IKKβ regulates VEGF expression and is a potential therapeutic target for ovarian cancer as an anti-angiogenic treatment

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Abstract

The prolongation of progression-free survival (PFS) in patients with advanced ovarian cancer by anti-angiogenic therapy has been shown in several clinical trials. However, although an anti-VEGF antibody (bevacizumab) is the only option currently available, its efficacy is limited and it is not cost-effective for use in all patients. Therefore, the development of a novel anti-angiogenic drug, especially composed of small molecule compounds, could be a powerful armament for ovarian cancer treatment. Since NF-κB signaling has the potential to regulate VEGF expression, we determined to identify whether VEGF expression is associated with NF-κB activation and to investigate the possibility of a novel IKKβ inhibitor, IMD-0354 (IMMD Inc. Tokyo, Japan), as an anti-angiogenic drug.

Tissue microarrays from 94 ovarian cancer tissues were constructed and immunohistochemical analyses performed. We revealed that IKK phosphorylation is an independent prognostic factor (PFS; 26.1 vs. 49.8 months, \(P=0.011\)), and is positively correlated with high VEGF expression. In in vitro analyses, IMD-0354 robustly inhibited adhesive and invasive activities of ovarian cancer cells without impairing cell viabilities. IMD-0354 significantly suppressed VEGF production from cancer cells, which led to the inhibition of angiogenesis. In a xenograft model, the treatment of IMD-0354 significantly
inhibited peritoneal dissemination with a marked reduction of intratumoral blood vessel formation followed by the inhibition of VEGF expression from cancer cells. IMD-0354 is a stable small molecule drug and has already been administered safely to humans in other trials. Anti-angiogenic therapy targeting IKKβ is a potential future option to treat ovarian cancer.

**Introduction**

The prognoses of ovarian cancer patients have remained much the same since the late 1990s, and for this reason, a large number of novel molecular targeted agents and innovative therapeutic associations of chemotherapy have been attempted and remain under investigation (1, 2). Among the new drugs studied for ovarian cancer, bevacizumab, an anti-vascular endothelial growth factor (VEGF) antibody, has shown promising activities in combination with standard chemotherapy in several Phase III trials, such as the ICON7 and GOG218 studies (3, 4).

Angiogenesis has been shown to be an important contributor to ovarian carcinogenesis and progression. Among various angiogenic factors, VEGF, the most endothelial cell-specific angiogenic factor characterized to date, induces endothelial cell proliferation, promotes cell migration and inhibits apoptosis. VEGF is firmly
believed to be a key regulator of physiological and pathological angiogenesis (5). Thus, it is likely that bevacizumab would be a powerful tool for ovarian cancer treatment. It has shown revolutionary efficacies not only as a first-line therapy combined with paclitaxel plus carboplatin but also as a treatment for relapsed cases. However, several concerns remain unresolved. In Phase III trials (ICON7 and GOG218), although maintenance treatment with bevacizumab extended the length of progression-free survival (PFS), this advantage stopped 10 months after the cessation of treatment and only ICON7 showed a trend towards improved overall survival (OS) in a subgroup of patients with high-risk disease (3, 4). In platinum-sensitive relapse, although bevacizumab has also been shown to improve PFS (OCEANS) (6), no overall survival benefit was found. In platinum-resistant relapse, a recent Phase III trial (AURELIA) reported an increase in response rate and a doubling of PFS (6.7 vs. 3.4 months) in those who received single agent chemotherapy plus bevacizumab compared with chemotherapy alone, while the effect on OS has not been reported (2, 7). These clinical data indicate that, in order to improve OS, long-term continuous anti-angiogenic maintenance therapy would be indispensable until patients show progressive disease. However, the pharmacoeconomic issue is obviously a major obstacle for clinical long-term use of bevacizumab in every patient. Hensley et al. commented that bevacizumab
costs $78.3 million for 3.8 progression-free months for 600 women (8). Hence, we believe that it would be meaningful to find better compounds that specifically inhibit angiogenesis with safety profiles, given that anti-angiogenic therapy is to be used in combination with cytotoxic chemotherapies. Indeed, anti-angiogenic molecules have been developed and many clinical trials are underway (9).

Nuclear factor-kappa B (NF-κB) is an inducible transcription factor that regulates a wide variety of gene expressions. Constitutive NF-κB signaling has been identified in tumors of epithelial origin, including breast, colon, lung, and ovarian carcinomas (10), and numerous in vitro and in vivo studies have suggested that NF-κB plays an important role in cancer progression including angiogenesis (11). Although various studies have suggested that NF-κB signaling correlates with angiogenesis, the importance of NF-κB in terms of VEGF expression remains controversial (12, 13, 14). In addition, the prognostic value of high NF-κB activity on ovarian carcinomas is also controversial. Kleinberg et al. reported that the nuclear localization of NF-κB/RelA (p65) is frequently expressed in advanced-stage serous ovarian carcinomas, and is associated with poor PFS (15), while Annunziata et al. wrote that high expression of cytoplasmic NF-κB1 (p50) was associated with poor survival in advanced-stage ovarian cancer patients (16). However, more recently, Yang et al. showed that the nuclear
accumulation of p65 in epithelial ovarian carcinomas is significantly associated with a good response to chemotherapy and can predict longer OS of patients (17). For these reasons, we were encouraged to analyze whether NF-κB signaling regulates VEGF expression in ovarian cancer tissues as well as whether targeting this signaling can be an alternative option for the inhibition of VEGF expression, leading to a new anti-angiogenic therapy.

The NF-κB/Rel family includes NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel. They can all form homo- and heterodimers, with the most abundant form being p50/p65 (18). These dimers are present in the cytosol in an inactive form, complexed with proteins of the IκB family. Upon stimulation, phosphorylation of IκB by IκB kinase (IKK) leads to degradation of IκB and renders NF-κB free to translocate into the nucleus. The nuclear NF-κB binds to promoters and enhancers, leading to gene transcription (11). Two IκB kinases, IKKα and IKKβ, have been well described. It is known that they have nonequivalent functions and that activation of IKK-β, rather than IKK-α, participates in the primary pathway by which proinflammatory stimuli activate NF-κB (18). Given that the indispensable roles played by NF-κB in many biological processes raise the concern that a complete shutdown of this pathway would have significant detrimental effects on normal cellular function, drugs that selectively target
only IKK-β activity could be of greater therapeutic value (19). IMD-0354 is a newly synthesized low-molecular-weight compound that selectively inhibits IKKβ in the IKK complex. IMD-1041 is a prodrug of IMD-0354 for oral administration and on its way to clinical application at present. A Phase 1 study was completed and up to 12 weeks of IMD-1041 administration proved to be sufficiently safe with very few adverse events ranked as mild (20). Therefore, we determined to elucidate the effect of this drug on ovarian cancer cells, focusing especially on VEGF expression.

With these goals in mind, we first constructed tissue microarrays (TMAs) from ovarian cancer samples and immunostained them with phosphorylated-IKK and VEGF antibody. We revealed that IKK phosphorylation is an independent prognostic factor and that its expression was positively correlated with high VEGF expression, suggesting that NF-κB activation regulates VEGF expression in ovarian cancer tissues. Further, we assessed the potential of a small molecular-weight IKK inhibitor, IMD-0354, as a novel therapeutic option for anti-angiogenic treatment against ovarian cancer.

Materials & Methods

Materials. IMD-0354, a synthetic IKKβ inhibitor, was supplied by IMMD Inc. (Tokyo, Japan). Growth factor reduced basement membrane proteins (Matrigel), human
fibronectin, collagen type 1 and Integrins Sampler Kits [#611435] were purchased from BD Biosciences (Bedford, MA). Antibodies against VEGF (A-20), integrin α1 (R-164), matrix metalloproteinase-2 (MMP-2; H-76) and CD31 (M-20) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against p-IKKα/β (Ser176/180), IκB-α, β-actin, and cleaved caspase-3 (Asp175) were obtained from Cell Signaling (Danvers, MA). Antibody against Ki-67 (RM-9106) was from Thermo (Waltham, MA).

**Cell culture.** SKOV3ip1 cells (serous adenocarcinoma, mutation; PIK3CA, ARID1A, amplification; ERBB2) (21) were kindly provided by Dr. Ernst Lengyel (University of Chicago, Chicago, IL) in 2007. RMUG-S cells (mucinous adenocarcinoma, mutation; TP53) (21) were obtained from the Health Science Research Resources Bank (Osaka, Japan) in 2008. CaOV3 (serous adenocarcinoma, mutation; TP53) and ES-2 cell lines (clear cell carcinoma, mutation; TP53, BRAF) (21) were purchased from ATCC (Manassas, VA) (CaOV3; in 2007, ES-2; in 2014). Cells were cultured in DMEM supplemented with 10% FBS and 1000 U/mL penicillin/streptomycin and incubated in 95% air/5% CO₂ at 37°C. Cells were authenticated by short tandem repeat DNA profiling at Takara-Bio Inc. (Otsu, Japan) and were used for this study within 6 months of resuscitation. Human umbilical vein endothelial cells (HUVECs) were collected as previously reported (22).
**TMA preparation and immunohistochemistry.** Ovarian carcinoma samples were collected from patients treated at Gifu University Hospital (Gifu, Japan) between 2006 and 2011 and used to construct the tissue microarray (TMA) slides. From the corresponding regions in paraffin blocks, tissue cores (diameter, 3 mm) were removed using a hollow needle, arrayed in paraffin blocks and sliced (4 μm) onto slides. Institutional Review Board approval was obtained. Satisfactory tissue cores were finally obtained from 94 patients, and the corresponding clinical data were collected. The slides were deparaffinized in xylene and rehydrated with 100% ethanol before antigen unmasking was performed by boiling in Target Retrieval Solution (pH9.0) (Nichirei Biosciences, Tokyo, Japan). After being placed in 3% H$_2$O$_2$ and being blocked with blocking solution (Dako, Carpinteria, CA), they were incubated with the primary p-IKKα/β antibody at 1:150 and VEGF-A antibody at 1:40 for one hour at room temperature. After washing with TBS containing 0.1% Tween-20 (TBST), they were stained using the Envision system (Dako) and then counterstained with Carrazzi's hematoxylin. Slides were intensively examined by two independent qualified pathologists (EM, SM) without knowledge of the clinical outcomes. The phospho-IKK staining sample was scored based on the intensity of the staining (0, none; 1, weak; 2, strong). “Positive” expression of p-IKK was defined if the score of intensity was 1 or 2.
“Negative” expression was defined if the score of intensity was 0. Human VEGF-A staining was scored based on the intensity of the staining (1, weak; 2, medium; 3, strong) and the percentage of positive cells (1, <10%; 2, 10 to 40%; 3, >40%). “High” VEGF expression was defined if the total score of intensity and density was $\geq 5$. “Low” VEGF expression was defined if the total score was $\leq 4$.

**Cell viability assessment.** Ovarian cancer cells ($3 \times 10^3$ cells) were seeded in 96-well plates and cultured in DMEM supplemented with 10% FBS with increasing concentrations of IMD-0354 ranging from 0.1 to 1.0 $\mu$M for 48 h. Cell viability was assessed using the CyQUANT cell proliferation assay kit (Molecular Probes, Eugene, OR). Cell viability was expressed as the ratio of the number of viable cells with IMD-0354 treatment to the number without treatment.

**Western blot analysis.** A total of $5 \times 10^5$ cells were plated onto 6-well plates and lysed with $1 \times$ Cell Lysis Buffer (Cell Signaling). Lysates (15 $\mu$g) were separated by 5-20% SDS-PAGE and transferred to polyvinylidene difluoride membranes, followed by incubation with the primary antibodies ($p$-IKK$\alpha/\beta$, 1:1000 in 5% bovine serum albumin (BSA); I\(\kappa\)B-$\alpha$, 1:1000 in 5% BSA; integrin $\alpha$1, 1:1000 in 5% BSA; integrin $\alpha$2, 1:1000 in 5% BSA; integrin $\alpha$5, 1:2500 in 5% BSA; integrin $\beta$1, 1:2500 in 5% BSA; integrin $\beta$3, 1:2500 in 5% BSA; MMP-2, 1:1000 in 5% BSA; $\beta$-Actin, 1:2000 in 5% BSA and then
with a corresponding secondary horseradish peroxidase conjugated IgG. The proteins were visualized with an electrochemiluminescent system (PerkinElmer Life Science, Waltham, MA).

**Immunofluorescent analysis.** 5 × 10³ cells were plated on 8-well chamber slides and allowed to attach overnight. After pretreatment with 0.1 μM of IMD-0354 for 24 h, the cells were stimulated by TNF-α (10 ng/ml, 30 min), fixed with 4% paraformaldehyde and stained with 1:200 rabbit anti-human NF-κB p65 (Cell Signaling) at room temperature for 1 h. After washing, samples were incubated with 1:50 Alexa Fluor 555-labeled goat anti-rabbit IgG (A-21429; Life Technologies, Carlsbad, CA) and finally stained with DAPI. The samples were observed using an FV1000-D Laser Scanning Confocal microscope (Olympus, Tokyo, Japan).

**In vitro adhesion assay.** Ovarian cancer cells (SKOV3ip1 or RMUG-S, 5 x 10⁴) were plated in a 96-well plate precoated with 50 μg/ml fibronectin or 50 μg/ml type 1 collagen. After incubation for 45 minutes at 37°C, cells were washed three times with PBS, fixed with methanol and stained with Giemsa solution. The number of adhesive cells was counted under a light microscope.

**Matrigel invasion assay.** *In vitro* cellular invasion assay was performed as described previously (23).
**Luciferase activity assay.** The pGL4-phVEGFA plasmid bearing human VEGF-A promoter sequence (position; 3853-5157 of human VEGF sequence (NG_008732)) into a firefly luciferase vector was obtained from RIKEN BioResource Center (Tsukuba, Japan). 5'-deletion mutant were made by deleting fragments using internal restriction sites for NheI (mut pGL4-phVEGFA, position; 5043-5157). 1 × 10^5 SKOV3ip1 cells were seeded in 12-well plates. After the replacement of culture media by Opti-MEM I Reduced Serum Media (Life Technologies), 1.0 µg of pGL4-phVEGFA and 0.1µg of pRL-TK renilla luciferase vector were co-transfected with Lipofectamine 2000 (Life Technologies). On the next day, various concentrations of IMD-0354 were treated and, 24 h later, luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Firefly luciferase values were normalized to renilla luciferase values.

**Enzyme linked immunosorbant assay (ELISA) of human VEGF-A.** 1 x 10^5 SKOV3ip1 cells were plated on 6-well plates. The cells were then starved in serum-free medium with various concentrations of IMD-0354 or the equivalent volume of DMSO for 24 hours. Thereafter, conditioned culture media were collected and stored at -80°C until analysis. Human VEGF-A Platinum ELISA kit (eBioscience, SanDiego, CA) was used to determine the concentration of VEGF-A.
**Wound healing assay.** HUVECs were cultured to subconfluence in 24-well culture plates and one wound of approximately 0.4 mm per well was made with a P-200 plastic tip. The cells were further cultured in serum-free conditions in the absence or presence of 5 ng/ml VEGF or concentrated SKOV3ip1 cells culture medium. Concentrated SKOV3ip1 cells culture medium was collected by culturing cells in serum-free DMEM with or without IMD-0354 in a subconfluent condition for 24 h and concentrated using Amicon Centrifugal Filter Units (Merck Millipore, Billerica, MA). 48 h after the incubation, the cells were fixed in 3.7% paraformaldehyde and stained with Giemsa solution. Migration was assessed by examining photographs of the cells that had migrated inside the wound area.

**Animal experiments.** Female athymic BALB/c nude mice (aged 4 to 5 weeks) were purchased from CLEA Japan Inc. (Tokyo, Japan) and were bred in aseptic conditions and kept at constant humidity and temperature (25 to 28°C). All animal experiments were approved by the Institutional Animal Care and Use Committee of Osaka University, in accordance with institutional and NIH guidelines. Ovarian cancer cells were suspended as single cells in a volume of 0.5 mL of PBS (SKOV3ip1; 1 × 10^6 cells, ES-2; 2 × 10^6 cells) and injected intraperitoneally into the mice. Mice were assessed daily for general health and development of ascites. After the inoculation (SKOV3ip1; 7
days, ES-2; 3 days), the mice were intraperitonally administered either 0.5 mL of 0.5% carboxymethylcellulose sodium salt (CMC-Na; Wako, Osaka, Japan) or IMD-0354 (30 mg/kg) every day for a total of 6 weeks to the SKOV3ip1 inoculated mice or 11 days to the ES-2 inoculated mice and finally sacrificed. The number of metastases and the volume of ascites in each mouse was counted, carefully dissected and the removed tumors weighed. Tumor tissues were immediately fixed and embedded in paraffin. The slides were prepared and incubated with p-IKKα/β antibody at 1:150, human VEGF antibody at 1:40, mouse CD-31 antibody, Ki-67 antibody, and cleaved caspase-3 antibody at 1:300 for 1 hour at room temperature. After washing with TBST, they were stained using N-Histofine Simple Stain MAX PO(R) (Nichirei Biosciences, Tokyo, Japan) and then counterstained with Carrazzi's hematoxylin.

**Statistical analysis.** Statcel version 3 (OMS-Publishing Inc., Saitama, Japan) and JMP version 10.0.2 (SAS Institute Japan Ltd., Tokyo, Japan) were used for statistical analyses. Data were expressed as means ± SEM. Differences were analyzed using the Mann-Whitney U test. Survival estimates were computed using the Kaplan-Meier method, and comparisons between groups were analyzed using the log-rank test. Multivariable analysis was performed using a Cox proportional-hazards regression model. Differences were considered statistically significant at $P < 0.05$. 
Results

Phosphorylation of IKK is an independent prognostic marker of ovarian cancer.

First, TMA slides from ovarian cancer patients were established. The characteristics of 94 patients are summarized in Supplemental Table 1. Phospho-IKK expression was evaluated by immunohistochemistry and each sample was scored based on the intensity of staining. Typically, clear cytoplasmic staining was seen in the cases of positive p-IKK expression (Fig. 1A). Of 94 patients, 58 (61.7%) showed strong p-IKK expression, 23 (24.4%) weak, and 13 (13.8%) cases were negatively expressed. In total, 81 (86.2%) cases showed positive p-IKK expression. Among patients with ovarian cancer, those who had positive p-IKK staining showed significantly worse PFS than those who had negative expression (PFS, 26.1 vs. 49.8 months, \(P=0.011\), Fig. 1B). Positive p-IKK staining also showed a trend toward worse OS compared with those with negative expression (OS, 34.0 vs. 50.9 months, \(P=0.073\), Fig. 1C). For a multivariate analysis, a backwards elimination approach was used to select a model for survival with multiple predictors. Age, FIGO stage, histological type, residual tumor at the time of surgery, and positive p-IKK expression were entered in this model. The final model included positive p-IKK expression as a significant independent predictor for
reduced PFS in ovarian cancer patients ($P=0.019$, $HR$: 5.85 (1.26-104.26), Supplemental Table 2).

**High VEGF expression is an independent prognostic marker of ovarian cancer patients and positively correlates with IKK phosphorylation.** Second, VEGF expression was evaluated by immunohistochemistry using anti-VEGF-A antibody. VEGF staining was scored based on the intensity of the staining and the percentage of positive cells. Representative pictures from four different histologies are shown in Fig. 2A. Forty-seven (50.0%) cases were defined as “high” VEGF expression. Patients with high VEGF expression had a significantly worse PFS than those with low VEGF expression (26.4 vs. 32.4 months, $P=0.014$, Fig. 2B). High VEGF expression also showed a trend toward worse OS compared with those with low VEGF expression (35.6 vs. 37.0 months, $P=0.086$, Fig. 2C). Forty-four of 81 cases (54.3%) in which IKKα/β was phosphorylated showed high VEGF expression, whereas only 3 (23.1%) of 13 cases in which p-IKKα/β expression was negative showed high VEGF expression. The correlation of IKK phosphorylation and VEGF expression was examined with the $\chi^2$ test, revealing that these expressions were significantly positively correlated (Fig. 2D, $P=0.037$).

**Constitutive activation of NF-κB in ovarian cancer cells was inhibited by IMD-**
Since IKK phosphorylation was correlated with VEGF expression in clinical samples and its expression was associated with poor prognosis, we were encouraged to analyze the effect of an IKKβ inhibitor on ovarian cancer cells. IMD-0354 [N-(3,5-bis-trifluoromethyl-phenyl)-5-chloro-2-hydroxy-benzamide], with MW 383.7 (Figure 3A), a specific IKK-β inhibitor selectively blocking IκBα phosphorylation (IC₅₀; ≤ 250 nM) (24) was used for the experiments. Although IMD-0354 at up to 1 μM did not affect the viabilities of SKOV3ip1 or RMUG-S ovarian cancer cells (Fig. 3B), it inhibited the constitutive phosphorylation of IKK (Fig. 3C). In order to determine the effect of IMD-0354 on NF-κB activation, the localization of NF-κB p65 was examined by immunofluorescence analyses. SKOV3ip1 cells were immunostained with an anti-p65 antibody and cell nuclei were identified by co-staining with DAPI. NF-κB p65 was dominantly located in the cytoplasm in the control and upon TNF-α stimulation, p65 drastically translocated into nuclei. The pretreatment of 0.1 μM IMD-0354 almost inhibited its nuclear translocation (Fig. 3D).

**IMD-0354 inhibits adhesion and invasion of ovarian cancer cells.** Next, we evaluated the effects of IMD-0354 on cell adhesion and invasion. IMD-0354 significantly inhibited the adhesion onto fibronectin (0.3 μM: SKOV3ip1, 51%; RMUG-S, 55%, respectively) as well as onto collagen type 1 (0.3 μM: SKOV3ip1, 62%; RMUG-S,
58%, respectively) in a dose dependent manner (Fig. 4A). In order to analyze how IMD-0354 inhibited cell adhesion, the expressions of various integrins were examined by Western blot (Fig. 4C). β1-integrin expression was inhibited by IMD-0354 treatment in each ovarian cancer cell line tested (SKOV3ip1, RMUG-S, and CaOV3), while the expressions of the other integrins tested (α1, α2, α5 and β3-integrin) were not altered.

Cell invasion follows adhesion. In vitro invasion assay revealed that IMD-0354 significantly impaired the invasion of cancer cells (0.3 μM: SKOV3ipi, 27%; RMUG-S, 33%, respectively) (Fig. 4D). The expression of matrix metalloproteinase 2 (MMP-2), known as an important molecule in ovarian cancer invasion, was inhibited by the treatment with IMD-0354 (Fig. 4E).

**IMD-0354 inhibits VEGF production from ovarian cancer cells, leading to the inhibition of angiogenesis.** Next, we examined the effect of IMD-0354 on VEGF production from cancer cells. Luciferase assay revealed that VEGF-A transcriptional activity was significantly inhibited by IMD-0354 in a dose dependent manner (Fig. 5A). Several possible NF-κB-like binding sites within the VEGF 5′-promoter region were previously reported (12) and pGL4-phVEGFA plasmid has two possible binding sites (4332-4341, 4992-4931) (Fig. 5B). Thus, 5′-deletion mutant (mut pGL4-phVEGFA) was made by deleting fragments containing these sites and a luciferase assay was
performed (Fig. 5C). Luciferase activity was drastically abolished and IMD-0354 did not show any inhibitory effects, indicating that NF-κB directly regulates VEGF promoter activity. Accordingly, IMD-0354 treatment of SKOV3ip1 cells resulted in a decrease in the levels of VEGF-A protein from 1.61 ng/ml to 0.97 ng/ml as evidenced by quantitative ELISA (Fig. 5D). To clarify the anti-angiogenic effect of IMD-0354 in vitro, the migratory activity of HUVECs was examined by a wound healing assay. Representative images are shown in Fig. 5E. VEGF-A 5 ng/ml was used as a positive control. The direct treatment of IMD-0354 to HUVECs did not affect the migration. While SKOV3ip1 culture media induced HUVECs migration and almost restored the scratched wound after 48 h of incubation, the pre-treatment of IMD-0354 to SKOV3ip1 cells inhibited HUVECs migration (Fig. 5F). The co-treatment of VEGF-A almost abolished the inhibitory effect of IMD-0354, indicating that IMD-0354 inhibited the migratory activity of HUVECs by inhibiting VEGF production from SKOV3ip1 cells.

**IMD-0354 inhibits peritoneal metastasis in an ovarian cancer xenograft model by inhibiting VEGF production from cancer cells.** Finally, we examined the therapeutic potential of IMD-0354 in an ovarian cancer xenograft model. SKOV-3ip1 (1 x 10⁶ cells) were injected intraperitoneally into female BALB/c nu/nu mice. One week after the inoculation, mice showed multiple tumor dissemination on the peritoneal surface, the
omentum, the surface of the liver and the small bowel mesentery. As preliminary experiments, dose finding study (7.5 mg, 15 mg or 30 mg/kg) was performed and 30 mg/kg showed the maximum effect without apparent adverse events on mice (Supplemental Fig. 1). Six weeks after treatment, both the tumor weight and the number of peritoneal implants were significantly inhibited in mice treated with IMD-0354 compared with controls (tumor weight: IMD-0354; 176.2 ± 136.9 vs. control; 518.6 ± 338.4 mg, number of peritoneal implants: 1.9 ± 2.7 vs. 26.1 ± 17.2, respectively, \( P < 0.001 \)) (Fig. 6A and 6B). Similarly, in another ovarian cancer xenograft model with ES-2, IMD-0354 significantly exerted anti-tumor effects on tumor weight, number of peritoneal metastases, and ascites formation compared with the control (tumor weight: IMD-0354; 227.6 ± 109.8 vs. control; 576.5 ± 180.6 mg, \( P < 0.001 \), number of peritoneal implants: 15.4 ± 6.7 vs. 31.8 ± 10.1, \( P < 0.001 \), ascites: 1.06 ± 0.72 vs. 2.84 ± 1.28 g, \( P < 0.01 \), respectively,) (Fig. 6C, 6D, and 6E). In order to analyze how IMD-0354 worked in vivo, immunohistochemical analyses from inoculated tumors were performed (Fig. 6F). Not only the phosphorylation of IKK but the expression of human VEGF was evidently inhibited in tumors treated with IMD-0354 compared with controls. Accordingly, the number of intratumoral vessels as presented by anti-mouse CD31 staining was significantly inhibited by the treatment with IMD-0354 (27.2 ± 5.7 vs. 96.0
± 36.1 /× 200 field, respectively, \( P < 0.01 \) (Fig. 6G). In contrast, the staining of peritoneal tumors with the proliferation marker Ki-67 and the apoptotic marker cleaved caspase-3 did not show significant difference between IMD-0354 treated mice and controls (Fig. 6H and 6I), suggesting that IMD-0354 inhibited ovarian cancer progression by inhibiting angiogenesis through the suppression of VEGF production from cancer cells.

**Discussion**

In this study, we revealed that, in more than 80 % of ovarian cancer cases, IKK is phosphorylated and its phosphorylation is an independent prognosis factor for patients and positively correlates with high VEGF expression. Furthermore, using a new IKKβ inhibitor, IMD-0354, we showed that this drug not only suppressed the adhesion and invasion of ovarian cancer cells but also transcriptionally inhibited VEGF production from cancer cells, which led to attenuation of the migration of endothelial cells induced by cancer cells. In a xenograft model, the treatment with IMD-0354 significantly inhibited peritoneal dissemination with a marked reduction of blood vessel formation. Thus, we suggest that an IKKβ inhibitor such as IMD-0354 can be considered to be an anti-angiogenic agent and a potential treatment for ovarian cancer, given that VEGF is
one of the most potent and specific angiogenic factors of tumor-induced angiogenesis.

Since it is clear that angiogenesis is an indispensable contributor to ovarian carcinogenesis and progression, we believe that research and development into new anti-angiogenic compounds should continue, to maximize the benefits and minimize the side effects as well as the costs. Following the success of bevacizumab, new molecules active against angiogenesis have been developed and several studies have been conducted or are underway. Some small molecule inhibitors of multitargeted tyrosine kinase (TKI) including VEGFR have been shown to be active in ovarian cancer (9). For example, Pazopanib is an oral angiogenesis inhibitor that inhibits VEGF receptor, platelet-derived growth factor receptor and c-Kit. In a Phase II study evaluating pazopanib in patients with recurrent ovarian cancer, the overall response rate was 18% and the median response duration was 113 days (25, 26). A recent Phase III clinical trial (AGO-OVAR16) showed that pazopanib extended PFS by an average of 5.6 months (17.9 months vs. 12.3 months), compared to a placebo, in women with advanced ovarian cancer who underwent initial successful treatment, while the interim analysis showed no difference in OS between the groups (27). Other TKIs such as sunitinib or sorafenib have shown limited results (28, 29). Angiopoietins are circulating protein growth factors that promote angiogenesis by interacting with Tie2
receptors. The anti-angiopoietin 1/2 peptibody, AMG386, given in combination with weekly paclitaxel demonstrated prolongation of PFS in a randomized Phase II trial with recurrent ovarian cancer patients (30). Although several candidates are under evaluation, none of the molecules inhibiting NF-κB pathways have been used as an angiogenesis inhibitor in the clinical setting so far. Herein, we suggested the potential of an IKK inhibitor, IMD-0354, for ovarian cancer treatment.

Angiogenesis in cancer has been shown to be associated with the chemokines (like monocyte chemoattractant protein, IL-8) or the growth factors (like TNFα). NF-κB activation is well known to play a role in the regulation of these angiogenesis inducing products (31). Although VEGF is a potent angiogenic factor, the mere inhibition of VEGF with bevacizumab has failed to demonstrate substantial prolongation of OS in ovarian cancer patients, indicating that there are several alternative pathways of angiogenesis other than the VEGF pathway. Thus, targeting NF-κB has the potential to inhibit various angiogenesis pathways and is likely to be promising as an anti-angiogenic therapy. While small molecule compound inhibitors of NF-κB have been proposed as a promising therapy for cancers with aberrant NF-κB activity, most classical NF-κB inhibitors are poorly selective and are known to have off-target effects and so few of them have progressed to Phase III clinical trials (32). Because
proteasome-mediated degradation of IκB is a required step in NF-κB signaling, the proteasome inhibitor, bortezomib, has been proposed as a general inhibitor of NF-κB; it is the only one clinically available and approved as the first line treatment for advanced multiple myeloma (33). However, there remains a concern that proteasome inhibition may affect other signaling pathways. IMD-0354 is a synthesized low-molecular weight compound that specifically inhibits IKKβ, inducing the inhibition of NF-κB activation only in inflammatory conditions, and it was proven that this drug does not inhibit other kinases, proteases or proteasome-related immune responses (34). No IKKβ inhibitor is known to be in the process of clinical application at present, except for IMD-1041, a prodrug of IMD-0354; a Phase 1 study with the IMD-0354 capsule confirmed sufficient safety (20). Since it appears obvious that the mere inhibition of NF-κB would be insufficient for a pronounced response unless combined with apoptosis-inducing drugs for ovarian cancer treatment, NF-κB inhibitors should be used as adjuvants along with cytotoxic chemotherapies in the clinical setting. For these reasons, we assume that IMD-0354 would be an ideal candidate because it is feasible for combination with current chemotherapies. Several previous studies have reported the effect of IMD-0354 on adult T-cell leukemia cells (20), breast cancer cells (35) or chronic lymphocytic leukemia cells (36). In these studies, at higher concentrations (1-50 μM), IMD-0354...
induced apoptosis of cancer cells. Indeed, in our experience, 10 μM of IMD-0354 comprehensively induced apoptosis of ovarian cancer cells tested by G0/G1-phase cell cycle arrest (data not shown). However, given that the IC$_{50}$ value for IKKβ of IMD-0354 is $\leq$ 250 nM, these apoptotic effects appear to be caused by a non-specific toxicity of the drug. Further elucidation would be required to identify how IMD-0354 works on cancer cells.

In immunohistochemical analyses, 81 of 94 (86%) ovarian cancer samples showed positive phosphorylated IKK expression. This is similar to previous findings (15, 16, 17). Although some in vitro studies using ovarian cancer cell lines suggested that NF-κB regulates VEGF expression (37), to our knowledge, none has reported the direct correlation of NF-κB activation and VEGF expression in clinical samples. In the present study, we reported that ovarian cancer tissues with positive p-IKK staining significantly expressed high levels of VEGF, proposing a clear rationale that targeting IKK phosphorylation can be considered an anti-angiogenic therapy for ovarian cancer treatment.

In conclusion, IKK phosphorylation is an independent prognostic factor and is associated with VEGF expression in cancer cancer tissues. IMD-0354 not only inhibited ovarian cancer invasion but suppressed VEGF production from cancer cells in
vitro and in vivo. In light of multiple publications highlighting the importance of angiogenesis in ovarian tumor biology and the results described here, we are supportive of clinical trials to study whether antagonizing IKKβ phosphorylation is a viable treatment strategy for ovarian cancer.

Acknowledgments

We thank Yuko Nishimura for her secretarial assistance, and Ayako Okamura for her technical assistance.

References


27. Bois AD, Floquet A, Kim JW, Rau J, Del Campo JM, Friedlander M, et al. Randomized, double-blind, phase III trial of pazopanib versus placebo in women who have not progressed after first-line chemotherapy for advanced epithelial ovarian,
fallopian tube, or primary peritoneal cancer (AEOC): Results of an international Intergroup trial (AGO-OVAR16).: Gynecologic Cancer 2013 ASCO Annual Meeting. J Clin Oncol 31, 2013 (suppl; abstr LBA5503)


Figure Legends

Figure 1. Phospho-IKK expression correlates with poor prognosis in patients with ovarian cancer. Immunohistochemical staining of tissue microarrays with malignant ovarian tissue sections (A). Representative areas of four different ovarian cancers stained using an anti-human phospho-IKK antibody and scored as 0, 1 and 2. No positive signal was observed by nonimmune area. Original magnification, ×200. Bar represents 100 μm. Kaplan-Meier curves of progression free survival (B) and overall survival (C) of ovarian cancer patients treated at Gifu University Hospital (n=94).

Figure 2. VEGF expression correlates with poor prognosis in patients with ovarian cancer. Immunohistochemical staining of tissue microarrays with malignant ovarian tissue sections (A). Representative areas of four different ovarian cancers stained using an anti-human VEGF-A antibody and scored as 1, 2 and 3. No positive signal was observed by nonimmune area. Original magnification, ×200. Bar represents 100 μm. Kaplan-Meier curves of progression free survival (B) and overall survival (C) of ovarian cancer patients (n=94). High VEGF expression was significantly more frequent in tumors that showed phosphorylated IKK expression than in those that had negative phosphorylated IKK staining (D). Statistical differences were examined by χ² test
Figure 3. IMD-0354 inhibits NF-κB activation of ovarian cancer cells. Molecular formula of IMD-0354 (A). Culturing of ovarian cancer cells (left; SKOV3ip1, right; RMUG-S) with up to 1 μM IMD-0354 had no effect on their viability (B). Data represent mean ± SEM, n=5 from triplicate independent experiments. Western blot (C). SKOV3ip1 and RMUG-S cells were incubated with IMD-0354 for 24 hours. Cell lysates were immunoblotted with anti–phosphorylated IKKα/β antibody (upper). The membranes were stripped and rehybridized with antibodies detecting IκBα (middle) or β-actin (lower). IMD-0354 inhibits NF-κB nuclear translocation induced by TNF-α (D). Representative confocal images of SKOV3ip1 cells stimulated by TNF-α (+) (10 ng/ml, 30 min) with or without pretreatment of 0.1 μM IMD-0354 are shown. Cells were immunostained with Alexa Fluor 555 labeled NF-κB p65 (red). In controls, NF-κB was dominantly located in the cytoplasm (upper). TNF-α stimulation (middle) induced the nuclear translocation of NF-κB p65. IMD-0354 inhibited its nuclear translocation (lower). Bar represents 50 μm.

Figure 4. IMD-0354 causes significant inhibition of adhesion and invasion of ovarian cancer cell lines. In vitro adhesion assay (A). 5 x 10^4 ovarian cancer cells (left; SKOV3ip1, right; RMUG-S) were plated onto 50 μg/mL fibronectin- or collagen type 1-
coated 96-well plates. After incubation for 45 minutes at 37°C, plates were washed to discard non-adherent cells and the number of adherent cells was counted under a light microscope. Data represent mean ± SEM, n=5 from triplicate independent experiments. Representative image of in vitro adhesion assay of SKOV3ip1 cells is shown (B). Bar represents 200 μm. Western blot (C). SKOV3ip1, RMUG-S, and CaOV3 cells were incubated with 0.3 μM of IMD-0354 or control for 24 hours. Cell lysates were immunoblotted with an antibody against integrin α1, α2, α5, β1 and β3. β-actin was used as a loading control. Each sample was run in triplicate. Numbers show the ratio between integrins indicated and β-actin expression. In vitro invasion assay (D). 5 x 10⁴ SKOV3ip1 (left) or RMUS-S (right) cells were placed on the top chamber in serum-free medium with IMD-0354, and allowed to invade for 24 hours. Non-invading cells were removed using a cotton swab, and invading cells on the underside of the filter were enumerated. Representative images are shown. Bar represents 100 μm. Data represent mean ± SEM, n=5 from triplicate independent experiments. Western blot (E). Cell lysates were immunoblotted with an antibody against MMP-2. β-actin was used as a loading control. Each sample was run in triplicate. Numbers show the ratio between MMP2 and β-actin expression. **; P < 0.01.

**Figure 5.** IMD-0354 inhibits VEGF production from ovarian cancer cells, which leads to
the inhibition of HUVECs migration induced by cancer cells. Effect of IMD-0354 on VEGF-A transcription activation (A). pGL4-hVEGFA firefly luciferase vector and renilla luciferase reporter, pRL-TK, were co-transfected into SKOV3ip1 cells. VEGF-A transcriptional activity was reduced in a dose dependent manner by the treatment with IMD-0354. Data represent mean ± SEM, n=5 from triplicate independent experiments. The schema of original and mutant pGL4-phVEGFA vector (mut pGL4-phVEGFA) (B). Possible NF-κB binding sites of VEGFA promoter are shown. Luciferase activity was drastically abolished with mut pGL4-phVEGFA and IMD-0354 did not show any inhibitory effects (C). Data represent mean ± SEM, n=4 from triplicate independent experiments. ELISA assay of VEGF-A (D). 1 x 10^5 SKOV3ip1 cells were cultured with 2 ml of 0.1% BSA/DMEM with or without IMD-0354 for 24 hours. Conditioned media were collected and the concentration of human VEGF-A was measured. Data represent mean ± SEM, n=4 from triplicate independent experiments. Effect of IMD-0354 on HUVECs migration examined by a wound healing assay (E). Endothelial cell monolayers were wounded at time 0, and cultures were incubated with serum free media (upper) or conditioned media from SKOV3ip1 cells (lower). 48 hours later, cells were fixed and representative pictures were taken. 5 ng/ml of human VEGF-A was used as a positive control. Bar represents 200 μm. Percentage wound recovery was
measured and compared with that at time 0 (F). Experiments were repeated 7 times and values are means ± SEM, n.s.; not significant, *; P < 0.05, **; P < 0.01, ***; P < 0.001.

**Figure 6.** Treatment of IMD-0354 inhibits peritoneal dissemination of ovarian cancer cells through the inhibition of VEGF production from cancer cells. Ovarian cancer cells (SKOV3ip1; 1 × 10^6 cells, ES-2; 2 × 10^6 cells) were injected i.p into female BALB/c nu/nu mice. After the injection (SKOV3ip1; 7 days, ES-2; 3 days), IMD-0354 (30mg/kg body weight) or an equal amount of 0.5% CMC-Na (control) was injected intraperitoneally daily for a total of 6 weeks to the SKOV3ip1 inoculated mice or for 11 days to the ES-2 inoculated mice. Effect of IMD-0354 on i.p. tumor weight (A), number of metastases (B) in SKOV3ip1 inoculated mice. Effect of IMD-0354 on i.p. tumor weight (C), number of metastases (D), and ascites formation (E) in ES-2 inoculated mice. Results were expressed as mean ± SEM, each n=18, respectively. Representative SKOV3ip1 tumor areas were stained with H&E, p-IKKα/β, human VEGF, the angiogenesis marker anti-mouse CD31, the proliferation marker Ki-67, and the apoptotic marker cleaved caspase-3 (F). Bar represents 50 μm. Number of microvessels per field by CD31 staining (G), the percentage of Ki-67 positive nuclei (H), and the percentage of cleaved caspase-3 positive cells (I). Results were
expressed as mean ± SEM, n=5, each. n.s.; not significant, **; \( P < 0.01 \), ***; \( P < 0.01 \).
Kinose Y, et al. Figure 1

A

Serous papillary adenocarcinoma

Clear cell carcinoma

Endometrioid adenocarcinoma

Mucinous adenocarcinoma

Negative control

B

Progression free survival

Probability of survival

Time (Mo)

0 10 20 30 40 50 60 70

P=0.011

p-IKK negative

p-IKK positive

C

Overall survival

Probability of survival

Time (Mo)

0 20 40 60 80 100 120 140

P=0.073

p-IKK negative

p-IKK positive
Kinose Y, et al. Figure 2

A

Serous papillary adenocarcinoma

B

Progression free survival

C

Overall survival

D

VEGF low  VEGF high

p-IKK negative  p-IKK positive

P=0.014

P=0.086

P=0.037

on June 21, 2017. © 2015 American Association for Cancer Research. mct.aacrjournals.org Downloaded from
A Molecular formula: C_{15}H_{8}ClF_{6}NO_{2}
Molecular weight: 383.67

B

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D

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TNFα (+)
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**Kinose Y, et al. Figure 4**

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**β-actin**

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Kinose Y, et al. Figure 5

**A**

![Graph showing relative luciferase activity vs. IMD-0354 concentration](image)

- **n.s.** indicates non-significant differences

**B**

![Diagram illustrating the effect of IMD-0354 on VEGF-A expression](image)

**C**

![Graph showing relative luciferase activity vs. IMD-0354 concentration](image)

- **n.s.** indicates non-significant differences

**D**

![Bar graph showing VEGF-A levels](image)

- **n.s.** indicates non-significant differences

**E**

![Images showing wound recovery rate](image)

- **n.s.** indicates non-significant differences

**F**

![Graph showing wound recovery rate vs. VEGF-A and IMD-0354](image)

- **n.s.** indicates non-significant differences

- **** indicates significant differences

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Kinase Y, et al. Figure 6

A. Tumor weight

B. Number of metastases

C. Tumor weight

D. Number of metastases

E. Ascites

F. Immunohistochemistry images:
   - H&E
   - p-IKKα/β
   - human VEGF-A
   - mouse CD31
   - Ki-67
   - Cleaved Caspase-3

G. Bar graph of number of vessels/200x field

H. Bar graph of Ki-67 positive cells (%)

I. Bar graph of apoptotic cells (%)

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Molecular Cancer Therapeutics

IKKβ regulates VEGF expression and is a potential therapeutic target for ovarian cancer as an anti-angiogenic treatment


Mol Cancer Ther Published OnlineFirst January 30, 2015.

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