Blockade of fatty acid synthase induces ubiquitination and degradation of phosphatidylinositol-3 kinase signaling proteins in ovarian cancer

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Running title: Fatty acid synthase controls ubiquitination of PI3K effectors
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Abstract

Aberrations within the phosphatidylinositol-3 kinase (PI3K) pathway occur in >45% of ovarian carcinomas (OCs). The PI3K cascade transmits signals from ErbB receptors downstream to S6 and 4EBP1, which are involved in protein biosynthesis. Many OCs reveal hyperactivation of ErbB1(EGFR) or ErbB2(HER2/neu). Unfortunately, the benefit of anti-ErbB drugs is yet rather limited in OC. Thus, novel targeting strategies are needed for OC. The lipogenic enzyme fatty acid synthase (FASN) is overexpressed in ~80% of OCs. It stimulates cell growth and signifies poor prognosis. FASN inhibition impedes (ErbB) membrane receptor signaling and sensitizes cells against anti-ErbB drugs. Here, we show that the FASN inhibitor C75 and FASN-targeting siRNAs abrogate growth, induce apoptosis and downregulate phosphorylation/expression of the PI3K effectors AKT, mTOR, p70S6K, S6 and 4EBP1. In contrast, FASN inhibition impairs expression, but only weakly affects phosphorylation of ERK1/2 mitogen-activated protein kinases in OC cells. Cycloheximide-mediated blockade of protein translation reveals that C75- or FASN siRNA-induced shutdown of FASN accelerates decomposition of signaling proteins. This effect is caused by C75- or FASN siRNA-dependent stimulation of ubiquitination followed by lysosomal-autophagosomal proteolysis. In contrast, PI3K inhibitor LY294002 blocks phosphorylation, but does not reduce expression/stability of PI3K effectors. Forced expression of hyperactive(HA-) AKT1, unlike HA-MEK1, impairs the growth-inhibitory action of C75. We provide first evidence that the anticancer action of FASN inhibitors is at least partially mediated by drug-dependent proteolysis of PI3K effectors. FASN is a promising cancer target, whose inhibition not only abrogates lipogenesis, which is indispensable for cancer growth, but also downregulates oncogenic PI3K signaling.
Introduction

The phosphatidylinositol-3 kinase (PI3K) signaling cascade controls many cellular functions including proliferation, differentiation, tumorigenesis, angiogenesis, autophagy and apoptosis. A high proportion of ovarian carcinomas (OCs) harbor molecular aberrations within the PI3K system including for instance PI3K itself, AKT, mTOR, and p70S6K (1, 2). The core route of the pathway from PI3K to AKT and mTOR diverges at mTOR (3), which activates either p70S6K and ribosomal protein S6 or the eukaryotic translation initiation factor 4E (eIF4E) inhibitor binding protein 1 (4EBP1). The S6 protein is a component of the 40S ribosomal subunit, which is required for the synthesis of polypeptides at the ribosome. 4EBP1, on the other hand, modulates the function of eIF4E, which is involved in cap-dependent translation of growth promoting genes. Thus, both S6 and 4EBP1 are involved in protein synthesis (4).

In addition, the PI3K system is well known to transmit oncogenic signals derived from membrane receptor tyrosine kinases such as ErbB1 (epidermal growth factor receptor, EGFR) or ErbB2 (HER2/neu) (5). Notably, both ErbB1 and -2 have been shown to be hyperactivated in 55% and 35% of OCs, respectively (6 – 9). This would suggest that blockade of ErbB1 and -2 would be a promising strategy for treatment of OC. Unfortunately, however, clinical studies yet reveal that ErbB1- or ErbB2-inhibitory drugs are largely inefficient in OCs (10). Definitive molecular explanations for these failures are still missing. Therefore, novel molecular targeting strategies for the treatment of OC are urgently needed.

Fatty acid synthase (FASN), the enzyme responsible for de novo synthesis of lipids from sugars, is overexpressed in up to 80% of OCs and signifies unfavorable clinical outcome (11, 12). FASN supports the formation of membrane-anchored lipid rafts.
These lipid rafts accommodate growth factor receptors including ErbB family proteins and facilitate receptor signaling (13). Thus, FASN is associated with oncogenic ErbB signaling. Most importantly, inhibition of FASN delays disease progression of human OC (14). Recently, we reported that the FASN inhibitor C75 induces growth arrest in OC cells, downregulates ErbB1 and ErbB2, and sensitizes the cells against ErbB blocking drugs (15). Here, we demonstrate that C75 affects OC cell growth by attenuating PI3K pathways downstream of ErbB receptors and we provide first evidence that this effect is caused by ubiquitin-mediated proteolysis of PI3K cascade molecules rather than by dephosphorylation of these proteins. In contrast, the PI3K inhibitor LY294002 blocks phosphorylation, but does not stimulate degradation of PI3K and its effector proteins. Therefore, targeting FASN not only abrogates lipogenesis in cancer cells, but also efficiently downregulates oncogenic PI3K signaling.
Materials and Methods

Cell culture and drugs

A2780 (kind gift from M. Krainer, Medical University Vienna, Austria), OVCAR-3, SKOV-3 (American Type Culture Collection, Manassas, VA) and HEY (originally from R. N. Buick, Univ. Toronto, ON, Canada) were maintained at 37°C, 5% CO₂, and 95% humidity in RPMI1640, alpha-MEM or DMEM, respectively, containing 10% fetal calf serum, 100IU(µg)/ml penicillin-streptomycin and 2mM glutamine (Gibco, Karlsruhe, Germany). They were diluted once or twice weekly at a ratio of 1:10 to 1:15 and cultured for up to 20 passages. The cell lines were tested for absence of Mycoplasma (Venor GeM, Minerva Biolabs, Berlin, Germany) and the species origins were confirmed as human by species PCR, and cell line identities and uniqueness were tested by fluorescent nonaplex PCR of short tandem repeat markers (DSMZ, Braunschweig, Germany). FASN inhibitor C75 (Sigma, St. Louis, MO), PI3K inhibitor LY294002 (Calbiochem, San Diego, CA), protein biosynthesis inhibitor cycloheximide (CHX, Sigma), and proteasome inhibitor bortezomib (BTZ, ChemieTek, Indianapolis, IN) were dissolved in DMSO and diluted 1:500 or 1:1,000 in culture media immediately before use. Chloroquine (CQ, Sigma), a lysosomotropic agent that blocks autophagic flux, is soluble in aqueous solutions and was used at final concentrations of 1.25, 2.5, 5, and 15µM.

Western blotting

Cells plated in media containing 5% fetal calf serum were treated with C75, LY294002, CHX, or BTZ, or with combinations of C75 with CHX or with BTZ. Proteins (20µg/lane) were subjected to SDS–PAGE, blotted onto PVDF membranes and
immunostained as described (16) using: anti-AKT, anti-phospho-AKT(Ser473), anti-mTOR, anti-phospho-mTOR(Ser2448), anti-p70S6K, anti-phospho-p70S6K(Thr421/Ser424), anti-phospho-p70S6K(Thr389), anti-S6 (clone 5G10), anti-phospho-S6(Ser240/244), anti-4EBP1, anti-phospho-4EBP1(Ser65), anti-MEK1/2, anti-ERK1/2, anti-phospho-ERK1/2(Thr202/Tyr204), anti-caspase-3, anti-poly(ADP-ribose) polymerase-1 (PARP-1), anti-Beclin 1, anti-Atg5 (clone D1G9), or anti-ubiquitin (clone P4D1) (all from Cell Signaling Technology, Boston, MA, and all at a dilution of 1:1,000, except anti-phospho-S6(Ser240/244), which was used at 1:2,000), or anti-actin (I-19, Santa Cruz Biotechnology, Santa Cruz, CA, at 1:1,000), or anti-LC3B (Genetex, Irvine, CA, at 1:2,000). Secondary antibodies were peroxidase-tagged donkey-anti-rabbit (Promega, Madison, WI), donkey-anti-goat (Santa Cruz Biotechnology) or chicken anti-mouse IgG (Santa Cruz Biotechnology) at 1:15,000. Detection was by enhanced chemiluminescence.

**Immunoprecipitation**

SKOV-3 cells (6x10^5/60 mm dish) grown in media containing 5% fetal calf serum were treated for 48 hours with 20µg/ml C75 and then processed for immunoprecipitation with anti-AKT or anti-actin using the Immunoprecipitation Kit (Protein G) from Roche Diagnostics (Mannheim, Germany). Sample preparation was performed essentially as described in the manufacturer’s protocol. Briefly, proteins from cell lysates were precleared and were immunoprecipitated overnight at 4°C on a rocking platform using specific antibodies bound to protein-G-agarose beads. Precipitated proteins were denatured (5 min, 95°C), separated from the beads and then subjected to SDS-PAGE and Western blotting using anti-ubiquitin, anti-AKT or anti-actin antibodies as described above.
Proliferation assays

Cells were plated in 96-well plates. After adhesion, media ± drugs were added. Cell numbers were estimated after indicated time periods using a formazan dye assay (Biomedica, Vienna, Austria) and results from triplicate determinations were given as means ± SD.

Cell viability assays

SKOV-3 cells grown in 35 mm dishes were exposed for 72 hours to 0-20 µg/ml C75. Adherent cells were trypsinized, pooled with nonadherent cells and stained with 0.01% trypan blue. Cell viability was then determined by direct counting of the cells under the microscope. For each drug concentration, the viable (pale) and the nonviable (blue) cell subsets were related to the sum of both populations, which has arbitrarily been set at 100%. Results from triplicate determinations were given as means ± SD.

Acridine orange staining

SKOV-3 cells grown to subconfluence in 35 mm dishes were exposed for 48 hours to 0.2% DMSO (solvent), to 20µg/ml C75 alone, to 2µM BTZ alone, or to C75 and BTZ together. For detection of acidic vesicles such as late endosomes and lysosomes the cells were stained for 20 min with acridine orange (Sigma, 1µg/ml) and then subjected to fluorescent microscopy. Chloroquine (CQ, 24h, 15µM) was used as a lysosomotropic positive control drug.
Transfection of small interfering RNA (siRNA) and detection of cell number, and of protein expression, phosphorylation and ubiquitination

A2780 were transiently transfected with 60nM FASN siRNAs and SKOV-3 with 75nM Beclin 1 (BECN1) or ATG5 siRNAs according to the manufacturer's instructions (Dharmacon, Lafayette, CO). Briefly, 3x10^5 cells/35mm dish were incubated in DharmaFECT 3 siRNA Lipid Transfection Reagent either containing 60 or 75 nM of siGENOME Non-Targeting siRNA #2 (D-001210-02-05) that targets firefly luciferase mRNA, but does not interact with any known human mRNA sequence, or containing a set of four siRNA species, each at a concentration of 15 or 18.75 nM, that target distinct sequences of the human FASN, Beclin 1 or ATG5 mRNAs, respectively (ON-TARGETplus SMARTpool FASN, L-003954-00, ON-TARGETplus SMARTpool BECN1, L-010552-00, ON-TARGETplus SMARTpool ATG5, L-004374-00). After 72 hours of culture at 37°C, 5% CO₂, and 95% humidity in RPMI1640 or alpha-MEM containing 5% fetal calf serum and 2mM glutamine, untreated or C75-exposed (10µg/ml, 72h) transfectants were subjected to a formazan dye cell proliferation assay (see above). Moreover, untreated transfectants were lysed and further processed for SDS-PAGE and Western blotting in order to detect protein expression, phosphorylation and ubiquitination (see above). All data were obtained from unselected polyclonal transfectant populations.

Transfection of cDNA constructs and detection of cell number, and of protein expression and phosphorylation

Human constitutively active(CA)-MEK1 lacking nuclear export signal domain and harboring activating amino acid substitutions at two phosphorylation sites (kindly provided by H. Kiyama, Osaka City Univ., Japan) and murine CA-AKT1 carrying the
myristoylated signal of Lck, which targets it to the membrane (kindly provided by D. Efremov, International Center for Genetic Engineering and Biotechnology, Rome, Italy) were transfected into SKOV-3 cells essentially as previously described (17). Specifically, pcDNA3 containing the gene for green fluorescent protein (GFP) was used as a negative control expression plasmid. For transient transfection, 5-8x10^5 SKOV-3 cells were plated in 6-well plates and allowed to attach. Then, 1µg plasmid DNA diluted in Opti-MEM I (Invitrogen, Paisley, UK) containing Lipofectamine LTX (Invitrogen) was incubated for 30 minutes at room temperature to allow formation of Opti-MEM-DNA-LTX transfection complexes, added to cells and left in cultures for 24 hours. Cells were then allowed to grow for another 24 hours in the absence of exogenous plasmid DNA. These transfected, polyclonal cell populations were not undergone any further clonal selection or enrichment, but were either directly processed for Western blot analysis or were trypsinized and subjected to C75-treatment and cell growth assays as described above.

**Statistical analyses**

The Student’s t-test for unpaired samples was applied to examine the significance in differences of protein expression/phosphorylation or of cell growth. p-values < 0.05 were considered significant.
Results

The effects of the FASN blocking drug C75 and the PI3K inhibitor LY294002 on PI3K and MAPK signaling pathways

Western blots shown in Fig. 1 demonstrate that the FASN inhibitor C75 downregulates the expression of the PI3K downstream proteins AKT, mTOR, p70S6K and 4EBP1 in A2780 OC cells in a time-dependent manner. The protein levels are reduced after 2 or 4 hours of drug exposure and remain lowered during the observation period (72 hours). Thus, C75 induces silencing of the PI3K pathway. Interestingly, when we determined the relationship between the phosphorylated and the corresponding total protein bands, we found that C75 also inhibits the specific phosphorylation/activity of the PI3K signaling proteins. This was particularly striking for 4EBP1 (Fig. 1). In contrast, C75 decreases total and phosphorylated ERK1 and -2 – the crucial signal effectors of the mitogen-activated protein kinase (MAPK) cascade – only slightly and only after prolonged periods of exposure (≥ 48h) (Fig. 1).

The effects of the FASN inhibitor C75 on PI3K and MAPK signaling were then compared with those induced by the direct PI3K antagonist LY294002. LY294002 blocks specific phosphorylation/activity of PI3K downstream effectors, but, unlike C75, it does not downregulate protein steady-state levels (Supplementary Fig. S1). Our data thus indicate that C75 and LY294002 affect PI3K signaling by crucially different mechanisms of action. While C75 inhibits the PI3K pathway primarily by downregulation of effector protein expression, LY294002 directly targets phosphorylation/activation of these proteins, but does not downregulate the protein expression levels. Dose-response experiments (not shown) revealed that C75 and...
LY294002 are equally efficient in growth inhibition at the concentrations used (7 µg/ml for C75 and 12 µg/ml for LY294002) and block A2780 cell growth completely (Supplementary Fig. S2).

**C75-induced downregulation of PI3K and MAPK proteins is not mediated by eIF4F-dependent translational control**

Unphosphorylated 4EBP1 associates with eIF4E. Thereby, 4EBP1 prevents eIF4E from binding to the eIF4F translation initiation complex and precludes eIF4F-dependent protein synthesis. Therefore, we speculated whether C75-mediated downregulation of PI3K and MAPK proteins might be caused by C75-dependent dephosphorylation of 4EBP1. However, two observations are against this idea. First, C75 suppresses AKT and p70S6K levels already after 2 hours of treatment, whereas dephosphorylation of 4EBP1 takes longer and requires at least 4 hours of exposure (Fig. 1). Second, LY294002 completely abrogates phosphorylation of 4EBP1, but does not downregulate PI3K and MAPK protein levels (Supplementary Fig. S1). Thus, these data suggest that PI3K and MAPK signaling proteins are not controlled by eIF4F-dependent translational mechanisms in A2780 OC cells.

**C75 stimulates degradation of PI3K and MAPK signaling proteins**

We then sought to determine whether the observed C75-mediated downregulation of PI3K and MAPK proteins might result from elevated protein degradation. To test this, A2780 cells were cotreated for 8 – 72 hours with C75 and with the translation inhibitor CHX, which is known to block de novo protein synthesis. Cell proteins were extracted after lysis of the cells and subjected to Western blotting. Separated proteins were then analyzed for the expression of AKT, mTOR, p70S6K, 4EBP1, and ERK1/2. Interestingly, as shown in Fig. 2, C75 markedly accelerates and aggravates CHX-
enabled downregulation of each signaling protein tested. Consequently, the FASN inhibitor C75 markedly shortened the period required for CHX to depress the steady-state levels of the PI3K and MAPK proteins to 50% of the original levels (half-lifes, IT$_{50}$) (Supplementary Table S1). Thus, inhibition of FASN silences the PI3K pathway mainly by reducing the stability of the downstream proteins rather than by direct dephosphorylation of these proteins.

**C75 stimulates protein ubiquitination**

Degradation of proteins is usually triggered by attachment of ubiquitin moieties to the client protein. Therefore, we hypothesized that the FASN inhibitor C75, but not the PI3K blocker LY294002, induces protein ubiquitination. To test this, A2780 cells were exposed to vehicle or drugs followed by Western blot analysis using a ubiquitin-specific antibody. Interestingly, inhibition of FASN causes a strong accumulation of ubiquitinated proteins, whereas direct blockade of PI3K does not promote protein ubiquitination (Fig. 3). Thus C75, in contrast to LY294002, stimulates protein ubiquitination. This is in agreement with the data showing that C75, but not LY294002, decreases the steady-state levels of PI3K and MAPK signaling proteins (Fig. 1 and Supplementary Fig. S1). Therefore, C75-mediated degradation of signaling proteins is at least in part caused by elevated ubiquitination of these proteins.

**Targeted knockdown of FASN by siRNA downregulates expression and phosphorylation of PI3K signaling proteins, and stimulates protein ubiquitination**

The FASN inhibitory drug C75 has been described as a malonyl-CoA analogue and is known to concomitantly activate fatty acid degradation by antagonizing the
allosteric inhibitory effect of malonyl-CoA on carnitine palmitoyltransferase-I (CPT-I), the major rate-limiting enzyme for long chain fatty acid oxidation (18 – 20). Therefore, we examined, whether the observed C75-induced effects on PI3K downstream protein expression, phosphorylation and stability were indeed due to blockade of FASN and not due to concurrent activation of CPT-I by transiently transfecting A2780 cells with a set of four different FASN-specific siRNAs. Notably, Bandyopadhyay et al. (21) recently reported that siRNA-mediated silencing of FASN expression, in contrast to pharmacological inhibition of FASN by C75, counteracts CPT-I function. In our studies, FASN-targeted RNA interference efficiently abrogated FASN protein expression and blocked cell growth. Most importantly, concordant with C75, FASN siRNAs dramatically downregulated the expression and phosphorylation of the PI3K downstream effectors AKT, mTOR, S6, and 4EBP1. On the other hand, while reducing overall expression of the MAPK proteins ERK1 and –2, they did not markedly impede phosphorylation of these signaling proteins. Moreover, corresponding to C75, FASN siRNAs stimulated protein ubiquitination (Fig. 4).

**C75 inhibits cell growth, survival and expression/phosphorylation of PI3K signaling proteins, and stimulates protein ubiquitination in all ovarian cancer cells studied**

Using in vitro growth assays and Western blot analyses we then examined the effects of C75 on cell growth, on apoptosis, on PI3K- and MAPK-signaling pathways, and on protein ubiquitination in a panel of additional OC cell lines (OVCAR-3, SKOV-3, HEY). Figure 5 demonstrates that C75 effectively reduced the cell numbers (Fig. 5A), downregulated the expression and phosphorylation of AKT and S6, and stimulated protein ubiquitination in all these cell lines. Moreover, similar to A2780, C75 did also lower the ERK1 and -2 protein levels but had less effect on the phosphorylation of
ERK1/2 in these cells (Fig. 5B, C). Immunoprecipitation for AKT followed by Western blotting against ubiquitinated proteins provided direct evidence that AKT indeed is a client protein of C75-induced ubiquitination in SKOV-3 cells (Fig. 5D). Cytotoxicity of C75 was demonstrated by a relative, dose-dependent increase of trypan blue stained (dead) cells (Fig. 5E) and by a decrease of caspase 3 and PARP-1, and a correlative occurrence of cleaved PARP-1 indicating apoptotic cell death (Fig. 5F).

C75 induces lysosomal protein degradation and autophagy

Unexpectedly, exposure of SKOV-3 cells to the proteasome inhibitor bortezomib (BTZ), which abrogates proteasomal proteolysis (22), did not upregulate the steady-state levels of AKT and ERK1/2. Instead, when given alone, BTZ down-regulated AKT just as C75 did. Moreover, AKT expression was almost completely shut down, when both BTZ and C75 were given together. In contrast, ERK1/2 expression was not significantly affected by BTZ (Fig. 6A). This suggests 1) that neither AKT nor ERK1/2 protein is subject to proteasomal degradation, and 2) that blockade of the proteasome by BTZ activates an alternative proteolytic mechanism that causes downregulation of AKT. This pathway can further be stimulated by coexposure to C75. Consequently, we wondered whether C75 might stimulate lysosomal activity. To this end, SKOV-3 cells were stained with the fluorescent dye acridine orange, which labels acidic cell compartments such as lysosomes (23). Figure 6B demonstrates that both BTZ and C75 alone or together strongly stimulate the formation of lysosomes in SKOV-3 cells. Moreover, Western analysis revealed that LC3B-I was converted by C75 to the smaller form LC3B-II, which is a marker for autophagy (Fig. 6C). Interestingly, the lysosomotropic agent CQ blocks autophagic flux after generation of LC3B-II but before lysosome-autophagosome fusion. Consequently, CQ also causes marked cellular accumulation of lysosomes (Fig. 6B). We observed that inhibition of
C75-mediated autophagy either by co-exposure of C75-treated SKOV-3 cells to the autophagic inhibitor CQ (Fig. 6D) or by siRNA knockdown of the key autophagic proteins Beclin 1 or Atg5 reverts C75-mediated growth inhibition (Figs. 6E – F). Thus, our data demonstrate that C75 stimulates lysosomal accumulation and formation of autophagosomes, and that induction of autophagy essentially contributes to the toxic effects of C75.

**Constitutively active AKT1 confers resistance against C75**

To assess the potential roles of the PI3K and the MAPK signaling pathways in mediating the anticancer action of C75, we transiently transfected constitutively active variants of AKT1 or MEK1 into SKOV-3 cells. Overexpression/hyperphosphorylation of transgenes or downstream effector molecules (AKT, MEK1/2, ERK1/2) was confirmed by Western blot analysis of the unselected polyclonal transfectant cell populations. Unlike wild-type SKOV-3 and GFP-transfected control cells, AKT1- and MEK1-transfectants exhibited an excess of the respective unphosphorylated and phosphorylated signal effectors (Fig. 7A). These transfectants were used to examine the relative contribution of MAPK and PI3K signaling to the growth inhibitory effect of C75. As shown in Figure 7B, C75 was found to strongly and equally suppress the growth of GFP- and MEK1-transfected SKOV-3 cells, whereas it was significantly less efficient in blocking growth of AKT1-transfected cells. Thus, our data demonstrate 1) that forced expression of dominant active AKT1 and MEK1 efficiently stimulates PI3K and MAPK pathways in SKOV-3 cells, respectively, and 2) that PI3K/AKT-, but not MAPK/MEK-hyperactivity, confers growth-resistance of the cells against C75. Thus, deactivation of the PI3K system seems to be crucial for C75 to exert its anticancer effect in these cells.
In summary, our data suggest that FASN activity is involved in the functionality of proteolytic pathways via a molecular cross-talk involving FASN and the ubiquitination system. Within this scenario, the deactivation of the PI3K system essentially contributes to the anticancer effect of C75 in OC cells.
Discussion

The phosphatidylinositol 3-kinase (PI3K) signaling pathway is crucial for survival and for progression of OC cells through the G1 phase of the cell cycle. The p110α catalytic subunit of PI3K (PIK3CA) is one of the most frequently altered oncogenes in cancer. Approximately 40% of ovarian tumors reveal amplification, whereas only 7% reveal mutation of PIK3CA. Conversely, the majority of breast cancers harbor mutations in PIK3CA (40%), whereas only a few reveal PIK3CA amplifications (9%) (1, 24, 25). We and others have recently shown that the PI3K signaling cascade interacts with lipogenic pathways in cancer cells. In fact, fatty acid synthase (FASN), the crucial enzyme in de novo generation of long chain fatty acids, is overexpressed in approximately 80% of OCs and is linked with the PI3K system in these tumors (13, 15). Unfortunately, the mechanisms of FASN-mediated regulation of PI3K signaling are still not fully characterized. Experimental evidence suggests that multiple pathways are involved (15). For instance, inhibition of FASN has been found to abrogate incorporation of phospholipids – the end products of FASN action – into cell membrane-anchored lipid rafts, which accommodate growth factor receptors. It is, therefore, thought that blockade of FASN indirectly impedes the formation of membrane signaling complexes, and prohibits recruitment and activation of downstream effectors such as PI3K (15). Moreover, persistent abrogation of FASN-dependent lipid biosynthesis causes accumulation of reactive oxygen species and of FASN substrates as well as depletion of FASN end products (15, 21). These stressful conditions impose a durable insult on the malignant cells, which consequently try to build up a compensatory unfolded protein response that is part of the endoplasmic reticulum stress response. These processes can lead to inhibition of PI3K and to cell
growth arrest (26, 27). Due to these properties FASN has been considered to represent a promising anticancer drug target. C75 is a derivative of the natural FASN blocker cerulenin. It was developed as an anti-FASN agent with a better chemical stability than cerulenin. Unfortunately, both compounds have been shown to affect nutrient uptake and body weight in mice, which hampers their use in clinical oncology (28). However, recently, several novel FASN drugs have been developed, which allegedly overcome these limitations including but not limited to C93, C247, FAS31, a series of 3-aryl-4-hydroxyquinolin-2(1H)-one derivatives, several analogs of epigallocatechin-3-gallate, and a panel of bisamide derivatives. Many of these promising substances are now subject to clinical evaluation and it is hoped that at least some of them will exploit the full potential of FASN as a cancer drug target in the years to come (reviewed in 29). Here we demonstrate that C75 and FASN-specific siRNAs impair both expression and phosphorylation of the PI3K downstream effector proteins AKT, mTOR, p70S6K, S6 and 4EBP1 in OC cells (Figs. 1, 4, 5). In contrast, the PI3K targeting drug LY294002 inhibits phosphorylation of the PI3K downstream effectors, but leaves expression of these proteins essentially unaffected (Supplementary Fig. S1). On the other hand, although abrogation of FASN by C75 or by siRNAs, and of PI3K by LY294002 does repress the steady-state levels of ERK1/2 protein to various degrees, phosphorylation of ERK1/2 is yet not consistently inhibited in FASN- or PI3K-blocked OC cells (Figs. 1, 4, 5, Supplementary Fig. S1). This uncoupling between ERK1/2 expression and phosphorylation is in line with reports demonstrating that the PI3K/AKT/mTOR cascade negatively cross-talks with MAPK signaling. For instance, it has been shown that hyperactive AKT curbs the function of C-Raf, which is the first kinase within the MAPK cascade (30). In addition, experimental data indicate that Rheb, which usually activates mTOR, also acts as an inhibitor of MAPK signaling (31). Importantly, exogenous expression of hyperactive
AKT1, but not of hyperactive MEK1, significantly lowered the sensitivity of SKOV-3 cells against C75 (Fig. 7). Thus our data suggest that the anticancer effects of FASN inhibition are at least in part mediated by inhibition of PI3K signaling.

Activation of the PI3K/AKT/mTOR cascade leads to phosphorylation and dislodgement of 4EBP1 from eIF4E. This enables eIF4E to bind to the scaffolding protein eIF4G, which contains the RNA helicase eIF4A as second binding partner. The assembly of eIF4A with eIF4E and eIF4G is designated eIF4F and represents the active translation initiation complex (4). PI3K/AKT/mTOR also activates ribosomal protein S6 via phosphorylation of p70S6K. Both events – activation of eIF4F and phosphorylation of S6 – stimulate translation of genes that regulate cell growth and apoptosis. Therefore, it is tempting to speculate that C75- and FASN siRNA-mediated downregulation of PI3K signaling occurs in two steps including a negative autoregulatory loop. Inhibition of FASN would first inhibit the phosphorylation/activity of PI3K downstream effectors. This would result in reduced activity of 4EBP1 and S6 and consequently in reduced translation of oncogenic genes including the PI3K signaling proteins. However, our findings argue against this idea, because we observed that the direct PI3K inhibitor LY294002 diminishes phosphorylation, but does not reduce the expression of PI3K downstream proteins (Supplementary Fig. S1). Instead, we actually demonstrate that inhibition of FASN rather stimulates the degradation of PI3K and MAPK signaling proteins in OC cells. Therefore, C75-induced downregulation of oncogenic signaling proteins likely is caused by increased proteolysis (Fig. 2). This is further supported by our finding that inhibition of FASN by C75 or by FASN-specific siRNAs markedly increases the ubiquitination of the proteins in OC cells and that AKT is a direct client for ubiquitination (Figs. 3 – 5). Coupling of the short modifier protein ubiquitin to a lysine residue of a protein targets
this protein for lysosomal or proteasomal degradation. In the OC cells analyzed, it
appears that the proteasome inhibitor BTZ does not stabilize AKT and ERK1/2.
Hence, these proteins are most likely degraded along the lysosomal rather than the
proteasomal route (Fig. 6A), which correlates with our finding that inhibition of FASN
causes striking accumulation of acidic vesicles such as late endosomes, lysosomes
and autophagosomes, and ultimately induces autophagy in OC cells (Figs. 6B, C).
Interestingly, blockade of C75-mediated autophagy by drugs such as CQ or by
siRNAs that silence crucial autophagic proteins such as Beclin 1 or Atg5 efficiently
abrogates the growth inhibitory action of C75 (Figs. 6D – F). This demonstrates that
autophagy is a cytotoxic rather than a cytoprotective response of OC cells to
inhibition of FASN. Generally, the activity of signaling pathways can be regulated
either by site-specific phosphorylation of the effector proteins or by modulation of
their expression level. Degradation of signaling proteins can occur either transiently
in response to activation (32) or constitutively, independent of the activation state of
the client protein. This has recently been demonstrated for several PI3K downstream
effectors including serum- and glucocorticoid-induced kinase-1 (SGK1) and p70S6K
(31, 33 – 36). Our data reveal for the first time that blockade of FASN stimulates
degradation of PI3K and MAPK signaling proteins, which is most likely caused by
induced ubiquitination and subsequent autophagic decomposition of the effector
proteins. This is corroborated by a recent report by Knowles et al. (37), who showed
that FASN activity is inversely related to the expression of several ubiquitination
enzymes. Thus, we provide first evidence that inhibition of FASN reduces PI3K
signaling not because of direct interference of FASN pathways with PI3K
downstream effector phosphorylation, but rather because of elevated ubiquitin-
dependent proteolysis of these PI3K effectors.
In summary, our data show that growth arrest and cytoreduction, which is induced by inhibition of FASN activity, is mediated at least in part by indirect silencing of the PI3K signaling pathway due to increased ubiquitination and degradation of the PI3K effector proteins. The observed molecular cross-talks between FASN-, PI3K- and proteolytic pathways provide the rationales for a number of novel combination targeting approaches that may ultimately improve the clinical management of OC.
References


Figure Legends

**FIGURE 1.** A, Inhibition of FASN by C75 downregulates the expression and phosphorylation of the PI3K downstream signaling proteins AKT, mTOR, p70S6K, and 4EBP1 in A2780 OC cells. Cells were treated for the indicated times with vehicle (≤ 0.1% DMSO) or with 7µg/ml C75 and subjected to Western blot analysis. One representative experiment. B, Autoradiographs were quantified by scanning densitometry. Each protein and phospho(p)-protein band was normalized to β-actin. In addition, each p-protein band was also normalized to its corresponding total protein band. The resulting ratios (Protein/Actin, p-Protein/Actin, p-Protein/Protein) represent measures for expression, phosphorylation and specific phosphorylation/activity of that particular protein, respectively. All calculated ratios were related to the ratios obtained from vehicle-treated control samples, which have been arbitrarily set at 1.0 (horizontal dashed line). Means ± SD, n = 3, Student’s t-test: * … p < 0.05, ** … p < 0.01, *** … p < 0.001 vs. control.

**FIGURE 2.** Inhibition of FASN accelerates the degradation of the PI3K downstream signaling proteins AKT, mTOR, p70S6K and 4EBP1, and of the MAPKs ERK1 and -2 in A2780 OC cells. A, Cell cultures were exposed for the indicated times (8, 24, 48, 72h) to vehicle (≤ 0.1% DMSO), to the FASN inhibitor C75 (7µg/ml), to the translation inhibitor cycloheximide (CHX, 15µg/ml), or to a combination of C75 and CHX, and the expression of the signaling proteins was determined by Western blotting. One representative experiment. B, Time-dependent decrease of the steady-state levels of the proteins was quantified by scanning densitometry. For each time point, the optical density values of the protein bands from cultures exposed either to CHX alone or to
CHX plus C75 were related to those obtained from vehicle treated controls. Results were expressed in % of control and were plotted against time in hours (h). Fifty % of control is marked by a horizontal dashed line. Means ± SD, n = 3, Student’s t-test: * … p < 0.05, ** … p < 0.01, *** … p < 0.001 vs. CHX alone.

**FIGURE 3.** Inhibition of FASN elevates protein ubiquitination in A2780 OC cells. A, Cells were treated for the indicated times (8, 24, 48, 72h) with vehicle (≤ 0.1% DMSO), with 7µg/ml C75, or with 12µg/ml LY294002. Whole cell lysates were subjected to SDS-PAGE followed by Western blot analysis using antibodies specific for ubiquitin or β-actin. One representative experiment. B, In each lane, ubiquitinated proteins and the corresponding β-actin band were quantified by scanning densitometry. The obtained integral optical density values for the ubiquitinated proteins were normalized to the corresponding optical densities of the β-actin bands and for each time point the resulting ratios were related to the corresponding vehicle-treated control, which has been arbitrarily set at 1.0 (horizontal dashed line). Means ± SD, n = 3, Student’s t-test: * … p < 0.05, ** … p < 0.01, *** … p < 0.001 vs. control.

**FIGURE 4.** Downregulation of FASN protein level in A2780 OC cells by transient transfection of FASN-targeting siRNAs causes cytoreduction (A), downregulates the expression and phosphorylation of the PI3K downstream signaling proteins AKT, mTOR, S6 and 4EBP1, and stimulates protein ubiquitination (B, C). A2780 cells were transfected with either a non-targeting control siRNA (60 nM) or with a set of 4 different FASN-targeting siRNAs (4x15 nM). After 72 hours cultivation, the transient transfectants were subjected to further experimentation. A, Cell growth analysis using a formazan dye assay as described in Materials and Methods. Means ± SD, n = 3,
Student’s t-test: *** … p < 0.001 vs. control siRNA. B, SDS-PAGE followed by Western blot analysis using antibodies against FASN, against the total or the phosphorylated (p-)forms of the PI3K downstream effectors AKT, mTOR, S6 or 4EBP1, against the MAPK proteins ERK1 and -2, or against ubiquitin. β-actin was used as loading control. One representative experiment. C, Bands from autoradiographs were quantified by scanning densitometry. Each protein and p-protein band, and the ubiquitinated proteins were normalized to β-actin. In addition, each p-protein band was also normalized to its corresponding total protein band. The resulting ratios (Protein/Actin, p-Protein/Actin, p-Protein/Protein) represent measures for expression, phosphorylation and specific phosphorylation/activity of that particular protein, or for protein ubiquitination, respectively. All calculated ratios were related to the ratios obtained from samples transfected with control siRNA, which have been arbitrarily set at 1.0 (horizontal dashed line). Means ± SD, n = 3, Student’s t-test: * … p < 0.05, *** … p < 0.001 vs. control siRNA.

FIGURE 5. The effects of the FASN blocker C75 on cell growth and survival (A, E, F), on expression/phosphorylation of PI3K and MAPK signaling proteins and on protein ubiquitination (B - D) are not restricted to A2780 OC cells. A, Three additional OC cell lines (OVCAR-3, SKOV-3, HEY) were exposed for 72 hours to 0-20 µg/ml C75 and were subjected to cell growth analysis using a formazan dye assay as described in Materials and Methods. Means ± SD, n = 3, Student’s t-test: 1 … OVCAR-3 p < 0.001, 2 … SKOV-3 p < 0.001, 3 … HEY p < 0.001 vs. 0 µg/ml C75 (Control). B, The OC cells were exposed for 72 hours to vehicle (≤ 0.1% DMSO) or to C75 (OVCAR-3: 6 µg/ml; SKOV-3: 20 µg/ml; HEY: 15 µg/ml) and were subjected to SDS-PAGE followed by Western blot analysis using antibodies against the total or
the phosphorylated (p-)forms of AKT, S6 or ERK1/2, or against ubiquitin. β-actin was used as loading control. One representative experiment. C, Bands from autoradiographs were quantified by scanning densitometry. Each protein and p-protein band, and the ubiquitinated proteins were normalized to β-actin. In addition, each p-protein band was also normalized to its corresponding total protein band. The resulting ratios (Protein/Actin, p-Protein/Actin, p-Protein/Protein) represent measures for expression, phosphorylation and specific phosphorylation/activity of that particular protein, and for non-specific protein ubiquitination, respectively. All calculated ratios were related to the ratios obtained from vehicle-treated control cells, which have been arbitrarily set at 1.0 (horizontal dashed line). Note that the y-axis has a log scale. Means ± SD, n = 3, Student’s t-test: * ... p < 0.05, ** ... p < 0.01, *** ... p < 0.001 vs. control. D, AKT is a direct client protein of C75-induced ubiquitination. Lysates of untreated (0.1% DMSO, 48h) and C75-treated (20µg/ml, 48h) SKOV-3 cells were immunoprecipitated with antibodies against AKT or β-actin, respectively, subjected to SDS-PAGE and immunoblotted with antibodies against ubiquitin, AKT or β-actin. β-actin was used as loading control. Note: A C75-dependent increase of ubiquitinated AKT can be seen despite concurrent downregulation of the AKT protein steady-state level. E,F, C75 induces cell death in OC cells. Treatment of SKOV-3 cells for 72h with C75 lowers the viable and elevates the nonviable cell population in a dose-dependent manner as demonstrated by trypan blue dye exclusion (E). Western blot analyses for expression of caspase 3 and cleaved PARP-1 demonstrates that OVCAR-3, SKOV-3 and HEY cells undergo apoptosis when exposed for 48h to 6, 20 or 15µg/ml C75, respectively. β-actin was used as loading control (F).
FIGURE 6. Inhibition of FASN activates the lysosomal-autophagosomal degradation system. A, SKOV-3 cells were exposed for 48h to vehicle (0.2% DMSO), to C75 (20µg/ml) alone, to bortezomib (BTZ, 0.07, 0.5, or 2µM) alone, or to C75 and BTZ together and were then subjected to Western blot analysis for detection of AKT and ERK1/2 expression. α,β-tubulin was used as loading control. B, SKOV-3 cells were exposed for 48h to vehicle (0.2% DMSO), to C75 (20µg/ml) alone, to BTZ (2µM) alone, or to C75 and BTZ together, stained with acridine orange, which labels acidic vesicles such as late endosomes and lysosomes and evaluated by fluorescence microscopy. The lysosomotropic agent chloroquine (CQ, 24h, 15µM) was used as a positive control drug. Note the strong accumulation of orange granules in the cytoplasm of all treated cells. C, OC cells were exposed for 48h to vehicle (≤ 0.1% DMSO) or to C75 (OVCAR-3: 6 µg/ml; SKOV-3: 20 µg/ml; HEY: 15 µg/ml) and then subjected to Western blot analysis for detection of post-translational conversion of cytosolic LC3B-I (18 kDa) into the autophagosome-associated form LC3B-II (16 kDa). β-actin was used as loading control. D, The autophagy inhibitor CQ counteracts C75-mediated growth inhibition. SKOV-3 cells were exposed for 72h to C75 (0-15µg/ml) or to the autophagy inhibitor CQ (1.25, 2.5, or 5µM) alone or together and were then subjected to cell growth analysis using a formazan dye assay as described in Materials and Methods. Means ± SD, n = 3. E, F, Transient transfection of Beclin 1- or Atg5-targeting siRNAs (75nM, 72h) efficiently abrogates the expression of the key autophagic proteins Beclin 1 or Atg5 in SKOV-3 cells as demonstrated by Western blot analysis, β-actin was used as loading control (E), and (F) counteracts C75-mediated growth inhibition. Non-targeting control-, Beclin 1- and Atg5-siRNA-transfected cells were exposed for 72h to vehicle (0.1% DMSO) or to 10µg/ml C75 and were then subjected to cell growth analysis using a formazan dye assay as
described in Materials and Methods. Means ± SD, n = 3, Student’s t-test: ** … p < 0.01, Beclin 1 siRNA vs. control siRNA in presence of C75, * … p < 0.05 Atg5 siRNA vs. control siRNA in presence of C75.

**FIGURE 7.** Hyperactivation of PI3K/AKT signaling induces resistance of SKOV-3 cells against the FASN inhibitor C75. Cells were transiently transfected with constitutively active(CA)-AKT1 or CA-MEK1. A plasmid harboring the green fluorescent protein (GFP) gene was used as negative control. A, Resultant unselected polyclonal transfectant cell populations were processed for Western blotting for detection of transgene expression and signal effector phosphorylation. B, Transfectants were exposed for 72 h to the indicated concentrations of C75 and subjected to cell growth analysis using a formazan dye assay as described in Materials and Methods. Means ± SD, n = 3, Student’s t-test: ** … p < 0.01, *** … p < 0.001 vs. GFP-transfected negative control cells.
**Figure 1** – Tomek et al.

**A**

- **p-AKT**
- **AKT**

**B**

- **p-ERK1/2**
- **ERK1/2**

**Time (h)**
- 2
- 4
- 8
- 24
- 48
- 72

**7µg/ml C75**
- -
- +

**Normalized Level (Relative to Control)**
- 0
- 2
- 4
- 8

**Exposure to C75 (h)**
- 2
- 4
- 8
- 24
- 48
- 72

- **p-mTOR**
- **mTOR**

- **p-70S6K(T389)**
- **p70S6K(T421/S424)**

**Time (h)**
- 2
- 4
- 8
- 24
- 48
- 72

**7µg/ml C75**
- -
- +

**Normalized Level (Relative to Control)**
- 0
- 2
- 4
- 8

**Exposure to C75 (h)**
- 2
- 4
- 8
- 24
- 48
- 72

- **p-4EBP1**
- **4EBP1**

**Time (h)**
- 2
- 4
- 8
- 24
- 48
- 72

**7µg/ml C75**
- -
- +

**Normalized Level (Relative to Control)**
- 0
- 2
- 4
- 8

**Exposure to C75 (h)**
- 2
- 4
- 8
- 24
- 48
- 72

- **p-ERK1/2**
- **ERK1/2**

- **p-70S6K(T389)**
- **p70S6K(T421/S424)**

**Time (h)**
- 2
- 4
- 8
- 24
- 48
- 72

**7µg/ml C75**
- -
- +

**Normalized Level (Relative to Control)**
- 0
- 2
- 4
- 8

**Exposure to C75 (h)**
- 2
- 4
- 8
- 24
- 48
- 72

- **p-AKT**
- **AKT**

- **p-Protein/Actin = Expression**
- **p-Protein/Actin = Phosphorylation**
- **p-Protein/Protein = Specific Phosphorylation/Activity**

- **Time (h)**
- 2
- 4
- 8
- 24
- 48
- 72

**7µg/ml C75**
- -
- +

**Normalized Level (Relative to Control)**
- 0
- 2
- 4
- 8

**Exposure to C75 (h)**
- 2
- 4
- 8
- 24
- 48
- 72

- **p-mTOR**
- **mTOR**

- **p-70S6K(T389)**
- **p70S6K(T421/S424)**

**Time (h)**
- 2
- 4
- 8
- 24
- 48
- 72

**7µg/ml C75**
- -
- +

**Normalized Level (Relative to Control)**
- 0
- 2
- 4
- 8

**Exposure to C75 (h)**
- 2
- 4
- 8
- 24
- 48
- 72

- **p-4EBP1**
- **4EBP1**

- **p-ERK1/2**
- **ERK1/2**

- **p-70S6K(T389)**
- **p70S6K(T421/S424)**

**Time (h)**
- 2
- 4
- 8
- 24
- 48
- 72

**7µg/ml C75**
- -
- +

**Normalized Level (Relative to Control)**
- 0
- 2
- 4
- 8

**Exposure to C75 (h)**
- 2
- 4
- 8
- 24
- 48
- 72

- **Actin**

- **p-Protein/Actin = Expression**
- **p-Protein/Actin = Phosphorylation**
- **p-Protein/Actin = Specific Phosphorylation/Activity**
Figure 2 – Tomek et al.

**A**

- **CHX (15µg/ml)**
- **CHX (15µg/ml) + C75 (7µg/ml)**

### AKT

- Protein Level (% of Control)
- Time (h)

### mTOR

- Protein Level (% of Control)
- Time (h)

### p70S6K

- Protein Level (% of Control)
- Time (h)

### 4EBP1

- Protein Level (% of Control)
- Time (h)

### ERK1/2

- Protein Level (% of Control)
- Time (h)
Figure 3 – Tomek et al. (Relative to Control)

1,2
1,4
1,6
1,8

2,0

12µg/ml LY294002
7µg/ml C75

* ***

7µg/ml C75
Ubiquitin
Actin

Time (h) 8 24 48 72
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Figure 4 – Tomek et al.

A) Bar graph showing the cell number (% of Control siRNA) for Control siRNA and FASN siRNA.

B) Western blot analysis for FASN, p-AKT, AKT, p-mTOR, mTOR, p-S6, S6, p-4EBP1, 4EBP1, p-ERK1/2, ERK1/2, Ubiquitin, and Actin.

C) Normalized level (Relative to Control siRNA) for FASN, AKT, mTOR, S6, 4EBP1, ERK1/2, Ubiquitin.
Figure 6 – Tomek et al.

A

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.07 µM</th>
<th>0.5 µM</th>
<th>2 µM</th>
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<th>0.5 µM</th>
<th>2 µM</th>
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<tr>
<td>C75</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BTZ (µM)</td>
<td>-</td>
<td>-</td>
<td>0.07</td>
<td>0.5</td>
<td>2</td>
<td>0.07</td>
<td>0.5</td>
</tr>
</tbody>
</table>

AKT

ERK1/2

α,β-Tubulin

DMSO  CQ

B

C75

OVCAR-3

SKOV-3

HEY

LC3B-I

LC3B-II

Actin

C

D

Cell Number (% of Untreated Cells)

C75 (µg/ml)

F

Cell Number (% of Untreated Cells)

C75

Control siRNA

Bclin1 siRNA

Atg5 siRNA

Beclin1

Atg5

Actin

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Figure 7 – Tomek et al.


Panel B: Dose-response curve showing the effect of C75 (µg/ml) on SKOV-3/GFP, SKOV-3/CA-MEK1, and SKOV-3/CA-AKT1 cell lines, with normalization to control. Statistical significance indicated by asterisks: **p < 0.01, ***p < 0.001.
Molecular Cancer Research

Blockade of fatty acid synthase induces ubiquitination and degradation of phosphatidylinositol-3 kinase signaling proteins in ovarian cancer

Katharina Tomek, Renate Wagner, Franz Varga, et al.

Mol Cancer Res Published OnlineFirst October 4, 2011.

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