for 3 days were examined by Western blotting.

FIGURE 6.

Proposed synergistic pathway to mitotic cell death in cells treated with a combination of paclitaxel and gefitinib or the PLK1 inhibitor BI2536. When cells are treated with gefitinib in combination with paclitaxel, the up-regulation of the active form of CDC25C enhances the transition of cells through G2 phase and into M phase by activating cdc2 via dephosphorylation. p53 normally suppresses CDC25C and enhances the G2/M checkpoint. However, this suppression is lost in p53 null Hec50co cells. In absence of functional p53, p38 MAPK signaling through MK2 is the primary mechanism which inhibits CDC25C under baseline conditions; however, in the presence of gefitinib and paclitaxel, CDC25C activity is further enhanced. PLK1 is required for both the G2/M phase transition and mitosis, and the effects of BI2536 in this study are most likely due inhibiting PLK1 in mitosis.