INTRODUCTION

Ovarian cancer is one of the lethal gynecologic malignancies and this is due in large part to the resistance of recurrent ovarian cancer cells to standard chemotherapeutic strategies (1). Resistance to apoptotic cell death is a fundamental characteristic of cancer cells, and a primary cause of treatment failure. Of the different chemotherapeutic agents in use for treating cancer, platinum-based chemotherapy is often used to treat recurrent ovarian cancers, but many of the ovarian cancer cells are resistant to the platinum-based agents (2). This resistance to chemotherapy results in recurrence and ultimately the loss of life. Therefore, there is an urgent need to improve the effectiveness of platinum-based chemotherapy.

Fatty acid synthase (FASN), the enzyme responsible for de novo synthesis of fatty acids, has been shown to be deregulated in several cancers, including epithelial ovarian carcinoma (EOC). In this study, we investigated the function of the FASN signaling pathway in a large series of Middle Eastern EOC patient samples, a panel of cell lines and nude mouse model. Using immunohistochemistry, we detected overexpression of FASN in 75.5% (114/151) of the tumor samples. Overexpression of FASN was associated significantly with tumor proliferative marker Ki-67 (P = 0.0009), activated AKT (P = 0.0117) and XIAP (P = 0.0046). Treatment of EOC cell lines with C-75, a selective inhibitor of FASN, caused inhibition of EOC cell viability via induction of apoptosis. Inhibition of FASN by C-75 led apoptosis via the mitochondrial pathway. FASN inhibition caused downregulation of activated AKT and its downstream targets. In addition, inhibition by FASN siRNA caused downregulation of FASN and activation of caspases, suggesting the role of FASN in C-75 mediated apoptosis. Furthermore, treatment of EOC cells with subtoxic doses of C-75 augmented the effect of cisplatin-mediated induction of apoptosis. Finally, treatment of EOC cell line xenografts with a combination of C-75 and cisplatin resulted in growth inhibition of tumors in nude mice through downregulation of FASN and activation of caspases. Altogether, our results show overexpression of FASN in Middle Eastern EOC, suggesting that FASN may be a potential therapeutic target in a subset of EOC, alone or in combination with other conventional chemotherapeutic agents.

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Overexpression of Fatty Acid Synthase in Middle Eastern Epithelial Ovarian Carcinoma Activates AKT and Its Inhibition Potentiates Cisplatin-Induced Apoptosis

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Fatty acid synthase (FASN), the enzyme responsible for de novo synthesis of fatty acids, has been shown to be deregulated in several cancers, including epithelial ovarian carcinoma (EOC). In this study, we investigated the function of the FASN signaling pathway in a large series of Middle Eastern EOC patient samples, a panel of cell lines and nude mouse model. Using immunohistochemistry, we detected overexpression of FASN in 75.5% (114/151) of the tumor samples. Overexpression of FASN was associated significantly with tumor proliferative marker Ki-67 (P = 0.0009), activated AKT (P = 0.0117) and XIAP (P = 0.0046). Treatment of EOC cell lines with C-75, a selective inhibitor of FASN, caused inhibition of EOC cell viability via induction of apoptosis. Inhibition of FASN by C-75 led apoptosis via the mitochondrial pathway. FASN inhibition caused downregulation of activated AKT and its downstream targets. In addition, inhibition by FASN siRNA caused downregulation of FASN and activation of caspases, suggesting the role of FASN in C-75 mediated apoptosis. Furthermore, treatment of EOC cells with subtoxic doses of C-75 augmented the effect of cisplatin-mediated induction of apoptosis. Finally, treatment of EOC cell line xenografts with a combination of C-75 and cisplatin resulted in growth inhibition of tumors in nude mice through downregulation of FASN and activation of caspases. Altogether, our results show overexpression of FASN in Middle Eastern EOC, suggesting that FASN may be a potential therapeutic target in a subset of EOC, alone or in combination with other conventional chemotherapeutic agents.

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INTRODUCTION

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Fatty acid synthase (FASN) is a multifunctional enzyme that catalyzes the terminal steps in the synthesis of the long-chain saturated fatty acid palmitate in normal cells (3). In normal cells, FASN expression levels are relatively low, since fatty acid is generally supplied by dietary fatty acid. In contrast, FASN is expressed at significantly higher levels in a variety of human epithelial cancers including breast, thyroid, colon, ovary, lung and prostate (4–9). Moreover, several reports have shown that FASN expression levels correlate with tumor progression, aggressiveness and metastasis (10–11). FASN appears to provide a selective proliferative advantage since its overexpression is shown to correlate with poor prognosis in breast and prostate cancers and is found to be elevated in the blood of cancer patients (9,12–13). Furthermore, inhibition of FASN activity is selectively cytotoxic to cancer cells in vitro and in vivo (14–16). Upregulation of FASN expression in cancer cells has been linked to phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathway (17–19). Activation of PI3-kinase pathway recruits a number of signaling proteins including AKT. During recruitment, AKT becomes phosphorylated/activated and exerts its...
antia apoptotic activity through phosphorylation of downstream targets such as Bad, FOXO and GSK3 (20–23). In addition, PI3K pathway has been shown to be capable of negatively regulating FASN-induced cell death (19).

In the current study, we investigated the expression of FASN and its correlation to other clinico-pathological parameters in a large cohort of Saudi EOC using tissue microarray (TMA) technology. We next examined the effect of C-75, a synthetic slow-binding inhibitor of FASN activity on a panel of EOC cell lines. In addition, we investigated whether subtoxic doses of C-75 can potentiate the anticancer effects of cisplatin in vitro and in vivo. Our findings strongly suggest that a tight functional association between FASN and AKT is taking place in a subset of EOC, and that FASN expression can be a useful biomarker. Furthermore, inhibition of FASN activity by C-75 induces apoptosis in EOC cells and combination treatment with C-75 and cisplatin augmented the apoptotic effects in vitro and in vivo, implicating therapeutic usefulness of FASN targeting in EOC.

MATERIALS AND METHODS

Patient Selection and Tissue Microarray Construction

One hundred fifty-six patients with ovarian carcinoma diagnosed between 1991–2007 were selected from the files of the King Faisal Specialist Hospital and Research Centre. All samples were analyzed in a tissue microarray (TMA) format. TMA construction was performed as described earlier (24). Two cores of ovarian carcinoma were arrayed from each patient. The Institutional Review Board of the King Faisal Specialist Hospital and Research Center approved the study. The King Faisal Specialist Hospital and Research Centre and the Department of Obstetrics and Gynecology, King Faisal Specialist Hospital and Research Center provided long-term follow-up data for these patients. The primary pathological diagnosis was serous in 225 patients (80.1%), endometrioid in 22 (14.1%), clear cell in 4 (2.6%) and undifferentiated/mixed epithelial in 5 (3.2%). The ages of the patients ranged from 19 to 86 years, with a median age of 56 years. The majority of patients underwent primary surgical staging or cytoreduction. In some patients who were not fit for primary surgery, primary neoadjuvant chemotherapy was followed by interval debulking surgery. The distribution by FIGO stage at diagnosis was: stage I–II in 22 patients (14.1%), stage III–IV in 137 (87.8%), and unknown in 11 (6.1%). The median follow-up time was 14.9 months (range, 2 to 130 months). Progression-free survival was computed from date of surgery for patients who underwent primary cytoreduction and from date of diagnosis by biopsy or cytology in those who underwent primary neoadjuvant chemotherapy. Since the majority of patients are lost to follow-up as their disease reaches its terminal stages, it was impossible to determine overall survival in this specific patient population.

Immunohistochemistry (IHC)

TMA slides were processed and stained manually. The streptavidin-biotin peroxidase technique with diaminobenzidine as chromogen was applied. For antigen retrieval, Dako Target Retrieval Solution pH 9.0 (Number S2368) was used, and the slides were boiled in a pressure cooker (Pascal Pressure Cooker, model: S2800, Dako Cytomation, Carpinteria, CA, USA). Primary antibodies used, their dilutions and antigen retrieval are listed in Supplementary Table 1. Endogenous peroxidase activity was quenched using 3% hydrogen peroxidase. Endogenous biotin was blocked and all slides were counterstained with hematoxylin, dehydrated, cleared, and cover slipped with premount. Only fresh cut slides were stained simultaneously to minimize the

| Table1. Clinico-pathological correlation of immunohistochemical expression of FASN in epithelial ovarian carcinomas. |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|
| Epithelial group                | Total          | High FAS-N     | Low FAS-N      | P value        |
|---------------------------------|----------------|----------------|----------------|----------------|
| Total number of cases           | 151            | 114            | 75.5           | 37             | 24.5           | 0.5703         |
| Age                             |                |                |                |                |                |
| ≤50 years                       | 59             | 39.1           | 46             | 78.0           | 13             | 22.0           | 0.9521         |
| >50 years                       | 92             | 60.9           | 68             | 73.9           | 24             | 26.1           |                |
| Tumor Stage                     |                |                |                |                |                |
| Stage I-II                      | 8              | 5.7            | 6              | 75.0           | 2              | 25.0           |                |
| Stage III-IV                    | 133            | 94.3           | 101            | 75.9           | 32             | 24.1           |                |
| Histopathology                  |                |                |                |                |                |
| Clear cell                      | 4              | 2.6            | 3              | 75.0           | 1              | 25.0           | 0.4235         |
| Endometrioid                    | 21             | 13.9           | 17             | 81.0           | 4              | 19.0           |                |
| Serous                          | 122            | 80.8           | 90             | 73.8           | 32             | 26.2           |                |
| Undifferentiated                | 4              | 2.6            | 4              | 100.0          | 0              | 0.0            |                |
| FIGO Grade                      |                |                |                |                |                |
| Well differentiated             | 26             | 17.2           | 18             | 69.2           | 8              | 30.8           | 0.6596         |
| Moderately differentiated       | 82             | 54.3           | 62             | 75.6           | 20             | 24.4           |                |
| Poorly differentiated           | 43             | 28.5           | 34             | 79.1           | 9              | 20.9           |                |
| Ki-67                           |                |                |                |                |                |
| Above 50                        | 54             | 36.7           | 49             | 90.7           | 5              | 9.3            | 0.0009         |
| Below = 50                      | 93             | 63.3           | 63             | 67.7           | 30             | 32.3           |                |
| PAKT (Ser473)                   |                |                |                |                |                |
| High (2-3)                      | 75             | 52.4           | 64             | 85.3           | 11             | 14.7           | 0.0117         |
| Low (0-1)                       | 68             | 47.6           | 46             | 67.7           | 22             | 32.3           |                |
| PFS-Median (months)             | 17.7           |                | 12.3           |                | 0.1784         |                |
influence of slide aging and maximize repeatability and reproducibility of the experiment. p-AKT scoring was done as described earlier (26). For purposes of statistical analysis, all cases staining at Level 0 or 1 were grouped as p-AKT negative and all cases staining at Level 2 and Level 3 were grouped as p-AKT positive. Two types of negative controls were used for p-AKT, one was the negative control in the kit in which the primary antibody was omitted and a preabsorption experiment using p-AKT Ser 473 blocking peptide (Cell Signaling Technology, Beverly, MA, USA, No. 1140) was used as the second negative control. Each TMA spot was assigned an intensity score from 0–3 (I0, I1–3) and proportions of the tumor staining for that intensity were recorded as 5% increments from a range of 0–100 (P0, P1–3). A final H score (range 0–300) was obtained by adding the sum of scores obtained for each intensity and proportion of area stained (H score = P1 + I2XP2 + I3XP3). Ovarian tumors were grouped into two groups using X-tile bioinformatics software: low FAS-N expression (H score < 0.05).

Reagents and Antibodies
C-75 was purchased from Calbiochem (San Diego, CA, USA) and cisplatin was purchased from Sigma. Antibodies against caspase 3, cleaved caspase 3, AKT, pAKT, FOXO1 and GSK3 antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA). FASN, cytochrome c, β-actin, and poly (ADP-ribose) polymerase (PARP) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). XIAP, cIAP1, and caspase 8 antibodies were purchased from R&D (Minneapolis MN, USA). Annexin V was purchased from Molecular Probes (Eugene, OR, USA). Apoptotic DNA laddering kit was obtained from Roche (Penzberg, Germany).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assays
One hundred four cells were incubated in triplicate in a 96-well plate in the presence or absence of indicated test doses of C-75, cerulein and C-75 in combination with cisplatin, a final volume of 0.20 mL for 48 h. The ability of C-75, cerulein and C-75 in combination with cisplatin to suppress cell growth was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assays, as described previously (28). Replicates of three wells for each dosage, including vehicle control, were analyzed for each experiment.

Statistics
All statistical analysis were performed using the Statview JMP software (version 7.0). The Fisher exact chi-square [χ2] test was used to assess associations between categorical variables. Kaplan-Meier survival analyses were carried out for progression free survival, using the log-rank test for differences between groups. Results were considered statistically significant when P from a two-tailed test was < 0.05.

Cell Culture
EOC cell lines MDAH2774 and SKOV3, OVCAR3 cells (purchased from American Type Culture Collection [ATCC, Manassas, VA, USA]), OVTOKO and OVISe (from Japanese Collection of Research Bioresources [Osaka, Japan]) were cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (ATCC), 100 units/mL penicillin, and 100 units/mL streptomycin (Sigma, St. Louis, MO, USA) at 37°C in humidiﬁed atmosphere containing 5% CO2. All experiments were performed in RPMI 1640 (ATCC) containing 5% serum.

DNA Laddering
A DNA laddering assay was performed as described earlier (28). Briefly, cells (2 × 106) were treated with cisplatin alone, C-75 alone and C-75 in combination with cisplatin for 48 h. The cells were then harvested and resuspended in 200 mL 1× PBS. Then, 200 mL lysis buffer containing 6 mol/L guanidine HCl, 10 mmol/L urea, 10 mmol/L Tris-HCl, and 20% Triton x (v/v; pH 4.4), were added to the cells and incubated for 10 min at room temperature. Isopropanol (100 mL) was added and shaken for 30 s on a vortex. Then, samples were passed through a filter and spun at 4,500g for 1 min, and the supernatant was discarded. The pellets were washed 3x with wash buffer containing 20 mmol/L NaCl, 2 mmol/L Tris-HCl, and 80% ethanol. The pellets then were transferred into a new 1.5-mL tube and eluted with 200 mL prewarmed elution buffer. After measuring the DNA, 2 mg DNA were electrophoresed on a 1.5% agarose gel containing ethidium bromide at 75V for 2h and visualized using an ultraviolet light source.

Gene Silencing Using siRNA
FASN siRNA and scrambled control siRNA were purchased from Qiagen (Valencia, CA, USA). Cells were transfected by Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) for 6 h after which the lipid and siRNA complex were removed and fresh medium was added. Cells were lysed 48 h after transfection and specific protein levels were determined by Western blot analysis with specific antibodies.

Cell Lysis and Immunoblotting
Cells were treated with FASN inhibitor C-75 as described in the legends and lysed as described previously (4). Proteins (15–20 mg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore, Billerica, MA, USA). Immunoblotting was done with different antibodies and visualized by the enhanced chemiluminescence (Amersham) method.
Measurement of Mitochondrial Membrane Potential

Cells were treated with C-75 for 48 h, washed twice with PBS, and suspended in mitochondrial incubation buffer. JC1 staining and flow cytometry was done as described previously (26).

Assays for Cytochrome c Release

EOC cells were treated with C-75 as described in figure legends and assayed for cytochrome c as described previously (26).

In Vivo Tumor Xenograft Studies

Six-week-old nude mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and maintained in a pathogen-free animal facility at least 1 wk before use. All animal studies were done in accordance with institutional guidelines. For xenograft study, mice were inoculated subcutaneously into the right abdominal quadrant with 5 million MDAH2774 cells in 200 mL PBS. After 1 wk, mice were assigned randomly into four groups receiving 3 mg/kg cisplatin, 10 mg/kg C-75, combination of cisplatin and C-75 or only 0.9% saline. The body weight and tumor volume of each mouse were monitored weekly. The tumor volume was measured as described previously (29). After 5 wks of treatment, mice were euthanized by cervical dislocation according to Animal Care and Use Committee (ACUC) guidelines (NIH Policy Manual: 3040-2 Animal Care and Use in the Intramural Program; http://oacu.od.nih.gov/ARAC/index.htm). Individual tumors were weighed, then snap frozen in liquid nitrogen for storage.

All supplementary materials are available online at www.molmed.org.

RESULTS

FASN Expression and its Correlation with Clinicopathological Parameters and p-AKT

Expression levels of FASN were examined by immunohistochemistry in a large cohort of 156 EOC patient samples. High levels of FASN expression were seen in 75.5% (114/151) of the epithelial ovarian carcinomas (Figure 1). Representative information for FASN expression was observed in 151 spots, and immunohistochemical analysis failure of the remaining five cases was due to missing spots or fixation artifacts. FASN overexpression was associated significantly with overexpression of p-AKT ($P = 0.0117$), XIAP ($P = 0.0046$) and proliferative marker Ki-67 ($P = 0.0009$) (Table 1). However, FASN overexpression was not associated with age, American Joint Cancer Committee (AJCC) stage, FIGO grade and histopathological subtype. FASN expression was also not associated with outcome ($P = 0.1784$). Ovarian cancer with high FASN had an overall survival of 17.7 months as compared with 12.3 months for EOC with low FASN.

Inhibition of FASN-Induced Loss of Cell Viability and Loss of Cell Proliferation and Apoptosis in EOC Cell Lines

We first sought to determine whether C-75, a selective FASN inhibitor, caused dose dependent inhibition of viability in EOC cells using MTT assay. Treatment of C-75 at increasing doses from 10 to 100 mmol/L caused significant loss of...
viability and was found to be statistically significant at most of the doses tested ($P < 0.05$, Student $t$ test) (Figure 2A). We further sought to determine whether the observed effect on C-75 growth inhibition was due to induction of cell cycle arrest or apoptosis. We, therefore, treated EOC cell lines with different doses of C-75 and determined cell cycle fractions by flow cytometry. As shown in (Figure 2B) the sub-G1 population of cells increased from 2.34% in the control to 54.66% at 50 mmol/L dosage in MDAH2774 cells. This increase in sub-G1 population was accompanied by loss of cells in G0/G1, S and G2/M phases, suggesting that the treated EOC cells were dying of apoptosis. Similar observation was made in like SKOV3, OVTOKO and OVISE cell lines.

C-75 treatment-induced apoptosis in EOC cells was confirmed further by annexin/PI dual staining assay (Figure 2C) suggesting that suppression of growth by C-75 in EOC cells was through apoptosis. To further confirm whether the cells were dying of apoptosis, DNA frag-
mentation status was confirmed by the DNA laddering assay (Figure 2D). Cerulenin, another FASN inhibitor, caused inhibition of proliferation via induction of apoptosis (Supplementary Figure 1).

**Constitutive Expression of FASN Associated with AKT Signaling Pathways in EOC Cell Lines**

Activation of AKT and overexpression of fatty acid synthase (FASN) frequently are observed in human ovarian cancer (19). To explore a possible connection between AKT and FASN, we have investigated the inhibition of FASN activity by C-75 on MDAH2774, SKOV3 and OVISE cells treated with 50 mmol/L of C-75 whose proteins were analyzed by Western blotting. All the cell lines expressed constitutive FASN expression and activated AKT, and C-75 treatment suppressed FASN and inactivated AKT in a dose-dependent manner (Figure 3A). Since FOXO1 transcription factors have been reported to be a downstream target of AKT and are known to promote transcription of genes involved in cell cycle arrest and apoptosis (30), we investigated the level of FOXO1 phosphorylation in C-75 treated and untreated EOC cells by Western blotting. Constitutive phosphorylation of FOXO1 was observed in all EOC cell lines and C-75 dephosphorylated it in a dose-dependent manner (Figure 3B). We next determined the activation of GSK3 in these cells, as it has been reported to be a downstream target of AKT involved in promoting cell survival (31). GSK3 dephosphorylation was seen more in EOC cells treated with C-75 (see Figure 3B). These observations suggest that FASN inhibition suppresses AKT activation and its downstream targets activated FOXO1 and GSK3 thereby inducing apoptosis. We sought to determine, by FASN-specific siRNA-targeting FASN, inhibited expression of FASN, dephosphorylation of AKT and induced apoptosis by cleavage of caspase 3 (Figure 3C). We next sought to determine whether FASN downregulation by FASN-specific siRNA caused inactivation of AKT. Inhibition of FASN by siRNA transfection downregulated FASN and dephosphorylated AKT and induced apoptosis by cleavage of caspase 3.

**C-75 Induced Apoptosis Through AKT Signaling via the Mitochondrial Pathway and Activation of Caspases**

Inactivation of AKT has been shown to induce apoptosis via the mitochondrial apoptotic pathway. We therefore sought to determine whether the observed apoptotic effect on EOC cells of C-75 involved the mitochondrial pathway. First, we tested the effect of C-75 therapy on the mitochondrial membrane potential in EOC cells. We treated EOC cells with 50 mmol/L of C-75 for 48 h, labeled with JC-1 dye and measured mitochondrial membrane potential by flow cytometry. Inhibition of FASN resulted in loss of mitochondrial membrane potential as measured by JC-1 red fluorescence depicting apoptotic cells (Figure 4A). The number of apoptotic cells increased in a dose-dependent manner in cells undergoing C-75 treatment. Next, we examined release of cytochrome c from the mitochondria. For this, mitochondria-free cytosolic lysates and mitochondrial extracts were
Figure 4. C-75-induced mitochondrial apoptotic pathway in EOC cells. (A) Loss of mitochondrial membrane potential by C-75 treatment in MDAH2774 and SKOV3 cells incubated with 25 and 50 mmol/L of C-75 for 48 h. Live cells with intact mitochondrial membrane potential (green bar) and dead cells with lost mitochondrial potential (red bar) were measured by JC1 staining and analyzed by flow cytometry as described in Materials and Methods. (B) C-75-induced release of cytochrome c. MDAH2774 and SKOV3 cells incubated with 25 and 50 mmol/L of C-75 for 48 h. Mitochondrial and cytoplasmic fractions were isolated and cell extracts were immunoblotted with antibodies against cytochrome c and β-actin. (C) Downregulation by FASN by C-75 inhibition causes downregulation of IAP1s, in MDAH2774, SKOV3 and OVISE cells incubated with 25 and 50 mmol/L of C-75 for 48 h. Cells were lysed and immunoblotted with antibodies against cIAP1, cIAP2, XIAP and survivin as described in Materials and Methods. (D) Activation of caspases induced by C-75 treatment in EOC cells. Cells incubated with 25 and 50 mmol/L of C-75 for 48 h. Cells were lysed and immunoblotted with antibodies against caspase 9, caspase 3 and cleaved caspase 3 and PARP as described in Materials and Methods. (E) MDAH2774 and SKOV3 cells were pretreated with 80 mmol/L of z-VAD-fmk and without for 2 h and subsequently with 25 and 50 mmol/L C-75 for 48 h. Cells were lysed and immunoblotted with antibodies against caspase 9, cleaved caspase 3 and PARP as described in Materials and Methods. (F) Effect of z-VAD/fmk on C-75-induced apoptosis in EOC cells. MDAH2774 and SKOV3 cells were pretreated with 80 mmol/L of z-VAD/fmk and without for 2 h and subsequently with 25 and 50 mmol/L C-75 for 48 h. Apoptosis was measured by annexin V/PI staining; z-VAD-fmk abrogates C-75-induced activation of caspase 3 in EOC cells.
XIAP expression was found to be associ-

ation (Figure 4B). mitochondria
cytoplasmic fractions after C-75 treat-
maintained and concurrently, there was a decrease in
cytoplasmic fractions after C-75 treatment (Figure 4B).

Additionally, in our clinical samples, XIAP expression was found to be associ-
ated with FASN expression (P = 0.0046) (Table 1). We therefore examined
cell response to apoptotic stimuli. EOC
treated with C7-5 for 48 h and the expression of XIAP, CIAP1 and
CIAP2 and survivin was determined using Western blotting. C-75 caused
downregulation of XIAP, CIAP1, CIAP2 in a dose-dependent manner (Figure 4C).

Inhibitors of the apoptotic proteins have been shown to affect the caspases di-
rectly (32). Cytochrome c release has been shown to cause activation of cas-
pases and cleavage of PARP. C-75 treat-
ment resulted in activation of caspase 9, caspase 3 and cleavage of caspase 3 and
PARP in MDAH2774, SKOV3 and OVISE cells (Figure 4D). These results are con-
istent with the data on cytochrome c release, and indicate that the activation of effector caspases are involved in C-75-
duced apoptosis in EOC cells. In addi-
tion, pretreatment of MDAH2774 and
SKOV3 cells with 80 mmol/L of z-VAD-
fmk, a universal inhibitor of caspases,
abrogated apoptosis and prevented apo-
potosis by C-75-induced apoptosis in
EOC cells (Figure 4E). This was further confirmed by annexin/PI staining (Figure 4F).

Inhibition of FASN Augments
Antiproliferative Effects of Cisplatin in
EOC Cells

The standard chemotherapy for EOC
patients currently is a combination of
taxane and platinum cisplatin (CDDP).
CDDP is a well-known anticancer agent that also is active against many types of
cancer (2). However cisplatin toxicity is a
major concern in treatment of EOC. Therefore, we sought to determine
whether C-75 augmented the antiprolif-
erative effect of cisplatin and the induc-
tion of apoptosis in EOC cells. A panel of
EOC cells, MDAH2774 and SKOV3, were
treated with subtoxic doses of C-75 in combination with subtoxic doses of cis-
platin for 48 h, and cell viability was as-
sayed using MTT assay (Figure 5A).

Combination treatment of 25 mmol/L C-
75 and 10 mmol/L cisplatin induced
growth inhibition, which was found to be statistically significant (P < 0.05) (Stu-
dent t test) in all cell lines. We further sought to determine if the observed
growth inhibition by MTT assay was due to induction of cell cycle arrest and ap-
optosis. We treated EOC cells with 25
mmol/L C-75 and 10 mmol/L cisplatin for 48 h, and cell cycle fractions were de-
termined by flow cytometry. The sub-G1
population of cells increased from 2.81% in the control to 3.69% with cisplatin alone and 36.97% with C-75 alone, how-
ever, combination treatment increased it
to 48.65% in MDAH2774 cells (Figure 5B.) This increase in sub-G1 population was accompanied by a loss of cells in
G0/G1, S and G2/M phases, suggesting that the treated EOC cells were dying of
apoptosis. Similar observation was also made in SKOV3 cells.

Combination treatment-induced apo-
potosis in EOC cells was further confirmed by annexin/PI dual staining assay (Fig-
ure 5C), suggesting that suppression of
growth by combination treatment in EOC cells is through apoptosis. To investigate
whether inhibition of FASN activity by the subtoxic doses of C-75 in combination with cisplatin could be via inactivation of
AKT pathway, MDAH2774, SKOV3 and
OVISE cells were incubated with subtoxic
doses of C-75 in combination with cis-
platin for 48 h, cells were lysed and pro-
teins were analyzed for Western blotting. As shown in Figure 5D, suppression of
FASN expression and dephosphorylation of
AKT was more effective when EOC
cells were treated with a combination of subtoxic doses of C-75 and cisplatin rather than when treated alone, thereby potentiating the effect of C-75.

We next investigated the activation of caspases in cells treated with subtoxic
doses of 25 mmol/L C-75 in combination with 10 mmol/L cisplatin in EOC cells
by Western blotting. Combination treat-
ment resulted in activation of caspase 9,
caspase 3 and subsequent cleavage of
caspase 3 and PARP in MDAH2774 and
SKOV3 cells (see Figure 5D).

FASN Inhibition Enhanced Cisplatin-
Mediated Antitumor Effects in Mice
Xenografts

To confirm whether C-75 in combina-
tion with cisplatin can inactivate AKT and its downstream targets, inducing ef-
icient apoptosis, we sought to determine
whether combination of C-75 with cis-
platin potentiates the inhibition of EOC xenograft tumor in nude mice as de-
scribed in Materials and Methods. After
5 wks of treatment, mice were eutha-
nized and the tumors were collected.
Combination treatment caused significant
regression of tumor volume at the end of
the fifth week (P < 0.05) (Figure 6A). A
significant reduction in the tumor
weight (Figure 6B) was observed in the
combination-treated mice when com-
pared with mice treated with C-75 or
with cisplatin alone. Furthermore, im-
ages of the tumor after necropsy showed
more shrinkage of tumor size in combi-
nation-treated tumors than in those tu-
mors treated alone (Figure 6C). Our data from in vitro experiments showed FASN
inhibition following combination treat-
ment with FASN inhibitor C-75 and cis-
platin and subsequent dephosphoryla-
tion of AKT of activated AKT. We
therefore examined whether FASN inhi-
bition in combination with cisplatin al-
tered the expression of these genes in
vivo. Western blot analysis was carried out to analyze FASN, activated AKT and the
caspase 3 levels in the primary tu-
mors derived from vehicle-treated mice
and in mice treated with C-75 alone or
with cisplatin alone or the two in com-
bination. FASN and its downstream target
activated AKT and the apoptotic marker
Caspase 3 was downregulated substantially in the combination-treated xenograft tumors (Figure 6D).

**DISCUSSION**

In light of recent evidence that links FASN activity and AKT activation for the promotion of tumorigenesis in various tumors (33–34), we sought to explore the relationship between FASN and AKT and its associated pathways in a cohort of Saudi EOC samples in a TMA format. Immunohistochemistry analysis of a large cohort of EOC samples showed an overexpression of FASN (75.5%) and its significant association with activated AKT and XIAP, linking its pathogenic role in tumorigenesis of Middle Eastern EOC. Sehdev et al. also have shown a higher incidence of FASN expression in ovarian carcinoma (7). Re-
Recently, it has been shown that AKT modulates the expression of FASN in a positive feedback manner in ovarian cancer cells (19). In this study, we have aimed to clarify this issue by investigating the effect of FASN inhibition on cell growth, proliferation and FASN/PI3K/AKT signal transduction in a panel of EOC cell lines. We demonstrated that inhibition of FASN activity by C-75, a selective inhibitor, resulted in downregulation of FASN, inactivation (dephosphorylation) of AKT, as well as downregulation of its downstream target, GSK3 and FOXO1, leading to induction of apoptosis. Our pharmacological inhibition and gene silencing studies suggest that inhibition of AKT does not affect the expression of FASN. On the other hand, C-75 treatment of EOC cells, C-75 alone and C-75 in combination, showed enhanced apoptosis and downregulation of caspase 3, cleaved caspase 3 and PARP. These findings suggest that FASN is an upstream effector of AKT and its downregulation induces cell death via modulation of AKT-mediated antiapoptotic genes such as XIAP, CIAPs and survivin in ovarian cancer cell lines. Apoptosis is a multistep process, and an increasing number of genes have been identified that are involved in the control or execution of apoptosis (33). Our study shows that FASN inhibition by C-75 in EOC cells caused apoptosis via disruption of the mitochondrial membrane, allowing activation of proapoptotic proteins and the release of cytochrome c into cytosol. Released cytochrome c results in the formation of apoptosome by interaction with APAF1 and caspase 9, leading to the activation of caspase 3, eventually resulting in cleavage of PARP in apoptotic cells, a hallmark of apoptosis by various antitumor agents (34). Furthermore, pretreatment of EOC cells with a broad-spectrum caspase inhibitor abrogated the C-75-induced apoptosis. These data suggest that inhibition of the FASN/AKT pathway in EOC-induced apoptosis via caspase cascade activation.

Cisplatin has been used to treat a variety of malignancies, however, acquired resistance prevents use of this chemotherapeutic agent owing to the escalation of doses to overcome cellular resistance (35). The increased doses of cisplatin can cause severe cytotoxicity to normal tissues, thereby posing major clinical challenges. The use of subtoxic doses of dual agents that target distinct molecules can be a useful alternative strategy of effective treatment with less toxicity. Our data showed that subtoxic doses of C-75 augmented the apoptotic response of cisplatin in EOC cells. Furthermore, combination treatment of C-75 and cisplatin significantly regressed the Xenograft tumor in the Nude mouse model, suggesting that FASN and cisplatin combination treatment has more effective antitumor effects in vivo. Altogether, these findings show that FASN/AKT signaling pathway plays a key role in the pathogenesis of Middle Eastern EOC. Inhibition of FASN/AKT sensitizes EOC cells to cisplatin-induced cell death via apoptosis in vitro and in vivo. Thus, effectiveness of the combination of inhibitors of FASN with conventional chemotherapeutic agents such as cisplatin offers a promising targeted therapy for the treatment of EOC.
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