An antagonist of the chemokine receptor CXCR4 induces mitotic catastrophe in ovarian cancer cells

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Abstract

The chemokine receptor CXCR4 is expressed by malignant cells in ovarian cancer and is implicated in their growth and spread. We report here a unique mechanism of action of a small peptide antagonist of CXCR4 on ovarian cancer cells: induction of cell death by mitotic catastrophe. CTCE-9908 inhibited ovarian cancer cell migration to CXCL12, but on longer incubation, caused cell death in CXCR4-positive cells. CTCE-9908 did not cause apoptosis or cellular senescence, but induced multinucleation, G2-M arrest, and abnormal mitosis in ovarian cancer cells. This suggests that cell death was caused by mitotic catastrophe. Using microarray and Western blot analysis, we showed that CTCE-9908 deregulated DNA damage checkpoint proteins and spindle assembly checkpoint proteins at G2-M phases of the cell cycle. Combination treatment of CTCE-9908 and the drug paclitaxel led to an additive cytotoxicity that also involved mitotic catastrophe. We conclude that CTCE-9908 has a unique mechanism of action in ovarian cancer cells that seems to be CXCR4 specific. [Mol Cancer Ther 2009;8(7):1893–905]

Introduction

Chemokines are chemotactic cytokines with a molecular mass of around 8 to 17 kDa and belong to a superfamily that is divided into four groups (CXCL, CX3CL, CCL, and XCL) according to the positioning of the first two closely paired and highly conserved cysteine (C) residues of their amino acid sequence. The specific effects of chemokines on their target cells are mediated by members of a family of seven-transmembrane G-protein–coupled receptors (1). To date, 20 human chemokine receptors have been identified (2). In inflammation and cancer, chemokines in the diseased tissues contribute to the rolling, tethering, and invasion of leukocytes from blood vessels, through the endothelial cell basement membrane, and into the parenchyma (3, 4).

In cancer, chemokine receptors are also expressed by malignant cells. The most commonly studied chemokine receptor on malignant cells is CXCR4. It is found on malignant cells of epithelial, mesenchymal, and hematopoietic origin in at least 23 different human malignancies (3). In some cancers, there is an association between levels of CXCR4 expression on malignant cells in primary human tumors and the extent of lymph node metastasis (5), and the CXCR4 ligand CXCL12 is frequently found at common sites of metastasis in these malignancies (2).

However, in glioma (6), astrocytoma (7), and ovarian cancer (8, 9) malignant cells, tumors produce both CXCL12 and its CXCR4 receptor. The biological significance of coexpression of CXCR4 and CXCL12 to cancer growth and spread is not understood.

In the human ovarian tumor microenvironment, chemokine CXCL12 stimulated the growth and invasion of tumor cells by establishing a protumor cytokine network in the tumor microenvironment (8). CXCL12 was also important for peritoneal dissemination of ovarian tumor cells because there was a CXCL12 gradient between ovarian tumor cells and peritoneal mesothelial cells (10). In addition, CXCR4 is expressed on the membrane and in the cytoplasm of malignant cells in a majority of ovarian cancer biopsies (11). Taken together, these findings suggest that CXCR4 is a potential target for ovarian cancer therapy.

CXCR4 antagonists include bicyclams (AMD3100; ref. 12), peptides derived from polyphemusin II proteins (T140 and TN14003; ref. 13), and peptides derived from the NH2-terminal region of CXCL12 (14). CTCE-9908 is a small peptide that comprises a dimerized sequence of the disordered NH2-terminal of CXCL12 and was designed to block the CXCR4 receptor (15, 16). Treatment with CTCE-9908 significantly reduced lung metastasis of osteosarcoma and melanoma cells in animal models (17). A phase I/II clinical trial of CTCE-9908 in patients with advanced solid cancers is being studied (16).

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4 Kulbe et al. personal communication.
The aim of this study was to investigate the activity of CTCE-9908 in ovarian cancer cells in vitro. We found that CTCE-9908 inhibited migration and induced cytotoxicity in CXCR4-expressing ovarian cancer cells in a CXCR4-specific manner. Such cytotoxicity was mediated through mitotic catastrophe. Cotreatment of CTCE-9908 and paclitaxel resulted in additive cytotoxicity in ovarian cancer cells.

Materials and Methods

Reagents

CTCE-9908 and scrambled peptide control (SC-9908) were obtained from Chemokine Therapeutics Corp. Anti-CXCR4, α-tubulin, Cdc2, Cdc25A, Cdc25C, Aurora A, Aurora B, Mad2L1, Plk1, NUMA, CENP-E, geminin, and survivin antibodies were purchased from Abcam; statinum antibody was from Cell Signaling; anti–phospho-MPF-2, Chk1, and Chk2 were from Upstate; CXCR4 inhibitory antibodies were from R&D Systems; AMD3100 octahydrochloride, paclitaxel, staurosporine, and calcein-AM were purchased from Sigma.

Cell Cultures

The ovarian cancer cell lines TOV21G and SKOV3 (all from American Type Culture Collection) and IGROV-1 (18) were cultured in DMEM supplemented with 10% fetal bovine serum. Two CXCR4 knockdown clones from IGROV-1 cells (shCXCR4 a9 and e10) and IGROV-1 cells with scrambled shRNA construct were cultured in DMEM supplemented with 10% fetal bovine serum and puromycin dihydrochloride (Sigma). IOSE20C2 and IOSE25C26 are hTERT-immortalized human ovarian surface epithelial cell lines from two different donors (19) that were cultured under endotoxin-free conditions. For CTCE-9908 and scrambled peptide control (SC-9908) Reagents, serum-starved cells were treated with CTCE-9908 (100 μg/mL) or SC-9908 (100 μg/mL) for 6 h. After treatment, cells were dissociated and resuspended in serum-free medium. Resupended cells were added to the upper chamber (5 × 10^5); complete medium with 10% fetal bovine serum was added to the lower chamber. Chambers were incubated overnight. Cells on the upper surface of the filter were removed using a cotton wool swab. Migrated cells on the lower surface were stained with calcein-AM and trypsinized. Trypsinized cell suspensions were collected and fluorescence was read at excitation λ 485 nm and emission λ 520 nm.

Flow Cytometry

For surface expression of CXCR4, cells were dissociated by enzyme-free PBS-based cell dissociation buffer (Invitrogen). Monoclonal antibodies against CXCR4 (clone 4G717) or IgG2B isotype control (R&D Systems) were applied to the cell suspension and incubated. After washing, Alexa Fluor 488 secondary antibody (Invitrogen) was incubated. Cells were counterstained with propidium iodide (PI) and analyzed on a FACSscan flow cytometer using CellQuest software (BD PharMingen). For apoptotic cell detection, cultured under endotoxin-free conditions. For CTCE-9908 Mig.

2. Endometrial cancer cell lines from two different donors (19) that were cultured under endotoxin-free conditions. For CTCE-9908 Fluo-3-Indolyl-β-d-Galactopyranoside Staining

Cellular senescence was detected with the Senescence Detection Kit (Biovision, Inc.).
DNA Laddering
Intraneososomal DNA fragmentation was detected with the Apoptotic DNA Ladder Detection Kit (Millipore).

Microarray
Total RNA was isolated using RNeasy Kit (Qiagen). RNA was quantified and assessed for integrity using a 2100 Bioanalyzer (Agilent Technologies). Expression profiles of all samples were compared with a commercial universal reference RNA (Clontech). The Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix) were used to define gene expression profiles in each sample. Probe synthesis and microarray hybridization were done according to standard Affymetrix protocols at the Institute of Cancer and the Cancer Research UK Clinical Centre microarray core facility.

Microarray Analysis
Three Affymetrix data sets were obtained from triplicate samples of IGROV control, IGROV cells treated with CTCE-9908 for 24 h (CTCE-9908 24 h), and IGROV cells with CTCE-9908 for 48 h (CTCE-9908 48 h). Data were analyzed using Bioconductor 1.95 running on R2.6.0. Probe set expression measures were calculated using the Affy package.

5 http://bioconductor.org
Robust Multichip Average default method. Differential gene expression was assessed between control (IGROV-1) and treated groups (CTCE-9908 24 h and CTCE-9908 48 h) using an empirical Bayes t test (limma package; ref. 21); P values were adjusted for multiple testing using the Benjamini-Hochberg method. Any probe sets that exhibited an adjusted P value of 0.05 were called differentially expressed. In addition, any probe sets that exhibited an absolute fold change of >2 were used to generate a heat map. Two-dimensional hierarchical clustering of expression data using differentially expressed genes across control and treated groups was done. Samples were clustered using a 1 – Pearson correlation distance matrix and average linkage clustering. Genes were clustered using a Euclidean distance matrix and average linkage clustering.

**Gene Set Enrichment Analysis Using Gene Ontology Processes**

Collections of gene sets based on Gene Ontology (22) term mapping to the Affymetrix human U133plus2 arrays were created using the GSEAbase package in Bioconductor (23). The function “geneSetTest” from the limma package was used to assess whether each Gene Ontology term had a tendency to be associated with up-regulation or down-regulation. The function used a Wilcoxon test to generate P values, ranking the data based on t statistics. Three collections comprising terms from the main branches of the Gene Ontology (Biological Process, Molecular Function, and Cellular Component) were tested separately. The Benjamini-Hochberg method was used to control the false discovery rate (24).

**Quantitative Real-time Reverse Transcription-PCR**

Total RNA was isolated by TRI reagent (Sigma) and the first-strand cDNA was synthesized by SuperScriptII RT Kit (Invitrogen). Predesigned probe and primer sets of human CXCR4 mRNA were obtained from TaqMan Gene Expression Assay (Applied Biosystems). Probe and primer sets for human 18S rRNA were used as endogenous controls. Real-time PCR reactions were done with the ABI PRISM 7700 Sequence Detector (Applied Biosystems). Data analyses were done using the ΔΔC_T method.

**Western Blotting**

For the experiments with CTCE-9908 treatment, cells were firstly synchronized at early S phase by double thymidine block. After the secondary thymidine (Sigma) block, cells were released by complete medium with or without CTCE-9908 for 3 d. Cell extract (30 μg) was run on 6%/10%/14% SDS-PAGE gel and transferred onto nitrocellulose membranes. The membranes were blocked in PBS with nonfat milk and probed with different primary antibodies. Horseradish peroxidase–conjugated secondary antibodies were detected using enhanced chemiluminescence Western blotting detection reagents (GE Healthcare). Protein concentration equivalence was confirmed after probing by β-actin antibody.

**CXCR4 siRNA**

For transient knockdown of CXCR4 in ovarian cancer cells, ON-TARGET plus SMART pool of CXCR4 gene was transfected into the cells by Dharmafect1 transfection reagent (all from Dharmacon). SiCONTROL Nontargeting siRNA Pool (Dharmacon) served as scrambled RNAi control. The cells were incubated for 72 h after transfection.

**Statistical Analysis**

Statistical analysis was evaluated unpaired t test with Welch correction (GraphPad Prism version 4 software).

**Results**

**CTCE-9908 Inhibits the Migration and Growth of CXCR4-Expressing Ovarian Cancer Cells**

We used three different ovarian cancer cell lines (IGROV-1, TOV21G, and SKOV3) and two hTERT immortalized ovarian surface epithelial (IOSE) cell lines. The ovarian cancer cell lines expressed varying levels of CXCR4. The IOSE cells (IOSE20 and IOSE25) were CXCR4 negative (Fig. 1A and B).

To test the effect of CTCE-9908, we treated IGROV-1 and TOV21G cells with 100 μg/mL CTCE-9908 and performed a migration assay. Scrambled peptide (SC-9908) was also used to test the specificity of the peptide sequence of CTCE-9908. The migration of IGROV-1 and TOV21G cells was significantly inhibited by CTCE-9908 but not by SC-9908 (Fig. 1C).

Having shown that CXCL12–CXCR4 signaling increases survival of ovarian cancer cells (8), we hypothesized that blockade of CXCR4 by CTCE-9908 would affect the growth of ovarian cancer cells. To test this, CXCR4-positive ovarian cancer and CXCR4-negative IOSE cell lines were treated with CTCE-9908 for 10 days and a cell viability assay was done. The number of viable cells in IGROV-1, TOV21G, and SKOV3 cell lines was significantly reduced by treatment with CTCE-9908, whereas IOSE cells (IOSE20 and IOSE25) were not affected by CTCE-9908. This result suggests that the effect of CTCE-9908 acts through CXCR4. In addition, the scrambled peptide had no activity on the cell lines (Fig. 1D).

To further confirm that CTCE-9908–mediated growth inhibition is CXCR4 dependent, two clones of IGROV-1 cells stably expressing a CXCR4-targeted small hairpin RNA construct, or mock scrambled shRNA construct (IGROV-1 scrambled), were used. Our FACS data showed that surface expression of CXCR4 was significantly down-regulated in IGROV-1 shCXCR4 clones when compared with the parental cells and the scrambled control. After CTCE-9908 treatment for 10 days, the number of viable cells in IGROV-1 shCXCR4 clones was higher than that of the scrambled control (Fig. 1E). Taken together, all the above results suggest that the growth inhibition induced by CTCE-9908 is CXCR4 specific.

**CTCE-9908 Induces Cytotoxicity of Ovarian Cancer but It Is Not Due to Acute Apoptosis and Cellular Senescence**

To determine whether CTCE-9908–mediated growth inhibition is a result of cell death, cells were stained with SYPRO10 and DEAD Red nucleic acid stains after 10-day CTCE-9908 treatment. Dead cells were detected in CTCE-9908–treated IGROV-1, TOV21G, and SKOV3 cells (Fig. 2A). The scrambled peptide SC-9908 did not cause cell death in any ovarian cancer and IOSE cells (data not shown).

To determine the mechanism for CTCE-9908 stimulated cytotoxicity, early and late events of apoptosis were
investigated at day 3 posttreatment. No increase in Annexin V–positive cells was found in CTCE-9908–treated IGROV-1, TOV21G, and SKOV3 cells (Fig. 2B); neither was DNA fragmentation detected in any CTCE-9908–treated ovarian cancer cells (Fig. 2C). The results indicate that acute apoptosis is not a mechanism for CTCE-9908 stimulated cytotoxicity.

To examine whether CTCE-9908 induces cellular senescence in ovarian cancer cells, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining was done at day 3 posttreatment. No positive X-gal staining was detected in untreated and CTCE-9908–treated IGROV-1 and SKOV3 cell lines. Endogenous β-galactosidase activity was found in some of untreated TOV21G cells; however, no induction of X-gal–positive cells was found in CTCE-9908–treated TOV21G cells (Fig. 2D).

CTCE-9908 Induces Multinucleation and G2-M Arrest in Ovarian Cancer Cells

Although neither apoptosis nor cellular senescence was found in CTCE-9908–treated ovarian cancer cells, CTCE-9908 induced significant morphologic changes in ovarian cancer cells. Giant multinucleated cells were observed in CXCR4-expressing IGROV-1, TOV21G, and SKOV3 cells (Fig. 3A) but not in CXCR4-negative IOSE cells after CTCE-9908 treatment (data not shown).

Flow cytometric analysis also revealed significant changes in DNA content and cell cycle distribution in CTCE-9908–treated ovarian cancer cells. The percentage of cells containing more than 4N DNA content (>4N cells, i.e., multinucleated cells) increased significantly in IGROV-1, TOV21G, and SKOV3 cells after CTCE-9908 treatment. G2-M arrest also occurred in CTCE-9908–treated IGROV-1,
TOV21G, and SKOV3 cells as indicated by an increase in the percentage of cells at these phases (Fig. 3B). However, multinucleated cells and G2-M arrest were not detected in CXCR4-negative IOSE cells after CTCE-9908 treatment (Fig. 3B and C).

CTCE-9908–induced multinucleation and G2-M arrest occurred in a concentration-dependent manner (Fig. 3C). The increase of >4N cells also occurred in a duration-dependent manner. When IGROV-1, TOV21G, and SKOV3 cells were treated with 100 μg/mL CTCE-9908 in a time course experiment, the percentage of >4N cells increased from 4 hours posttreatment and reached a peak at 48 hours posttreatment (Fig. 3D). The scrambled peptide SC-9908 did not induce multinucleation and G2-M arrest in ovarian cancer and IOSE cells (data not shown).

CTCE-9908 Induces DNA Re-replication and Abnormal Mitoses in Ovarian Cancer Cells

To determine whether these multinucleated cells resulted from DNA re-replication of 4N cells, we monitored the BrdUrd incorporation after CTCE-9908 treatment by flow cytometry analysis. The results show that the CTCE-9908–induced >4N cells were positive for BrdUrd (Fig. 4A). This suggests that DNA re-replication is a mechanism by which the multinucleated cells are formed. To determine whether the multinucleation results from cell-cell fusion between two 4N cells, we performed a cell-cell fusion assay. Similar to many other tumor cell lines, IGROV-1, TOV21G, and SKOV3 cells are able to fuse spontaneously in tissue culture (25). However, we found no significant increase of cell-cell fusion when treating IGROV-1 cells with 100 μg/mL CTCE-9908. The percentage of fused cells was significantly decreased when treating IGROV-1 cells with 300 μg/mL CTCE-9908 (Fig. 4B). Similar observations were also evident in TOV21G and SKOV3 cells after CTCE-9908 treatment (data not shown). Therefore, our observations indicate that the CTCE-9908–induced multinucleation is not due to cell-cell fusion.

Multinucleation may be caused by abnormal mitosis. Indeed, multinucleation and abnormal mitosis are morphologic characteristics of mitotic catastrophe, a cell death that occurs during mitosis or results from abnormal mitosis (26). To test this, we examined mitosis in CTCE-9908–treated ovarian cancer cells by DAPI staining. We found abnormal mitoses with multipolar metaphase and anaphase plates in IGROV-1, TOV21G, and SKOV3 cells after CTCE-9908 treatment. Using quantitative analysis of normal and abnormal mitoses, we found that the percentage of the normal mitoses decreased in IGROV-1, TOV21G, and SKOV3 cells after

Figure 3. CTCE-9908 induces multinucleation and G2-M arrest in ovarian cancer cells. A, fluorescent staining for plasma membrane, lipid vesicles, and nuclei following 100 and 300 μg/mL CTCE-9908 treatment for 3 d in IGROV, TOV21G and SKOV3 cells. Green, plasma membrane and lipid vesicles; blue, nuclei. B, flow cytometric analysis of DNA content in IGROV, TOV21G, SKOV3, and IOSE25C26 cells treated with CTCE-9908 (100 and 300 μg/mL) for 3 d. A representative cell cycle profile of each treatment was shown. G1: G1 phase of cell cycle; G2/M: G2-M phase of cell cycle. >4N, cells with more than 4N DNA content. C, quantification of cell cycle distribution (sub-G1, G1, S, and G2-M) and >4N cells in IGROV, TOV21G, SKOV3, and IOSE25C26 cells after 3-d treatment with increasing concentration of CTCE-9908 (0–300 μg/mL). D, quantification of cell cycle distribution and >4N cells in IGROV, TOV21G, and SKOV3 cells treated with CTCE-9908 (100 μg/mL; top) for 4 to 72 h.
Figure 4. CTCE-9908 induces DNA re-replication and abnormal mitoses in ovarian cancer cells. A, flow cytometric analysis for bromodeoxyuridine (BrdUrd) incorporation in IGROV, TOV21G, and SKOV3 cells treated with CTCE-9908 (100 and 300 μg/mL) for 3 d. The quadrant shows the limits for replicating cells (top left; BrdUrd-positive 2N and 4N cells) and re-replicating cells (top right; BrdUrd-positive >4N cells). Percentage of the re-replicating cells is indicated. IgG1 isotype was used as negative control. B, Cell-cell fusion was detected by flow cytometry in IGROV cells after treatment with CTCE-9908. Top, unstained IGROV cells (No label), DiO-labeled cells (DiO only), DiI-labeled cells (DiI only), and double-labeled cells (DiO & DiI) were cultured without CTCE-9908 for 3 d. Bottom, mixture of DiO- and DiI-labeled IGROV cells were treated with CTCE-9908 (100 and 300 μg/mL) for 3 d and subsequently analyzed by flow cytometry. R1 represents a region for double positive (DiO and DiI positive) cells. Percentage of double positive cells is indicated. C, left, fluorescent staining of DNA in IGROV, TOV21G, and SKOV3 cells after treatment of CTCE-9908 (0 or 100 μg/mL) for 3 d. White circle indicates abnormal mitoses with multipolar metaphase or anaphase. Blue, DAPI stain. Right, quantification of normal and abnormal mitoses in IGROV-1, TOV21G, and SKOV3 after treatment of CTCE-9908. Fifteen to thirty of 400× objective images of each treatment were captured. Number of normal mitoses, abnormal mitoses, and total number of nuclei were counted on each image. D, immunofluorescent staining for phosphorylated MPM2 (pMPM2) following 100 μg/mL CTCE-9908 treatment for 3 d in IGROV, TOV21G, and SKOV3 cells. pMPM2 was labeled in green, F-actin was labeled in red by phalloidin, and nuclei were counterstained in blue by DAPI. White circle indicates abnormal mitoses with multipolar metaphase and multiple spindles. E, immunofluorescent staining for α-tubulin in IGROV, TOV21G, and SKOV3 cells after CTCE-9908 treatment (100 μg/mL) for 3 d. α-Tubulin was labeled in green, F-actin was labeled in red by phalloidin, and nuclei were counterstained in blue by DAPI. White circle indicates abnormal mitoses with multipolar metaphase and multiple spindles.
treatment with CTCE-9908, whereas the percentage of abnormal mitoses increased significantly after the CTCE-9908 treatment (Fig. 4C). To ensure that those cells with multipolar chromosome segregation were in mitosis, phosphorylated MPM2 (a mitosis-specific marker) immunostaining was done. Ovarian cancer cells with multipolar chromosome segregation were positive for phosphorylated MPM2 (Fig. 4D). Staining for α-tubulin revealed multiple spindles in the abnormal mitoses in CTCE-9908–treated IGROV-1, TOV21G, and SKOV3 cells (Fig. 4E). Taken together, our results show that CTCE-9908 induced abnormal mitoses in ovarian cancer cells. Thus, we suggest that CTCE-9908 stimulated the cytotoxicity of ovarian cancer cells by mitotic catastrophe in a CXCR4-dependent manner.

To know whether this is a phenomenon restricted to ovarian cancer cells, we examined the effect of CTCE-9908 on endometrial cancer cells. Two endometrial cancer cell lines (HEC1B and AN3CA) were used in this study. Real-time reverse transcription-PCR and flow cytometric analyses showed that HEC1B cells strongly expressed CXCR4, whereas

### Table 1. Significant Gene Ontology categories after CTCE-9908 treatment in IGROV-1 cells

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Abbreviations: GO, Gene Ontology; FDR, false discovery rate.
AN3CA cells were CXCR4 negative (Supplementary Fig. S1A and B). HEC1B and AN3CA cells were treated with CTCE-9908 for 3 days and a cell viability assay was done. The number of viable HEC1A cells was significantly reduced by CTCE-9908 treatment whereas AN3CA cells were not affected by CTCE-9908 (100 μg/mL). Giant multinucleated cells were found in CTCE-9908-treated HEC1B cells but not in CTCE-9908-treated AN3CA cells (Supplementary Fig. S1D). G2-M arrest and induction of >4N cell population were also detected in HEC1B cells but not in ANC3A cells after treatment with CTCE-9908 (Supplementary Fig. S1E). These results suggest that CTCE-9908 acts in a similar CXCR4-dependent fashion on other malignant cells.

To investigate whether other CXCR4 antagonists or inhibitors also induce G2-M arrest and multinucleation, ovarian cancer cells were treated with CXCR4 inhibitory antibodies, CXCR4-targeted siRNA, or nonpeptide bicyclam CXCR4 antagonist (AMD3100). None of these treatments induced multinucleation, although AMD3100 was growth inhibitory (Supplementary Fig. S2). Based on these data, we conclude that CTCE-9908 exhibits a unique cytotoxicity in ovarian cancer cells that seems to be CXCR4 specific.

**CTCE-9908 Affects DNA Damage Checkpoint and Spindle Assembly Checkpoint at G2-M Phases**

To explore the mechanism of CTCE-9908–induced mitotic catastrophe in ovarian cancer cells, genes found to be differentially expressed in IGROV-1 cells after treatment with 100 μg/mL CTCE-9908 (after 24 and 72 hours) versus untreated IGROV-1 control were analyzed with Gene Set Enrichment Analysis to identify the Gene Ontology categories most represented in down-regulated genes (Table 1; Supplementary Table S1). These results show that CTCE-9908 directly or indirectly affected the expression of many genes involved in G2 and M phases of cell cycle. In particular, genes involved in the G2-M transition checkpoints (such as Cdc2, Cdc25A, Cdc25C, and CENP-F) were significantly down-regulated in

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6 Supplementary material for this article is available at Molecular Cancer Therapeutics Online [http://mct.aacrjournals.org/].
CTCE-9908–treated IGROV-1 cells. These data suggest that CTCE-9908 might affect DNA damage checkpoint and spindle assembly checkpoint at G2-M phases of ovarian cancer cells.

To evaluate the integrity of DNA damage checkpoint and spindle assembly checkpoint in ovarian cancer cells after CTCE-9908 treatment, we examined the expression level of several key proteins involved in these two checkpoints. In this experiment, IGROV-1, TOV21G, and SKOV3 were first synchronized by double thymidine block. The synchronized cells were then treated with 100 μg/mL CTCE-9908 for 3 days. Because Cdc2 (cyclin-dependent kinase 1), Cdc25A, Cdc25C, Chk1, and Chk2 are essential to the DNA damage checkpoint, which controls cell cycle progression from G2 to M phase (27), Western blots for these DNA damage checkpoint proteins were done. Our results showed that Cdc2, Cdc25A, Cdc25C, and Chk1 were down-regulated in CTCE-9908–treated IGROV-1, TOV21G, and SKOV3 cells. Chk2 expression was also decreased in TOV21G and SKOV3 after CTCE-9908 treatment (Fig. 5A).

In mitosis, spindle assembly checkpoint is governed by several mitotic kinases, such as Aurora A, Aurora B, Mad2L1, Plk1, and BubR1 (26). Down-regulation of Plk1 was found in CTCE-9908–treated TOV21G and SKOV3 cells (Fig. 5B), whereas up-regulation of BubR1 was detected in CTCE-9908–treated IGROV-1 cells (Fig. 5C). Other mitotic kinases, such as Aurora A, Aurora B, and Mad2L1, were not affected by CTCE-9908 (Fig. 5B).

Besides mitotic kinases, several nuclear matrix and spindle pole proteins are also associated with spindle assembly checkpoint. NUMA (nuclear mitotic apparatus protein 1) is a spindle pole protein in mitosis (28). Centromeric protein F (CENP-F/mitosin) is a large human nuclear protein transiently associated with the outer kinetochore plate in M phase (29). Our results show that NUMA and CENP-F were down-regulated in CTCE-9908–treated IGROV-1, TOV21G, and SKOV3 cells. Chk2 expression was also decreased in TOV21G and SKOV3 after CTCE-9908 treatment (Fig. 5A).

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Combination Treatment of CTCE-9908 and Paclitaxel Results in Additive Cytotoxicity by Mitotic Catastrophe

Paclitaxel (Taxol) is widely used in the treatment of breast and ovarian cancers. A low concentration (10 nmol/L) of paclitaxel was found to induce mitotic arrest, multinucleation, and eventually cell death in HeLa cells (31). To examine whether low concentrations of paclitaxel also induced mitotic arrest and multinucleation in ovarian cancer cells, IGROV-1, TOV21G, and SKOV3 cells were treated with 10 nmol/L paclitaxel for 24 hours. DNA content FACS analysis revealed that G2-M arrest and multinucleation were found in IGROV-1, TOV21G, and SKOV3 cells after treatment with 10 nmol/L paclitaxel (Fig. 6A). Therefore, we hypothesized that combining CTCE-9908 with low concentration of paclitaxel might result in additive cytotoxicity. We analyzed the cell survival after combining 100 μg/mL CTCE-9908 with 10 nmol/L paclitaxel. An additive cytotoxic effect was found between CTCE-9908 and paclitaxel in IGROV-1, TOV21G, and SKOV3 cells after treatment with both drugs for 3 days (Fig. 6B).

To confirm that this additive cytotoxicity is due to mitotic catastrophe, cell cycle distribution was analyzed after combination treatment with CTCE-9908 and paclitaxel. Additive accumulation of G2-M arrest and multinucleation were detected in IGROV-1 cells when treated with CTCE-9908 and paclitaxel. Similarly, additive G2-M arrest was found in TOV21G cells after cotreatment of CTCE-9908 and paclitaxel (Fig. 6A). However, no additive G2-M arrest or multinucleation was observed in SKOV3 cells after combination treatment. This might be because SKOV3 cells are more sensitive to 10 nmol/L paclitaxel. Taken together, our results show that combination treatment with CTCE-9908 and paclitaxel conferred additive cytotoxicity by mitotic catastrophe.

Discussion

Because the signals of CXCL12 in tumor microenvironment are important for the growth, metastasis, and survival of malignant cells (32), blocking the signals of CXCL12 by inactivating its receptor, CXCR4, has been considered as a potential antitumor therapy. Preclinical studies showed that the major antitumor activities of CXCR4 antagonists (AMD3100, AMD3465, T140, and TN14003) are inhibition of CXCL12-induced tumor growth and cell migration (33–37). However, these CXCR4 antagonists seldom induced cell death in majority of tumors. Our preliminary results also showed that bicyclams and CXCR4 inhibitory antibodies did not induce cell death in ovarian cancer cells. There are only few articles that showed AMD3100-induced apoptosis in glioblastoma and medulloblastoma (38, 39). Different tumor cell types may account for the dissimilarity of AMD3100-induced cell death.

CTCE-9908 exhibits antitumor activity. Treatment of osteosarcoma cells in vitro with CTCE-9908 led to decreases in growth rate, adhesion, migration, and invasion of the tumor cells. Using tail vein injection of osteosarcoma cells, mice that were treated with CTCE-9908 had a significant reduction in pulmonary metastasis (17). We showed in the present study that CTCE-9908 induced cell death in ovarian cancer cells by mitotic catastrophe. This is a unique cytotoxicity induced by CTCE-9908 because other CXCR4 inhibitors, including AMD3100, CXCR4 inhibitory antibody, and CXCR4-targeted siRNA, did not show any mitotic catastrophe in ovarian cancer cells.

CTCE-9908 induced multinucleation, G2-M arrest, and abnormal mitosis in ovarian cancer cells. These changes may be due to aberrant regulations of cell cycle protein or cell death signals induced by CTCE-9908-CXCR4 signaling. There are some data to suggest that different interactions with the CXCR4 receptors may result in different biological signals. CXCL12 down-regulated the phosphorylation of Rb and the transcription of E2F-1 in neuronal cells through CXCR4, whereas the binding of HIV envelope glycoprotein gp120 to CXCR4 exerted opposite effects on Rb and E2F-1 activities in neurons (40). Moreover, CXCL12 induced homing and development of T lymphocytes, whereas the binding of gp120 to CXCR4 induced autophagy and apoptosis in T lymphocytes (41). Most recently, CXCR4-gp120IIIB interactions were found to induce apoptosis and inhibit tumor growth of prostate cancer (42).

CXCR4 is also expressed on primordial germ cells, neuronal precursors, and hemopoietic stem cells (43). It is possible that our study has uncovered an unknown action of CXCR4 signaling in maintaining the integrity of the mitotic spindle—an action that could be important in stem and progenitor cells.

To better understand the mechanism of the CTCE-9908-induced mitotic catastrophe, we investigated the checkpoints of G2-M transition. Mitotic catastrophe occurs as a result of DNA damage or deranged spindle formation coupled to the debilitation of different checkpoint mechanisms that would normally arrest progression into mitosis and hence suppress catastrophic events until repair has been achieved. The “DNA damage checkpoint” arrests cells at the G2-M transition in response to unreplicated DNA or DNA damage, and the “spindle assembly checkpoint” prevents anaphase until all chromosomes have obtained bipolar attachment. The combination of checkpoint deficiencies and specific types of damage (such as DNA damage or deranged spindle formation) would lead to mitotic catastrophe (26).

Our results showed that several G2-M checkpoint proteins (Cdc2, Cdc25A, Cdc25C, Chk1, and Chk2) and spindle checkpoint proteins (Plk1, NUMA, CENP-F, and stathmin) were down-regulated in ovarian cancer cells after CTCE-9908 treatment. Depletion of Chk1 led to premature activation of Cdc2-cyclin B and mitotic catastrophe (44). Inhibition of Chk2 would stabilize centrosomes, maintain high cyclin B1 levels, and allow for a prolonged activation of cyclin-dependent kinase 1. Under these conditions, multinuclear HeLa syncytia did not arrest at the G2-M boundary and rather entered mitosis and subsequently died during the metaphase of the cell cycle (45). Silencing of Plk1 caused induction of mitotic arrest and further apoptosis in prostate cancer cells (46). Inactivation of NUMA by immunodepletion would lead to
a failure of normal spindle assembly and production (47). Silencing of CENP-F induced misaligned chromosomes, premature chromosome decondensation before anaphase onset, and mitotic cell death (29). Inhibition of statin expression would lead to accumulation of cells in the G2-M phases and was associated with severe mitotic spindle abnormalities and difficulty in the exit from mitosis (30). Based on the above evidence, we suggested that CTCE-9908 weakened the DNA damage checkpoint and spindle assembly checkpoint in ovarian cancer cells.

Mitotic catastrophe is a common phenomenon occurring in tumor cells with impaired p53 function following exposure to various cytotoxic and genotoxic agents. It is interesting to know whether CTCE-9908 is genotoxic and whether the action of CTCE-9908 is related to inactivation of p53 or BRCA1 in ovarian cancer cells. Our results showed that CTCE-9908 did not induce DNA damage in ovarian cancer cells, as indicated by no positive H2AX signal being detected after treatment with CTCE-9908 (data not shown). This result suggests that CTCE-9908 is not genotoxic. Moreover, the p53 and BRCA1 status of the three ovarian cancer cell lines we used is as follows: mutated p53 and BRCA1 were found in IGROV-1; wild-type p53 and BRCA1 in TOV21G; and null p53 and wild-type BRCA1 in SKOV3. Based on these observations, we do not believe that the action of CTCE-9908 is related to p53 or BRCA1 inactivation in ovarian cancer cells.

Paclitaxel is a microtubule-stabilizing agent that causes deranged spindle formation in tumor cells, a possible explanation for the additive cytotoxicity between CTCE-9908 and paclitaxel relating to the combination of weakened spindle assembly checkpoints and deranged spindle formation in ovarian cancer cells. This hypothesis is supported by a report showing that silencing of Plk1 in breast cancer cells improved sensitivity toward paclitaxel in a synergistic manner (48).

HER2 enhances the expression of CXCR4 in breast cancer, which is required for HER2-mediated invasion and metastasis. HER2 also inhibits CXCL12-induced CXCR4 degradation (49). It will be interesting to investigate the action of CTCE-9908 on CXCR4-positive breast cancer cells in the future. Although the prognostic influence of HER2/neu is not dependent on the CXCR4/CXCL12 signaling pathway in ovarian cancer (50), it would be interesting in the future to study the action of CTCE-9908 on HER2 activity in ovarian cancer cells.

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

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References


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