Inhibition of endothelial cell proliferation and angiogenesis by orlistat, a fatty acid synthase inhibitor

Cecille D. Browne,[1] Elizabeth J. Hindmarsh,[1,2] and Jeffrey W. Smith[3]
Cancer Research Center, Burnham Institute for Medical Research, La Jolla, California, USA

ABSTRACT **Orlistat, an antiobesity drug, is cytostatic and cytotoxic to tumor cells (1). The antitumor activity of orlistat can be attributed to its ability to inhibit the thioesterase domain of fatty acid synthase (FAS). The objective of the present study was to test the effect of orlistat on endothelial cell proliferation and angiogenesis. Orlistat inhibits endothelial cell FAS, blocks the synthesis of fatty acids, and prevents endothelial cell proliferation. More significantly, orlistat inhibits human neovascularization in an *ex vivo* assay, which suggests that it may be useful as an antiangiogenic drug. The mechanism of these effects can be traced to the fact that orlistat prevents the display of the vascular endothelial growth factor (VEGF) receptor (VEGFR2/KDR/Flk1) on the endothelial cell surface. Thus, orlistat is an antiangiogenic agent with a novel mechanism of action.—Browne, C. D., Hindmarsh, E. J., and Smith, J. W. Inhibition of endothelial cell proliferation and angiogenesis by orlistat, a fatty acid synthase inhibitor. *FASEB J.* 20, 2027–2035 (2006)**

Key Words: VEGF · KDR · neovascularization

Tumor angiogenesis is initiated when vascular endothelial growth factor (VEGF) binds to its receptor, VEGFR2/KDR/Flk1 (hereafter referred to as KDR), and activates a proliferative signaling cascade through the MAPK pathway (2, 3). The importance of this pathway as a drug target has been validated by the recent success of new antiangiogenesis drugs. For example, AVASTIN is a monoclonal antibody (mAb) that binds to VEGF and prevents this growth factor from binding to its cellular receptor (KDR) (4, 5). AVASTIN has been approved for treating patients with metastatic colon cancer (6). Another example is SUTENT (SU11248), a small molecule antagonist of the kinase domain of KDR (7). This drug has shown efficacy in both gastrointestinal stromal tumors (8) and metastatic renal cell carcinoma (9). Along with results from other VEGF and KDR antagonists, these findings provide clinical validation of the VEGF/KDR pathway as a drug target for halting tumor progression.

In a series of seemingly unrelated studies, we and others have begun to explore fatty acid synthase (FAS) as a drug target in tumor cells. FAS is the enzyme responsible for cellular synthesis of palmitate, the precursor of long-chain nonessential fatty acids (10–13). FAS, which contains seven separate enzymatic pockets, is situated as a head-to-tail dimer with the ketoacyl synthase and malonyl/acetyl transferase domains of one monomer working together with the dehydratase, enoyl reductase, ketoacyl reductase, acyl carrier protein, and thioesterase domains on the adjacent monomer (10–13). These enzymatic domains act sequentially to condense acetyl-coenzyme A with malonyl-coenzyme A to form a four-carbon intermediate. Six additional turns of the enzyme's cycle convert this intermediate to palmitate, which is then liberated from FAS by the action of the thioesterase domain (14).

Initially, FAS attracted interest as a drug target in oncology because it is up-regulated in most solid tumors, including those of the breast (15–17), prostate (18–20), and ovary (21, 22). Further validation of FAS as a target comes from a number of studies showing that a pharmacologic blockade of FAS is cytostatic and cytotoxic to tumor cells. A synthetic analog of cerulenin, c75, targets the condensing enzyme of FAS (23) and can suppress tumor cell proliferation and in some cases induce tumor cell apoptosis (23–29). We recently showed that orlistat, an approved antiobesity drug, is an unanticipated inhibitor of the thioesterase domain of FAS. Like c75, orlistat is cytotoxic and cytostatic to tumor cells *in vitro* and can inhibit tumor growth *in vivo* (1).

Here we show for the first time, that orlistat has antiproliferative effects on endothelial cells. We find that orlistat's antiproliferative effects on endothelial cells can be traced to its ability to prevent the cell surface expression of KDR, a mechanism of action that is distinct from other antiangiogenic agents. Our findings indicate that further development of orlistat as an

[1] These authors contributed equally to this work.

[2] Present address: Immunology and Genetics Division, John Curtin School of Medical Research, The Australian National University, Canberra ACT 0200, Australia. E-mail: liesa.hindmarsh@anu.edu.au

[3] Correspondence: Cancer Research Center, Burnham Institute for Medical Research, 10901 North Torrey Pines Rd., La Jolla, CA 92037, USA. E-mail: jsmith@burnham.org

doi: 10.1096/fj.05-5404com

antiangiogenic drug is warranted. Such drugs could provide important therapeutic options, different pharmacologic profiles, and perhaps improved efficacy over currently approved antiangiogenic agents.

MATERIALS AND METHODS

Materials

Orlistat (XENICAL, Roche Pharmaceuticals, Nutley, NJ) was extracted by solubilizing a tablet in ethanol (1 ml). The extract was centrifuged at 14,000 rpm for 5 min to remove insoluble excipients. Aliquots of orlistat (250 mM) were stored at −80°C until used.

Cell culture

Human umbilical vein endothelial cells (HUVEC; Clonetics, Cambrex Bioproducts, NJ) were maintained in Clonetics EGM-MV (normal growth medium) at 37°C in 5% CO_2. Cells were maintained in 150 mm tissue culture plates and used in assays between cell passages 3 and 8.

Probing FAS activity with activity-based profiling

HUVECs were washed twice, harvested into ice-cold PBS, and collected by centrifugation at 1000 rpm for 5 min. Cell pellets were stored at −80°C until needed. Pellets were thawed on ice, resuspended in 50 mM Tris-HCl, 150 mM NaCl (pH 8.0), and lysed by sonication. Cell fractions were separated by ultracentrifugation at 64,000 rpm for 1 h at 4°C. Soluble fractions were measured spectrophotometrically for protein content at 280 nm.

Serine hydrolase activity profiles were determined using fluorophosphonate (FP)-TAMRA; polyethylene glycol (PEG)-6-carboxytetramethylrhodamine (provided by ActivX Biosciences, La Jolla, CA) (30). Soluble protein fractions (25–60 μg) were incubated with orlistat (0–10 μM) or vehicle control for 30 min at room temperature. Fractions were then treated with 5 μM FP-TAMRA, for 1 h at room temperature. Reactions were stopped by addition of Laemmli buffer followed by boiling for 5 min. Nonspecific binding of the probe was assessed by using a protein sample preheated to 95°C for 10 min prior to labeling with FP-TAMRA. Labeled protein samples were resolved by SDS-PAGE, and fluorescent bands were visualized by scanning the gel with a Hitachi flatbed fluorescent scanner at 585 nm.

Effect of orlistat on the incorporation of acetate and malonyl-coenzyme A into cellular fatty acids

Inhibition of FAS activity in whole cells was measured by the incorporation of [^{14}C] acetate into fatty acids. HUVECs (1×10^5 cells/well in 24-well plates) were cultured in EGM-MV for 24 h and subsequently serum-starved for 16 h in EBM containing VEGF (40 ng/ml; Chemicon International, Temecula, CA). Serum-starved cells were treated with orlistat for 4 h. [^{14}C] acetate (50 μCi; Amersham Biosciences, Piscataway, NJ) was added to each well and incubated for 3 h at 37°C. Fatty acids were isolated by an initial hypotonic lysis of cells in 50 mM Tris, pH 7.5, followed by extraction in chloroform: water (1:1) for 30 min at room temperature. Lipid phases were dried under N_2 and extracted with butanol:water (1:1). The butanol phase was evaporated under N_2; radiolabeled lipids were counted by scintillation.

FAS inhibition in cell lysates was measured by fatty acid incorporation of [^{14}C] malonyl coenzyme A (CoA) (24). HUVECs were grown for 24 h in 150 mm dishes in EGM-MV. Cells were washed and harvested as before, centrifuged at 2000 rpm for 5 min and stored at −80°C. Cell pellets were lysed in hypotonic buffer (pH 7.5; 20 mM Tris, 1 mM DTT, 1 mM EDTA). Lysates were homogenized, sonicated, and spun at 14,000 rpm for 15 min at 4°C. Lysates (80 μl volumes) were treated with orlistat or vehicle for 1 h at room temperature. Treated lysates were added to a reaction mixture (1 ml; 500 μM NADPH, 167 μM acetyl CoA, 100 mM KCl) containing [^{14}C] malonyl CoA (0.4 μCi, Amersham Biosciences) and incubated at 37°C for 25 min. Subsequently, unlabeled malonyl CoA (100 μM) was added, and mixtures were reincubated for 30 min. Lipids were extracted, dried, and counted by scintillation as before.

Cell proliferation assays

HUVECs were plated at 2×10^3 cells/well in 96-well plates and cultured for 24 h in EGM-MV (100 μl/well). Where noted, cells were washed twice with PBS and incubated in serum-starvation medium (EBM with 0.2% FBS) for 24 h. Medium was replaced with EGM-MV or with starvation medium containing VEGF (40 ng/ml) for 24 h. Cells were incubated in the presence of orlistat (10 μM). Bromodeoxyuridine (BrdU) (100 μM) was added, and its uptake was measured using colorimetric Cell Proliferation ELISA (Roche).

Western blotting and immune-precipitation

HUVECs were seeded in 150 mm TC dishes at $1–2 \times 10^6$ cells per plate in EGM-MV. After 48 h, subconfluent monolayers were starved with 0.2% EBM basal medium containing 15 μM orlistat and incubated for an additional 18–20 h. Cells were then stimulated for 2–40 min with VEGF (50 ng/ml). Monolayers were washed three times with ice-cold PBS and lysed in 50 mM Tris-HCl, pH 7.5 (1% Triton X-100, 150 mM NaCl, 1.0 mM EGTA, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, and 1 mM sodium orthovanadate). Cell lysates were spun at 14,000 rpm for 15 min at 4°C. Clarified lysates were immune-precipitated with anti-KDR (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Immune-precipitates and lysates were immune-blotted with antibodies to KDR (Santa Cruz; Abcam, Cambridge, MA) and phosphorylated KDR (Santa Cruz). FAS immune-precipitation and immune-blotting were performed using antibodies against the FAS N-terminal region (Pharmingen, BD Biosciences, San Jose, CA).

Angiogenesis assays

This procedure is a modification of the placental vessel neovascularization assay developed by Brown et al. (31). Placentas were obtained within 48 h of delivery, having been stored at 4°C under sterile conditions. Superficial vessels, ~1–2 mm in diameter and 2–5 cm in length, were excised from the apical placenta surface and dissected into 1–2 mm fragments (but for each assay a consistent length was used). Medium 199 (M199; 3 mg/ml fibrinogen) was added to 48-well tissue culture plates. One vessel fragment was placed in the center of each well. Thrombin (0.25 U; Amersham) was added to induce clot formation. Gels were overlaid with M199 supplemented with 20% FBS, 0.1% ε-aminocaproic acid, 2 mM L-glutamine, and 1% penicillin/streptomycin/fungizone, plus orlistat (25 or 50 μM) or an equivalent volume of ethanol (vehicle control). Fumagillin (200 nM), a known inhibitor of angiogenesis (32), was also overlaid in designated

wells as a positive control. Vessels were cultured for 14–21 d, with daily medium replacement. Vessel fragments were stained with Phalloidin-AlexaFluor 594 (Molecular Probes, Eugene, OR) and quantified at 645 nm using the Cytofluor Fluorescence Plate Reader (Applied Biosystems, Foster City, CA). In pilot studies we found that, prior to the growth of explants, vessels embedded in fibrin typically yielded a baseline of fluorescence between 1000 and 2000 relative fluorescence units. Experiments on the growth of vessel explants were considered to be valid only if fumagillin could block the increase in fluorescence to baseline levels.

Vessel fragments were fixed in Carnoy solution and sectioned for immunostaining. Endogenous peroxidase activity was inhibited by incubation in 3% H_2O_2 in PBS for 10 min, and slides were trypsinized to enhance subsequent immunostaining (Digest-All 2, Zymed Laboratories, South San Francisco, CA). To demonstrate neovascularization, slides were blocked (Avidin/Biotin Blocking Kit, Vector Laboratories, Burlingame, CA), incubated overnight at 4°C with primary CD31 MAb (33.5 μg/ml; DakoCytomation, Carpinteria, CA), with biotinylated secondary antimouse IgG (10 μg/ml; Jackson IR USA, West Grove, PA) for 45 min at room temperature, and with Vectastain avidin-biotin complex (ABC) peroxidase-conjugated biotin–avidin complex (Vector) for 30 min. For FAS visualization, slides were incubated overnight at 4°C with rabbit anti-human FAS (2 μg/ml; IBL-America, Minneapolis, MN) and then with secondary anti-rabbit HRP conjugate (DakoCytomation) for 45 min at room temperature. Peroxidase activity was visualized with diaminobenzadine tetrahydrochloride substrate [3,3′-diaminobenzidine (DAB); Vector] for 10 min; slides were counterstained with hematoxylin.

Flow cytometry

HUVECs were grown in EGM-MV for 48 h and starved an additional 24 h in 0.2% EBM basal medium containing vehicle control or 15 μM orlistat. Monolayers were released from the culture plate with PBS containing EDTA, aliquoted at ~1 million cells per tube, and collected by microcentrifugation at 1000 rpm for 5 min. Prior to addition of primary antibody (Ab), nonspecific binding sites on cells were blocked with PBS containing 1% BSA. Cells were then incubated with primary Ab or an irrelevant isotype control (25 μg/ml) for 40 min on ice. Cells were spun at 2000 rpm for 2 min at 4°C and then resuspended in 100 μl PBS-BSA. Cell suspensions were incubated for 20 min at 4°C with 20 μg/ml of FITC-conjugated goat anti-mouse Ab (Santa Cruz). Following addition of PBS-BSA (100 μl) containing propidium iodide (10 μg/ml; Molecular Probes), samples were analyzed using BD FACSort 3-color Analyzer and BD CellQuest software.

RESULTS

Orlistat blocks the FAS thioesterase, fatty acid synthesis, and proliferation in HUVECs

The ability of orlistat to inhibit the FAS thioesterase in HUVECs was measured by activity-based profiling of the active site of the FAS thioesterase (1). HUVEC lysates were incubated with a concentration range orlistat (0–10 μM) and with FP-PEG-TAMRA, a probe that reacts with the serine nucleophile of the active site of the thioesterase (33), and therefore competes with orlistat for binding (1). The FAS thioesterase was covalently tagged with the activity-based probe (**Fig. 1*A***, *lane 1*) and orlistat competed for the labeling in a dose-dependent manner (Fig. 1*A*). In the HUVEC lysates, half-maximal inhibition occurred at ~130 nM orlistat, as determined by densitometry scanning. The TAMRA-labeled protein at ~230 kDa (Fig. 1*A*) has been identified as FAS in other cells lines using mass spectrometry (1). In this study we confirmed its identity in HUVECs by immune-precipitation with anti-FAS Ab (Fig. 1*B*), as we have done previously with tumor cells (1).

The effect of orlistat on the function of FAS in HUVECs was tested by measuring the incorporation of metabolic precursors into palmitate, the end product of FAS (1). Two types of studies were done. First, we measured the incorporation of [^{14}C] malonyl CoA into palmitate in lysates of HUVECs. The activity of FAS was measured in cell lysates because whole cells are not permeable to malonyl CoA. In this case, where the cell membrane was not a barrier to orlistat, the IC_{50} for inhibition of the incorporation of [^{14}C] malonyl CoA into palmitate fell between 250 and 500 nM (Fig. 1*C*), a value that is close to the IC_{50} of orlistat for inhibition of activity-based labeling of FAS (Fig. 1*A*).

In the second type of assay, we measured the effects of orlistat on the incorporation of [^{14}C] acetate into palmitate in live HUVECs. Unlike malonyl CoA, acetate can traverse the plasma membrane. In live cells orlistat had minimal effect on the incorporation of acetate into fatty acids, unless cells were stimulated with VEGF. VEGF stimulated the incorporation of [^{14}C] acetate into fatty acids, suggesting that an increase in fatty acid synthesis may be a consequence of VEGF signaling. Importantly, 15 μM of orlistat completely ablates the VEGF-induced increase in the incorporation of acetate into palmitate in whole cells (Fig. 1*D*).

The effect of orlistat on endothelial cell proliferation was measured by assessing the incorporation of BrdU in response to VEGF. Proliferation of HUVECs was challenged with a concentration range of orlistat, with the IC_{50} equaling ~1–3 μM (Fig. 1*E*). We also tested the effects of orlistat on apoptosis in HUVECs. In this instance, we compared the effects of orlistat on cell death in proliferating vs. contact-inhibited HUVECs. Cells were treated with a dose range of orlistat, and cell death was measured 48 h following treatment (Fig. 1*F*). The level of cell death was normalized to values obtained with untreated cultures of HUVECs. While orlistat elicits extensive cell death in proliferating HUVECs, the drug is without effect on nonproliferating HUVECs. Importantly, cell death is not observed until 48 h after treatment with orlistat, and the effects of orlistat on other cell processes were made well before this time. Therefore, the effects of orlistat on fatty acid synthesis and proliferation are not due to a toxic effect of the drug.

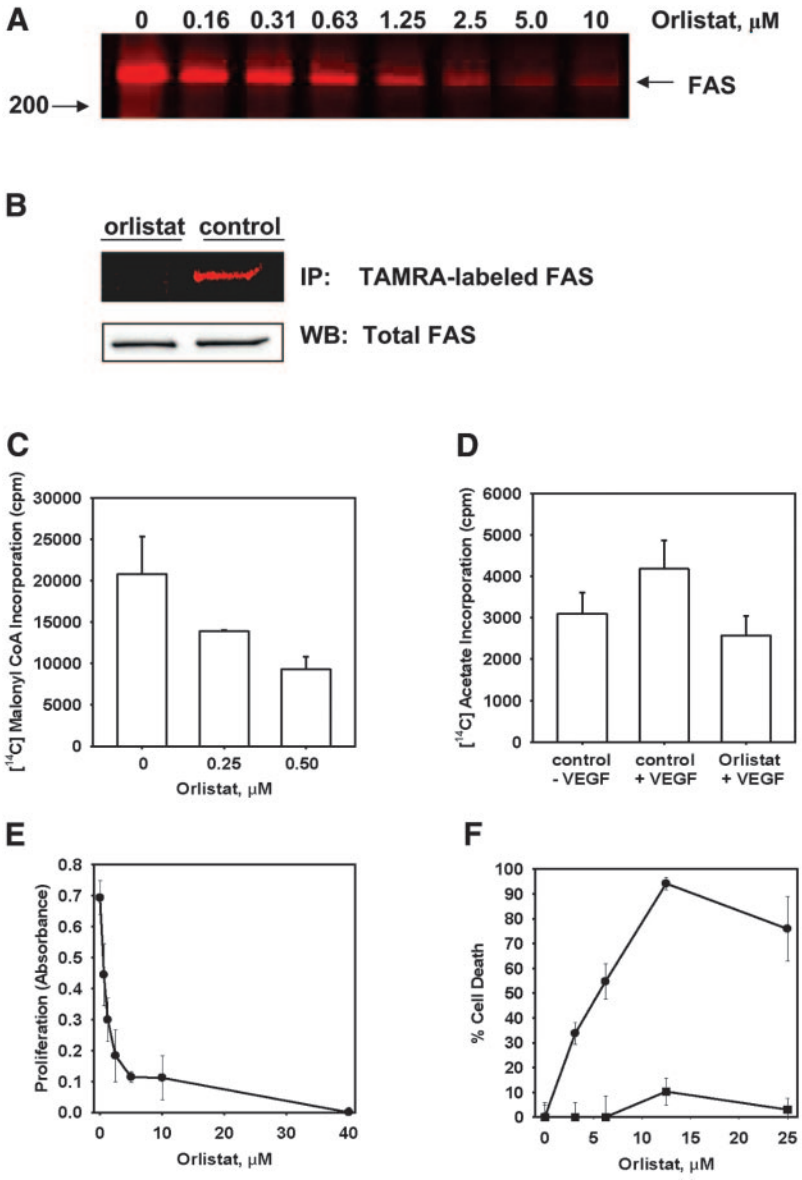
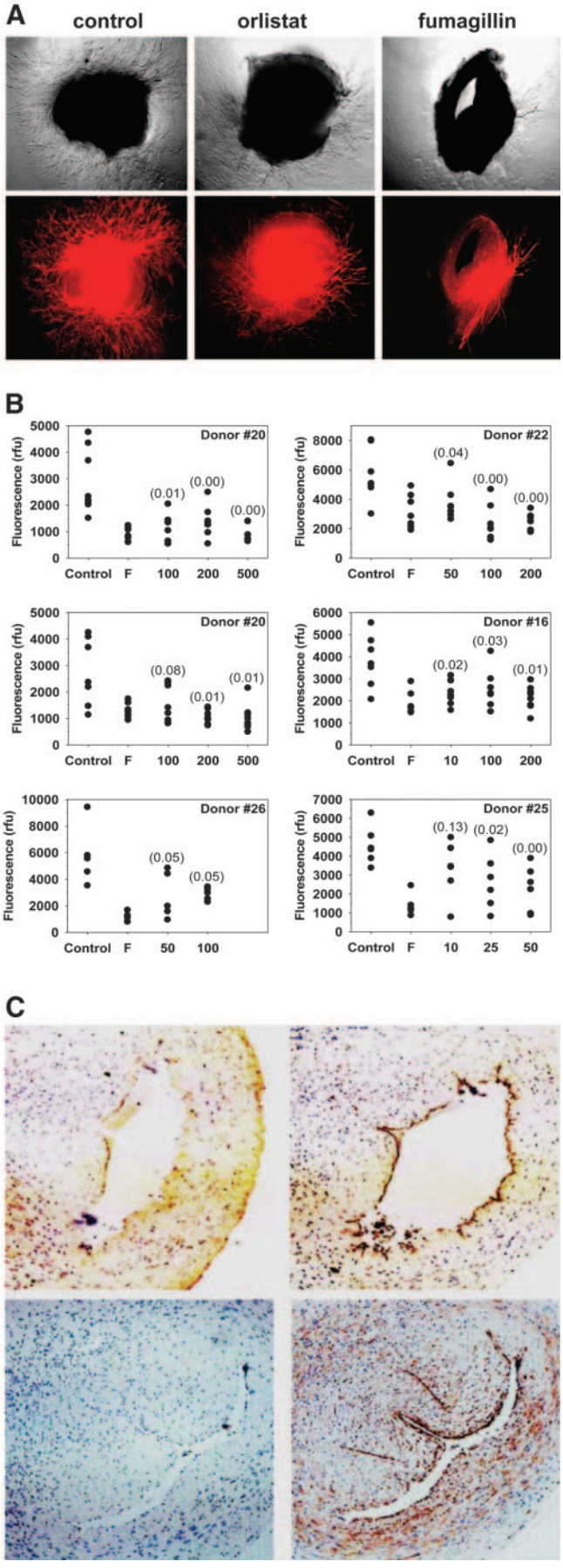


Figure 1. Orlistat inhibits the thioesterase domain of fatty acid synthase and inhibits the metabolic function of FAS in human umbilical vein endothelial cells. *A)* The effect of orlistat on the FAS thioesterase was probed in HUVEC lysates that were treated with orlistat (0–10 μM) or vehicle control for 30 min. The treated lysates were exposed to the activity-based probe, FP-TAMRA (5 μM), resolved using SDS-PAGE and visualized by scanning with a Hitachi fluorescent gel scanner. *B)* Lysates treated with orlistat or control and exposed to FP-TAMRA (5 μM) were immune-precipitated with antibodies against FAS and resolved using SDS-PAGE. Bands were visualized by scanning with a Hitachi fluorescent gel scanner and the gel was subsequently transferred onto nitrocellulose paper and immunoblotted with antibodies against FAS. *C)* The effect of orlistat on the incorporation of [^{14}C] malonyl CoA was measured in lysates of HUVECs. The lysates, containing FAS, were treated with orlistat or vehicle for 1 h. The lysates were subsequently incubated with [^{14}C] malonyl CoA for 25 min at 37°C to allow the malonyl CoA to incorporate into palmitate. Fatty acids were extracted from each sample and quantified by scintillation counting. Triplicate wells were assigned for each condition and statistically significant differences were observed between control treated lysate and each of orlistat-treated lysate ($P<0.2$ for 0.25 μM and $P<0.05$ for 0.5 μM). *D)* The effect of orlistat on the incorporation of acetate into fatty acids was probed in live HUVECs. The cells (1×10^5 cells/well in 24-well plates) were cultured in EGM-MV for 24 h and subsequently serum-starved for 16 h in EBM containing VEGF (40 ng/ml; Chemicon International, Temecula, CA). Serum-starved cells were treated with 15 μM orlistat for 4 h. [^{14}C] acetate was added to each well and incubated for 3 h at 37°C. After incubation with [^{14}C] acetate, fatty acids were extracted and quantified by scintillation counting. Six wells were designated as control + VEGF; three wells were designated as orlistat + VEGF. Statistically significant differences were observed between these two conditions ($P<0.05$). Three wells were grown without VEGF as growth control. *E)* The effect of orlistat on the proliferation of HUVECs was determined by measuring the incorporation of BrdU following treatment of cells with the drug. HUVECs were seeded in 96-well plates at a density of 2000 cells/well and cultured in endothelial growth media for 24 h at 37°C. Cells were subsequently cultured in media containing EBM and 2% FCS for 24 h. Culture media was replaced with EBM containing 2% FCS and VEGF (20 ng/ml) to stimulate proliferation. At this time cells were treated with orlistat (0–40 μM) and BrdU (100 μM) was added to track DNA synthesis. After a 24-h incubation the incorporation of BrdU uptake was measured using colorimetric Cell Proliferation ELISA (Roche) as described in Materials and Methods. Triplicate wells were used for each condition. SE in three repetitions of this experiment was typically 10%. *F)* The effect of orlistat on endothelial cell death was also measured across a concentration range of the drug. HUVECs were grown to 60% confluency (proliferating ●) or 100% confluency (contact-inhibited ■) and then exposed to orlistat for 48 h. Cell death was measured with the Roche Cell Death ELISA kit. Each point is the average of triplicate data points. SE was less than 12% for each point.

Orlistat inhibits neovascularization in a model of human angiogenesis

The antiproliferative effect of orlistat on HUVECs led us to test its effects on neovascularization in an *ex vivo* model of explanted human blood vessels (31). Human placental vessels were embedded within fibrin clots, and the sprouting of new vessels from the explants was monitored. Vessel explants were cultured in the presence or absence of orlistat or fumagillin, a compound known to inhibit angiogenesis (32). When visualized by bright-field microscopy, the degree of new vessel sprouts was lower in inhibitor-treated vs. control vessels (**Fig. 2*A***, *upper panel*). To quantify the degree of vessel spouting, the vessels were stained with fluorescent phalloidin, which binds to actin within the newly sprouted vessels (Fig. 2*A*, *lower panel*). We found that staining of explants immediately after embedding them



in fibrin, and prior to the growth of explants, yielded far less staining with fluorescent phalloidin, with the relative fluorescence measures being closer to 500–2000 RFU rather than the values of 2500–8000, obtained when new vessels had grown.

The effect of orlistat on the growth of new vessels was measured with explants obtained from five donors (resulting in six repetitions). In each case fumagillin was used as a positive control, which decreased the labeling fluorescent phalloidin and indicated the inhibition of new vessel growth. In all cases, the inhibitory effect of fumagillin was statistically significant with P values < 0.01. In 30–40% of the placentas that we obtained, vessel growth was absent or was not inhibited by fumagillin. Results from these placentas are not included in our analysis. The growth of new vessels was also challenged with multiple concentrations of orlistat (Fig. 2*B*). For each condition, five to seven explants were studied. The effects of orlistat on vessel growth were generally dose-dependent and were statistically significant (P values for the Student's t test are shown above each data set, Fig. 2*B*). The concentrations of fumagillin and orlistat required to elicit effects were substantially higher than reports in other types of angiogenesis assays (1, 32), almost certainly reflecting poor penetration of these drugs into the solid fibrin clot. Altogether, the results in Fig. 2*B* strongly suggest that orlistat can block angiogenesis in human vessels.

To confirm the expression of FAS in placental vessel explants, cross sections of vessel fragments were stained with antibodies against FAS and CD31, an endothelial marker. Positive staining of CD31 (Fig. 2*C*, *upper right panel*) highlights the position of a vessel within surrounding placental tissue. Immunostaining with anti-FAS Ab showed FAS in the endothelium and in the surrounding placental vessel cells (*lower right panel*).

Figure 2. Inhibition of neovascularization in human placental vessels by orlistat. *A)* A model of sprouting of placental vessels implanted in fibrin was used to determine if orlistat treatment blocked neovascularization. Vessels were cultured for 14–21 d in medium containing vehicle (*left panel*), orlistat (50 μM), or fumagillin (200 nM). Vessels were visualized by staining actin within the vessels with phalloidin. *B)* The effect of orlistat on neovascularization was measured in vessels from five separate donors. Two experiments were performed with explants from Donor #20. Placental explants were embedded in fibrin as described in Materials and Methods and then treated with vehicle, fumagillin as a positive control, or with orlistat. The growth of explants was measured by staining vessel fragments with Phalloidin-AlexaFluor 594 and subsequent quantification at 645 nm as described in Materials and Methods. Each data point (●) represents the extent of neovascularization from a single explant. The statistical significance of each treatment was measured by comparing the control group to the treated groups with the Student's t test. P values are noted above each data set in parentheses. *C)* The expression of FAS was assessed in cross sections of excised human placental blood vessels by immunohistochemistry. Vessels were stained with an anti-CD31 (*upper right*) or anti-FAS Ab (*lower right*), or with irrelevant isotype control (*left panels*).

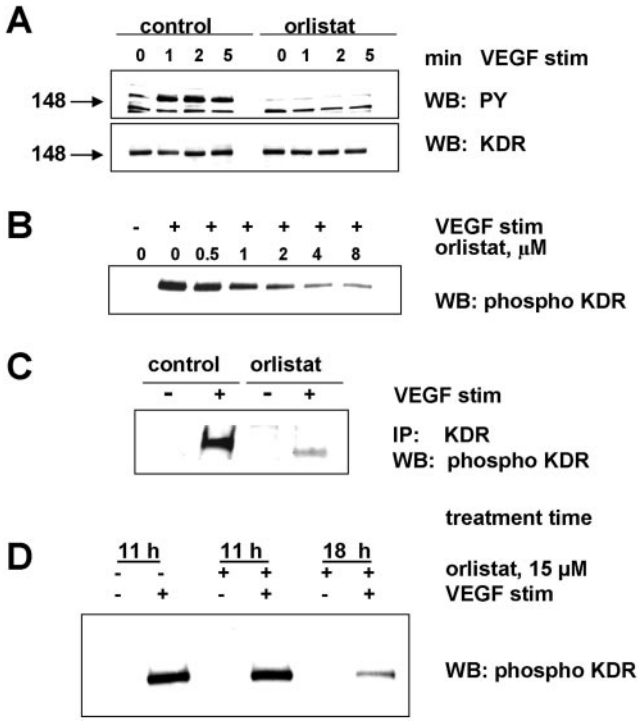


Figure 3. Orlistat inhibits the activity of KDR in human endothelial cells. *A)* HUVECs were serum-starved with EBM plus 0.2% FBS with or without orlistat (15 μM) for 18 h. Cells were stimulated with VEGF (50 ng/ml) for 1, 2, and 5 min. Lysates were immunoblotted with antiphosphotyrosine (*upper panel*) and with anti-KDR (*middle panel*). *B)* The effect of orlistat on KDR phosphorylation was examined in HUVECs treated with orlistat (0.5–8 μM) for 18 h. Cells were then stimulated for 5 min with VEGF (50 ng/ml, and lysed as described in Materials and Methods). Cell lysates were immunoblotted with an antiphospho-KDR Ab. *C)* To confirm the observations in (*A*), the effect of orlistat on KDR phosphorylation was examined as described for (*A*) and the cell lysates were immune-precipitated with anti-KDR Ab. These precipitates were transferred to nitrocellulose and then probed with antiphospho KDR Ab. *D)* The effect of orlistat on KDR phosphorylation was examined as a function of time. HUVECs were incubated with orlistat for either 11 or 16 h, and then stimulated with VEGF as described above. Cells were lysed, separated by SDS-PAGE, and transferred to nitrocellulose. The blots were probed with Ab against the phosphorylated form of KDR.

Specific staining was absent with irrelevant isotype control antibodies (*upper and lower left panels*).

Orlistat inhibits KDR function in HUVECs

To examine the mechanism by which orlistat prevents endothelial cell proliferation, we measured the phosphorylation of KDR in response to VEGF. Endothelial cells were stimulated with VEGF, lysed, and probed with a general Ab against phosphotyrosine and, subsequently, with Ab against KDR. Orlistat blocked the VEGF-stimulated phosphorylation of a band with the mass equivalent to KDR (**Fig. 3*A***, *top panel*) but was without effect on the total levels of KDR in cells (Fig. 3*A*, *lower panel*). To determine if the band phosphorylated in response to VEGF is KDR, a similar blot was probed with antibodies specific for phosphorylated KDR (Fig. 3*B*). As shown in this figure, orlistat blocked the VEGF-stimulated phosphorylation of KDR in a dose-dependent manner, with an apparent cellular IC_{50} of ~1 μM (Fig. 3*B*). These findings were confirmed by immunoblotting an immune-precipitation of KDR, followed by immunoblotting with Ab specific for phosphorylated KDR (Fig. 3*C*). The effects of orlistat were time-dependent; an ~18 h treatment with the drug was required to block the phosphorylation of KDR in HUVECs (Fig. 3*D*).

Orlistat inhibits the cell-surface display of KDR in HUVECs and its association with Gαq11

The effects of orlistat on the cell surface distribution of KDR were examined by flow cytometry (**Fig. 4**). Orlistat reduced expression of KDR on the cell surface (*left panel*), but had no effect on other membrane markers like CD31 (*middle panel*) or the αvβ3 integrin (*right panel*). Therefore, orlistat elicits a rather selective clearance of KDR from the cell surface.

To further probe the effect of orlistat on KDR function at the cell surface, we examined its association with Gαq11, a member of the heterotrimeric G protein family that is required for KDR signaling. In fact, the physical association between KDR and Gαq11 is required for VEGF-induced proliferation (34). Coimmune-precipitation experiments were conducted to determine if orlistat disrupts the interaction of Gαq11 with KDR. HUVECs were stimulated with VEGF, which induces the association of KDR and Gαq11, and then lysed at various times after treatment. The lysates were immune-precipitated with Ab against Gαq11, separated

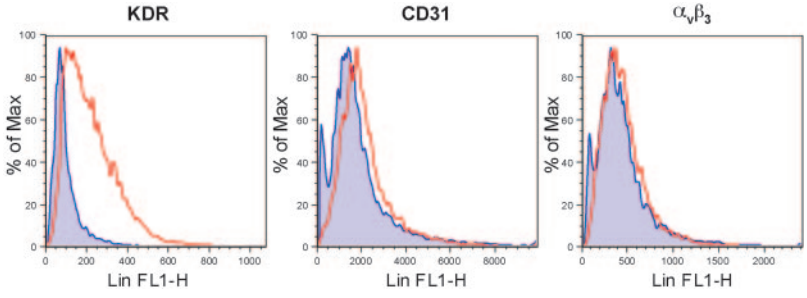


Figure 4. Orlistat prevents the display of KDR on the cell surface. Flow cytometry was used to measure the effects of orlistat on the cell surface expression of KDR. HUVECs were treated with orlistat, harvested from dishes, and then stained with antibodies against KDR, CD31, and αvβ3 as described in Materials and Methods. FITC-conjugated secondary Ab was used to detect cell surface staining for each protein. Staining was performed in the absence of fixation. Staining of control HUVECs is shown in red, and staining of orlistat-treated cells is shown with blue shading.

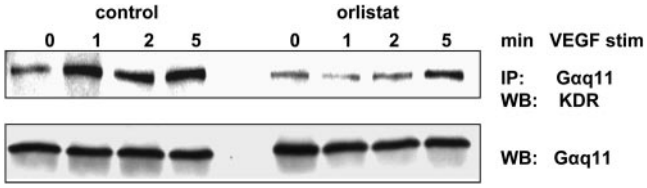


Figure 5. Orlistat disrupts the association between KDR and Gαq11. The association between KDR and Gαq11 was examined by coimmune-precipitation studies. HUVECs were temporarily starved by culturing them in EBM containing 0.2% FBS with or without orlistat (15 μM) for 18 h. Then, cells were stimulated with VEGF (50 ng/ml) and lysed at various times (0–5 min). HUVEC lysates were immune-precipitated with anti-Gαq11 Ab, and the lysates were transferred to nitrocellulose membranes. The blots were probed with antibodies against KDR or Gαq11.

on SDS gels, and transferred to nitrocellulose. Western blots were performed to detect the presence of KDR in the immune-precipitates. In the absence of VEGF we observed a basal level of association between KDR and Gαq11. In the minutes following stimulation with VEGF, the levels of KDR associated with Gαq11 increased (**Fig. 5**, *left panel*). In contrast, the VEGF-stimulated increase in this association was less profound and delayed in orlistat-treated cells (Fig. 5, *right panel*). The same blot was probed with antibodies against Gαq11, showing that the amount of this protein that was immune-precipitated is essentially equivalent in all samples (Fig. 5, *lower panel*). These observations lend additional support to the idea that orlistat disrupts the proper display and subsequent protein associations at the cell surface.

DISCUSSION

In this study we find that orlistat, a FAS inhibitor, is an antiangiogenic agent. Orlistat: 1) blocks the FAS thioesterase and inhibits fatty acid synthesis in endothelial cells; 2) prevents endothelial cell proliferation and neovascularization; and 3) blocks VEGF-induced activation of KDR by clearing the receptor from the cell surface. The current drugs that antagonize this signaling pathway block either the binding of VEGF to KDR (AVASTIN, Genentech), or block the tyrosine kinase activity of KDR (e.g., SUTENT, Pfizer). Therefore, orlistat defines a new class of antiangiogenic agent that prevents the cell surface expression of KDR.

Orlistat blocks VEGF-induced proliferation of HUVECs and induces apoptosis in proliferating HUVECs at concentrations in the low micromolar range. Because orlistat blocked fatty acid biosynthesis in whole cells in the same concentration range, the simplest interpretation of these findings is that the antiproliferative and proapoptotic effects of orlistat result from its inhibition of FAS. This conclusion is also supported by our observation that an siRNA knock down of FAS in HUVECs also blocks VEGF-stimulated proliferation (data not shown). While similar concentrations of orlistat (~1–5 μM) halt proliferation and induce apoptosis, the two effects are observed at different times. A halt to proliferation is observed within 24 h after treatment with drug, whereas the apoptotic effects are not observed until 48 h after treatment. These observations are consistent with our prior finding in tumor cells showing that orlistat-induced apoptosis also follows a halt in cell proliferation (1). In that report we also showed that orlistat fails to elicit apoptosis in normal human fibroblasts. Altogether, these findings argue against a general toxicity of orlistat and suggest that its effects are the result of interception of specific cellular pathways.

In endothelial cells, one of these pathways involves signaling by VEGF through its cell surface receptor KDR. In the presence of orlistat, very little KDR is detected on the HUVEC surface by flow cytometry, even though the surface display of other common cell surface proteins is not affected. The fact that orlistat disrupts the VEGF-induced association between Gαq11 and KDR is also consistent with the idea that the drug prevents the proper display of KDR on the cell. It was previously reported that the G protein Gαq11 physically associates with KDR in response to VEGF and that this association is necessary for VEGF-mediated proliferation of endothelial cells (34). Interestingly, we find that orlistat diminishes the level of association between Gαq11 and KDR, and substantially delays the increase in this association that results from treatment with VEGF. Our findings differ slightly from previous analysis of the association of KDR with Gαq11. We observed a basal association between the two proteins in the absence of VEGF stimulation. This type of ligand-independent interaction was not observed in previous reports (34). Our findings on the links between KDR and Gαq11 are qualitatively similar to prior reports (34), but differ in that we observed association at slightly different times after VEGF stimulation. Although there is no explanation for these distinctions, we suspect they may relate to differences in protocol. Moreover, the differences that are observed have no bearing on the interpretation of the present study.

The observation that orlistat disrupts the cell surface expression of KDR generally parallels ideas put forth by others showing that a pharmacologic blockade of FAS interferes with cellular localization of the human growth factor (HER2) receptor, $p185^{HER2}$, in tumor cells (35). Consequently, it has been suggested that FAS inhibition disrupts targeting of lipid domain-associated $p185^{HER2}$ to the cell membrane (36). Altogether then, these observations suggest that orlistat disrupts the cellular trafficking, or cellular localization, of growth factor receptors. It is tempting to speculate that such misregulation is linked to the reduction in palmitate synthesis. While a precise mechanism for improper trafficking will require further study, it may relate to the inability of orlistat-treated cells to assemble lipid rafts, or the disruption of protein palmitoylation, which targets many signaling proteins to the inner leaflet of the plasma membrane.

The work presented here shows, for the first time, a requirement for FAS in VEGF-stimulated endothelial cell proliferation and angiogenesis. Moreover, orlistat, an inhibitor of FAS, is identified as a potential antiangiogenic agent. The drug blocks VEGF-stimulated proliferation of endothelial cells by a unique mechanism of action; the down-regulation of KDR from the cell surface. Orlistat can block neovascularization in explants of human placental vessels, an *ex vivo* model of human angiogenesis. In the original report characterizing the growth of vessel explants from placental vessles (31), the authors showed a clear contribution of both the VEGF and basic fibroblast growth factor (bFGF) pathways. Consequently, the inhibitory effects of orlistat in this assay may extend beyond the drugs ability to down-regulate the display of KDR on the cell surface. Therefore, we cannot exclude the possibility that orlistat has similar effects of the bFGF pathway. Nevertheless, our findings provide an alternative therapeutic route for antiangiogenic therapy. In addition, the fact that orlistat kills proliferating HUVECs, but not contact-inhibited HUVECs, suggests that the drug could selectively hit the pathogenic formation of new vessels *in vivo*. FJ

We thank the staff of Scripps Encinitas Birth Pavilion for providing placenta. This work was supported by grants from the Department of Defense Breast Cancer Center of Excellence, DAMD17–02-0693, and from the National Institutes of Health (U54 RR020843–02, P01 CA82713–06, R 01 CA106582–02). E. J. H. was supported by a fellowship (6FB-0037) from the California Breast Cancer Research Program.

REFERENCES

1. Kridel, S. J., Axelrod, F., Rozenkrantz, N., and Smith, J. W. (2004) Orlistat is a novel inhibitor of fatty acid synthase with antitumor activity. *Cancer Res.* **64,** 2070–2075
2. D'Angelo, G., Struman, I., Martial, J., and Weiner, R. I. (1995) Activation of mitogen-activated protein kinases by vascular endothelial growth factor and basic fibroblast growth factor in capillary endothelial cells is inhibited by the antiangiogenic factor 16-kDa N-terminal fragment of prolactin. *Proc. Natl. Acad. Sci. U. S. A.* **92,** 6374–6378
3. Witte, L., Hicklin, D. J., Zhu, Z., Pytowski, B., Kotanides, H., Rockwell, P., and Bohlen, P. (1998) Monoclonal antibodies targeting the VEGF receptor-2 (Flk1/KDR) as an anti-angiogenic therapeutic strategy. *Cancer Metastasis Rev.* **17,** 155–161
4. Kim, K. J., Li, B., Houck, K., Winer, J., and Ferrara, N. (1992) The vascular endothelial growth factor proteins: identification of biologically relevant regions by neutralizing monoclonal antibodies. *Growth Factors* **7,** 53–64
5. Ferrara, N., Hillan, K. J., Gerber, H. P., and Novotny, W. (2004) Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat. Rev. Drug Discov.* **3,** 391–400
6. USFDA (2004) FDA approves first angiogenesis inhibitor to treat colorectal cancer. *FDA News*, PO4–23, www.fda.gov/bbs/topics/newss/2004/new01027.html
7. Mendel, D. B., Laird, A. D., Xin, X., Louie, S. G., Christensen, J. G., Li, G., Schreck, R. E., Abrams, T. J., Ngai, T. J., Lee, L. B., Murray, L. J., Carver, J., Chan, E., Moss, K. G., Haznedar, J. O., Sukbuntherng, J., Blake, R. A., Sun, L., Tang, C., Miller, T., Shirazian, S., McMahon, G., and Cherrington, J. M. (2003) In vivo antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacodynamic relationship. *Clin. Cancer Res.* **9,** 327–337
8. Demetri, G. D. v. O., A. T., Blackstein, M., Garrett, C., Shah, M., Heinrich, M., McArthur, G. Judson, I, Baum, C. M., and Casali, P. G. (2005) Phase 3, multicenter, randomized, double-blind, placebo-controlled trial of SU11248 in patients (pts) following failure of imatinib for metastatic GIST. In 2005 ASCO Annual Meeting, Orlando, FL
9. Motzer, R. J. R., B. I, Michaelson, M.D., Redman, G.G., Hudes, G.R., Wilding, G., Bukowski, R.M., George, D.J., Kim, S. T., and Baum, C. M. (2005) Phase 2 trials of SU11248 show antitumor activity in second-line therapy for patients with metastatic renal cell carcinoma (RCC). In 2005 ASCO Annual Meeting, Orlando, FL
10. Tsukamoto, Y., Wong, H., Mattick, J. S., and Wakil, S. J. (1983) The architecture of the animal fatty acid synthetase complex. IV. Mapping of active centers and model for the mechanism of action. *J. Biol. Chem.* **258,** 15312–15322
11. Chirala, S. S., Jayakumar, A., Gu, Z. W., and Wakil, S. J. (2001) Human fatty acid synthase: role of interdomain in the formation of catalytically active synthase dimer. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 3104–3108
12. Rangan, V. S., Joshi, A. K., and Smith, S. (2001) Mapping the functional topology of the animal fatty acid synthase by mutant complementation in vitro. *Biochemistry* **40,** 10792–10799
13. Joshi, A. K., Witkowski, A., and Smith, S. (1997) Mapping of functional interactions between domains of the animal fatty acid synthase by mutant complementation in vitro. *Biochemistry* **36,** 2316–2322
14. Mattick, J. S., Nickless, J., Mizugaki, M., Yang, C. Y., Uchiyama, S., and Wakil, S. J. (1983) The architecture of the animal fatty acid synthetase. II. Separation of the core and thioesterase functions and determination of the N-C orientation of the subunit. *J. Biol. Chem.* **258,** 15300–15304
15. Alo, P. L., Visca, P., Marci, A., Mangoni, A., Botti, C., and Di Tondo, U. (1996) Expression of fatty acid synthase (FAS) as a predictor of recurrence in stage I breast carcinoma patients. *Cancer* **77,** 474–482
16. Nakamura, I., Kimijima, I., Zhang, G. J., Onogi, H., Endo, Y., Suzuki, S., Tuchiya, A., Takenoshita, S., Kusakabe, T., and Suzuki, T. (1999) Fatty acid synthase expression in Japanese breast carcinoma patients. *Int. J. Mol. Med.* **4,** 381–387
17. Wang, Y., Kuhajda, F. P., Li, J. N., Pizer, E. S., Han, W. F., Sokoll, L. J., and Chan, D. W. (2001) Fatty acid synthase (FAS) expression in human breast cancer cell culture supernatants and in breast cancer patients. *Cancer Lett.* **167,** 99–104
18. Swinnen, J. V., Roskams, T., Joniau, S., Van Poppel, H., Oyen, R., Baert, L., Heyns, W., and Verhoeven, G. (2002) Overexpression of fatty acid synthase is an early and common event in the development of prostate cancer. *Int. J. Cancer.* **98,** 19–22
19. Rossi, S., Graner, E., Febbo, P., Weinstein, L., Bhattacharya, N., Onody, T., Bubley, G., Balk, S., and Loda, M. (2003) Fatty acid synthase expression defines distinct molecular signatures in prostate cancer. *Mol. Cancer Res.* **1,** 707–715
20. Bandyopadhyay, S., Pai, S. K., Watabe, M., Gross, S. C., Hirota, S., Hosobe, S., Tsukada, T., Miura, K., Saito, K., Markwell, S. J., Wang, Y., Huggenvik, J., Pauza, M. E., Iiizumi, M., and Watabe, K. (2005) FAS expression inversely correlates with PTEN level in prostate cancer and a PI 3-kinase inhibitor synergizes with FAS siRNA to induce apoptosis. *Oncogene* **24,** 5389–5395
21. Pizer, E. S., Wood, F. D., Heine, H. S., Romantsev, F. E., Pasternack, G. R., and Kuhajda, F. P. (1996) Inhibition of fatty acid synthesis delays disease progression in a xenograft model of ovarian cancer. *Cancer Res.* **56,** 1189–1193
22. Gansler, T. S., Hardman, W., 3rd, Hunt, D. A., Schaffel, S., and Hennigar, R. A. (1997) Increased expression of fatty acid synthase (OA-519) in ovarian neoplasms predicts shorter survival. *Hum. Pathol.* **28,** 686–692
23. Kuhajda, F. P., Pizer, E. S., Li, J. N., Mani, N. S., Frehywot, G. L., and Townsend, C. A. (2000) Synthesis and antitumor activity of an inhibitor of fatty acid synthase. *Proc. Natl. Acad. Sci. U. S. A.* **97,** 3450–3454
24. Kuhajda, F. P., Jenner, K., Wood, F. D., Hennigar, R. A., Jacobs, L. B., Dick, J. D., and Pasternack, G. R. (1994) Fatty acid synthesis: a potential selective target for antineoplastic therapy. *Proc. Natl. Acad. Sci. U. S. A.* **91,** 6379–6383

25. Furuya, Y., Akimoto, S., Yasuda, K., and Ito, H. (1997) Apoptosis of androgen-independent prostate cell line induced by inhibition of fatty acid synthesis. *Anticancer Res.* **17,** 4589–4593
26. Pizer, E. S., Jackisch, C., Wood, F. D., Pasternack, G. R., Davidson, N. E., and Kuhajda, F. P. (1996) Inhibition of fatty acid synthesis induces programmed cell death in human breast cancer cells. *Cancer Res.* **56,** 2745–2747
27. Pizer, E. S., Chrest, F. J., DiGiuseppe, J. A., and Han, W. F. (1998) Pharmacological inhibitors of mammalian fatty acid synthase suppress DNA replication and induce apoptosis in tumor cell lines. *Cancer Res.* **58,** 4611–4615
28. Li, J. N., Gorospe, M., Chrest, F. J., Kumaravel, T. S., Evans, M. K., Han, W. F., and Pizer, E. S. (2001) Pharmacological inhibition of fatty acid synthase activity produces both cytostatic and cytotoxic effects modulated by p53. *Cancer Res.* **61,** 1493–1499
29. Heiligtag, S. J., Bredehorst, R., and David, K. A. (2002) Key role of mitochondria in cerulenin-mediated apoptosis. *Cell Death Differ.* **9,** 1017–1025
30. Patricelli, M. P., Giang, D. K., Stamp, L. M., and Burbaum, J. J. (2001) Direct visualization of serine hydrolase activities in complex proteomes using fluorescent active site-directed probes. *Proteomics* **1,** 1067–1071
31. Brown, K. J., Maynes, S. F., Bezos, A., Maguire, D. J., Ford, M. D., and Parish, C. R. (1996) A novel in vitro assay for human angiogenesis. *Lab. Invest.* **75,** 539–555
32. Sin, N., Meng, L., Wang, M. Q., Wen, J. J., Bornmann, W. G., and Crews, C. M. (1997) The anti-angiogenic agent fumagillin covalently binds and inhibits the methionine aminopeptidase, MetAP-2. *Proc. Natl. Acad. Sci. U. S. A.* **94,** 6099–6103
33. Liu, Y., Patricelli, M. P., and Cravatt, B. F. (1999) Activity-based protein profiling: the serine hydrolases. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 14694–14699
34. Zeng, H., Zhao, D., Yang, S., Datta, K., and Mukhopadhyay, D. (2003) Heterotrimeric G alpha q/G alpha 11 proteins function upstream of vascular endothelial growth factor (VEGF) receptor-2 (KDR) phosphorylation in vascular permeability factor/VEGF signaling. *J. Biol. Chem.* **278,** 20738–20745
35. Menendez, J. A., Vellon, L., Mehmi, I., Oza, B. P., Ropero, S., Colomer, R., and Lupu, R. (2004) Inhibition of fatty acid synthase (FAS) suppresses HER2/neu (erbB-2) oncogene overexpression in cancer cells. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 10715–10720
36. Menendez, J. A., Vellon, L., and Lupu, R. (2005) Targeting fatty acid synthase-driven lipid rafts: a novel strategy to overcome trastuzumab resistance in breast cancer cells. *Med. Hypotheses.* **64,** 997–1001

Received for publication November 15, 2005.
Accepted for publication May 25, 2006.