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# The Fatty Acid Synthase Inhibitor Orlistat Reduces the Growth and Metastasis of Orthotopic Tongue Oral Squamous Cell Carcinomas

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## Abstract

Fatty acid synthase (FASN) is the biosynthetic enzyme responsible for the endogenous synthesis of fatty acids. It is downregulated in most normal cells, except in lipogenic tissues such as liver, lactating breast, fetal lung, and adipose tissue. Conversely, several human cancers, including head and neck squamous cell carcinomas (HNSCC), overexpress FASN, which has been associated with poor prognosis and recently suggested as a metabolic oncoprotein. Orlistat is an irreversible inhibitor of FASN activity with cytotoxic properties on several cancer cell lines that inhibits tumor progression and metastasis in prostate cancer xenografts and experimental melanomas, respectively. To explore whether the inhibition of FASN could impact oral tongue squamous cell carcinoma (OTSCC) metastatic spread, an orthotopic model was developed by the implantation of SCC-9 ZsGreen LN-1 cells into the tongue of BALB/c nude mice. These cells were isolated through *in vivo* selection, show a more invasive behavior *in vitro* than the parental cells, and generate orthotopic tumors that spontaneously metastasize to cervical lymph nodes in 10 to 15 days only. SCC-9 ZsGreen LN-1 cells also exhibit enhanced production of MMP-2, ERBB2, and CDH2. The treatment with orlistat reduced proliferation and migration, promoted apoptosis, and stimulated the secretion of $VEGFA_{165b}$ by SCC-9 ZsGreen LN-1 cells. *In vivo*, the drug was able to decrease both the volume and proliferation indexes of the tongue orthotopic tumors and, importantly, reduced the number of metastatic cervical lymph nodes by 43%. These results suggest that FASN is a potential molecular target for the chemotherapy of patients with OTSCC. *Mol Cancer Ther; 13(3); 585–95. ©2013 AACR.*

## Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide (1, 2), with a 5-year survival rate of 50% that remains relatively unchanged for the past three decades (2, 3). The oral tongue squamous cell carcinoma (OTSCC) is the most common cancer of the oral cavity (4) characterized by a high incidence of metastasis to the draining lymph nodes (5). Currently, the presence of metastasis in the neck lymph nodes has been considered the most important prognostic factor for OTSCC and responsible for a decrease in the overall survival rates by nearly 50% (6–8). Of note, 10% to 50% of the patients with OTSCC without clinical evidence of neck lymph node metastasis (N0) may present occult metastatic foci (8–10). Local recurrences and second primary tumors also negatively impact the prognosis of patients with OTSCC, which usually present significantly worse prognosis than those with squamous cell carcinomas of the oropharynx, larynx, hypopharynx, and other oral cavity sites (11–13).

In contrast to normal cells, cancer cells synthesize *de novo* substantial amounts of fatty acids, irrespective of the circulating lipid levels, and as a result may acquire growth and survival advantages as well as drug resistance (14, 15). The human fatty acid synthase (FASN) is a 270 kDa cytosolic dimeric enzyme responsible for the production of palmitate that is upregulated in several malignancies (16), including OTSCC (17–19). In OTSCC, FASN expression seems to be associated with overall survival rates and microscopic characteristics that influence disease progression and prognosis, such as histologic grade, lymphatic permeation, perineural infiltration, and nodal metastasis (20). Furthermore, high immunohistochemical expression

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of FASN and ERBB2 is associated with OTSCC thickness and lymphatic embolization (21). Importantly, FASN has been suggested as an oncoprotein in breast and prostate cancers (22, 23). Experimental studies have shown that FASN pharmacologic inhibition or knockdown with specific siRNAs reduce cancer cell proliferation by blocking DNA replication, induce apoptosis, and decrease the size of prostate, ovarian, and breast cancer xenografts (24–27). Orlistat (tetrahydrolipstatin), initially described as an inhibitor of pancreatic and gastric lipases and acts as a tight-binding irreversible inhibitor of the FASN thioesterase domain (26). This drug exhibits both *in vitro* and *in vivo* antitumor properties against melanoma, breast, and prostate cancer cells, with no evident adverse effects on normal cells (26–29). Our group has shown that orlistat is able to reduce by 52% the number of mediastinal lymph node metastasis in experimental B16-F10 melanomas (29). Moreover, we demonstrated that the drug changes the fatty acid composition of the mitochondrial membranes and promotes apoptosis in B16-F10 cells through the intrinsic pathway, independent of p53 activation or mitochondrial permeability transition (30, 31).

Here, we describe that orlistat reduces the growth and promotes apoptosis in both SCC-9 and SCC-9 ZsGreen LN-1 cells. In addition, this drug enhances the production of $VEGFA_{165b}$ and reduces migration of SCC-9 ZsGreen LN-1 cells. Importantly, both the size and proliferation indexes of experimental primary tongue tumors were significantly decreased by the drug, whereas the number of metastatic cervical lymph nodes was reduced by 43%. These findings strongly suggest that FASN might be a potential molecular target to improve OTSCC chemotherapy by preventing tumor growth and metastasis.

## Materials and Methods

### Cell culture

SCC-9 cells (American Type Culture Collection; ATCC), derived from a human OTSCC, was maintained in DMEM/F-12 (Invitrogen) supplemented with 2% or 10% of FBS (Cultilab), 400 ng/mL hydrocortisone, and antibiotic/antimycotic solution (Invitrogen). HaCat cells (ATCC), spontaneously immortalized human keratinocytes from morphologically normal skin, were cultured in DMEM (Invitrogen) supplemented with 10% of FBS. Orlistat (Xenical, Roche) was added to the culture medium at the concentrations described in the figure legends. Labeled SCC-9 cells were obtained by the transduction with the pLNCX2 retroviral vector (Clontech) containing the coding sequence of the ZsGreen protein, as described by Harrell and colleagues (32). For the Oil red O staining, SCC-9 or HaCat cells ($5 \times 10^4$) were seeded in two-well chamber slides (BD Falcon) and after 24 hours the medium replaced by fresh medium supplemented or not with 10% FBS and cultured for additional 48 hours. Oil red O staining was performed using the isopropanol method (33). SCC-9 and SCC-9 ZsGreen cells were authenticated through investigation of the loci D2S1338, D19S433, CSF1PO, TPOX, TH01, vWA, D16S539, D7S820, D13S317, D5S818, FGA, D3S1358, D18S51, D8S1179, D21S11, and amelogenin. The genetic profile was the same described by ATCC.

### Total lipid biosynthesis

A total of $1 \times 10^6$ SCC-9 cells treated with 200 μmol/L of orlistat or its vehicle in DMEM/F-12 2% FBS for 48 or 72 hours or $1 \times 10^6$ SCC-9 ZsGreen LN-1 cells in DMEM/F-12 10% FBS were incubated in 1 mL of DMEM/F-12 2% FBS containing 1 mmol/L of acetate and 1 μCi of $^{14}$C-acetate (Perking Elmer) for 2 hours at 95% $O_2$/5% $CO_2$ and 37°C in a shaker water bath. Lipid extractions were performed according to Dole's method (34). The results (mean of three values expressed in cpm) were divided by the DNA content of each cell pellet (expressed in μg/μL).

### Estimation of FASN activity by NADPH oxidation

Cells were collected and lysed in homogenizers with a buffer containing 1 mmol/L EDTA, 50 mmol/L Tris-HCl pH 7.3, 150 mmol/L NaCl, and 1 mmol/L phenylsulfonylmethylfluoride. FASN activity was measured in a reaction solution containing 200 mmol/L sodium phosphate buffer (pH 7.4), 0.1% Triton X-100, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 0.35 mmol/L NADPH, 0.060 mmol/L acetyl-CoA (Sigma), 0.1 mmol/L malonil-CoA (Sigma). NADPH conversion to NADP was monitored by the loss of absorbance at 340 nm with the aid of an UltroSpec 3000 UV/Visible Spectrophotometer (Pharmacia Biotech). The reactions were started by the addition of 100 μg of protein and FASN-specific activity defined as μmoles of NADPH consumed $\times$ $min^{-1}$ $\times$ $mg^{-1}$.

### Orlistat solutions

For cell culture, orlistat (Fig. 1) was extracted from Xenical capsules according to Knowles and colleagues (35). Mice were treated with orlistat solutions prepared according to Kridel and colleagues (26).

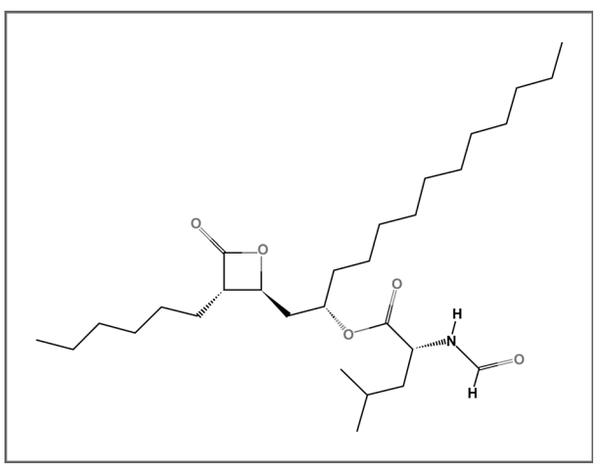


Figure 1. Orlistat structure.

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### RNA interference-mediated silencing of FASN expression

The 25-mer RNA molecules were synthesized, annealed, and purified by the manufacturer (Stealth RNAi, Invitrogen). The sequence targeting human FASN (NM_004104.4) corresponds to nucleotides 158–182 (5′ CAGAGTCGGAGAACTTGCAGGAGTT 3′). SCC-9 or SCC-9 ZsGreen LN-1 cells grown at 50% confluence were transfected with 100 nmol/L of siRNAs using liposomes (Lipofectamine 2000, 1 μg/mL, Invitrogen). As negative controls, cells were transfected with nonspecific control oligos (Stealth RNAi Negative Control Duplexes, Medium GC Duplex, Invitrogen).

### Flow cytometry

Samples were analyzed in a FACSCalibur flow cytometer (BD Biosciences) equipped with an argon laser and CellQuest Pro software. Ten thousand events were collected for each sample. For the analysis of cell death, cells were harvested following orlistat treatment or FASN silencing in DMEM/F-12 supplemented with 2% FBS, washed with PBS, and resuspended in a binding buffer containing Annexin V-FITC (1:500, Invitrogen) or Annexin V-PE and 7-AAD-PerCP (1:100, BD Pharmingen). For the cell-cycle analysis, SCC-9 cells were seeded, serum starved for 48 hours, and treated with orlistat or transfected with FASN-specific siRNAs in DMEM/F-12 supplemented with 10% FBS. After fixation in cold 70% ethanol, cells were treated with RNAse (10 μg/mL) during 1 hour at 37°C and stained with 50 μg/mL of propidium iodide during 2 hours at 4°C. The distribution of cells in the cell cycle was analyzed by the software ModFit (Verity Software House).

### Protein extractions and Western blotting

Protein lysates and Western blotting reactions were performed as previously described (29). Forty micrograms of each protein lysate were probed with antibodies against FASN (1:3,000; Transduction Laboratories), ERBB2 (1:2,000; Dako), SKP2 (1:1,000; Santa Cruz Biotechnology), CDKN1B ($p27^{kip1}$, 1:500; Transduction Laboratories), CTNNB1 (β-catenin, 1:1,000; clone 14/β-catenin, BD Biosciences), CDH1 (E-cadherin, 1:2,500, clone 36/E-cadherin; BD Biosciences), CDH2 (N-cadherin, 1:2,500, clone 32/N-cadherin; BD Biosciences), and ACTB (β-actin, 1:60,000, AC-15; Sigma).

### *In vivo* selection of a SCC-9–derived cell line with increased metastatic potential

SCC-9 ZsGreen cells ($5 \times 10^5$ in 20 μL of PBS) were implanted subcutaneously into the footpads of the left front limb of five BALB/c nude mice. The animals were sacrificed 60 days later and the axillary lymph nodes collected, maintained in DMEM/F-12, and analyzed in an epifluorescence microscope (Nikon Eclipse TI). Fragments of positive lymph nodes were used for explant cultures, from which the SCC-9 ZsGreen LN-1 cell line was isolated. Importantly, this new cell line metastasizes to cervical lymph nodes approximately 10 days after its implantation into the tongue of BALB/c nude mice, in contrast with about 60 days of the parental cells.

### Invasion assay

The myoma organotypic invasion assay was performed as described by Nurmenniemi and colleagues (36) and the culture tissues harvested at day 10.

### Scratch assay

A total of $4 \times 10^5$/mL SCC-9 ZsGreen LN-1 cells grown in DMEM/F12 with 5% FBS were plated in each well of 6-well plates. After 15 hours, the confluent monolayer was scraped with a sterile 200 μL pipette tip to create cell-free areas. After two washes with PBS, medium containing orlistat (100 and 200 μmol/L) or ETOH was added and cells incubated for 18 hours. Images were obtained at 0, 8, 12, and 18 hours. Cell migration was analyzed with the ImageJ software by counting the number of cells in the scratched areas.

### Zymography

Zymographic analysis was performed as previously described (37). Briefly, SCC-9 ZsGreen or SCC-9 ZsGreen LN-1 cells were plated ($1 \times 10^5$ cells) in DMEM/F-12 containing 2% FBS and after 24 hours the medium was changed by fresh DMEM/F-12 without FBS. After additional 24 hours, medium was collected and cells harvested and counted. The volume of medium used for zymography was proportional to the number of harvested cells.

### ELISA for $VEGFA_{165b}$

The concentration of $VEGFA_{165b}$ was determined with the Human VEGF165b DuoSet ELISA (R&D Systems) following the manufacturer's instructions and normalized by the protein concentration of the cell culture medium from each well after the treatment with orlistat (in DMEM/F-12 containing 2% FBS for 48 hours) or transfection with siRNAs specific for FASN.

### Orthotopic murine model of oral squamous cell carcinoma

SCC-9 ZsGreen LN-1 cells were grown until 60% to 70% confluence in DMEM/F-12 with 10% FBS, resuspended ($10^5$ in 20 μL of PBS), and implanted into the right lateral portion of the tongue of 6- to 8-week-old male BALB/c nude mice (School of Medicine, University of São Paulo, São Paulo, Brazil) by using a syringe with a 30 gauge disposable needle (BD Biosciences). The animals were previously anesthetized with intraperitoneal injections of ketamine (100 mg/kg) and xylazine. Twenty-four hours later, the animals started to be treated with daily intraperitoneal injections of orlistat (240 mg/kg) or its vehicle (60 μL of 33% ethanol). Fifteen days following the implantation, 1 mL/100 g of bromodeoxyuridine (BrdUrd; GE Healthcare) was administered (intraperitoneally) and after 2 hours the animals were sacrificed

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by $CO_2$ inhalation, dissected, and the tongue, cervical lymph nodes, and livers collected. The size of the primary tumors was determined with a caliper and their volumes calculated with the formula volume = 0.5 × length × width$^2$. Tissue samples were fixed in 10% formalin for immunohistochemistry or frozen in liquid nitrogen for Oil red O staining (38). Cervical lymph nodes were observed with an epifluorescence microscope for the detection of metastatic foci and images acquired for each positive lymph node. The area of the metastatic foci was measured with the software ImageJ. This experiment was performed 3 times independently with a total of 52 mice and the approval of the Committee for Ethics in Animal Research of the State University of Campinas (1500–1).

### Immunohistochemistry for BrdUrd and FASN

The immunohistochemical reactions for bromodeoxyuridine (BrdUrd) or FASN were done according to manufacturer's instructions (GE Healthcare) and as described elsewhere (19).

## Results

### SCC-9 endogenously synthesize lipids

To compare the endogenous lipid synthesis by the tumorigenic SCC-9 cells with the nontumorigenic HaCat cells, we performed Oil Red O staining. As depicted in Fig. 2A and C, cytoplasmic lipid droplets were clearly evidenced in both SCC-9 and HaCat cells grown in the presence of 10% FBS. In contrast, after serum starvation, lipids were observed in SCC-9 cells only (Fig. 2B and D). These results indicate that SCC-9 cells endogenously produce lipids while the nontumorigenic HaCat cells utilize lipids provided, at great extent, by the serum.

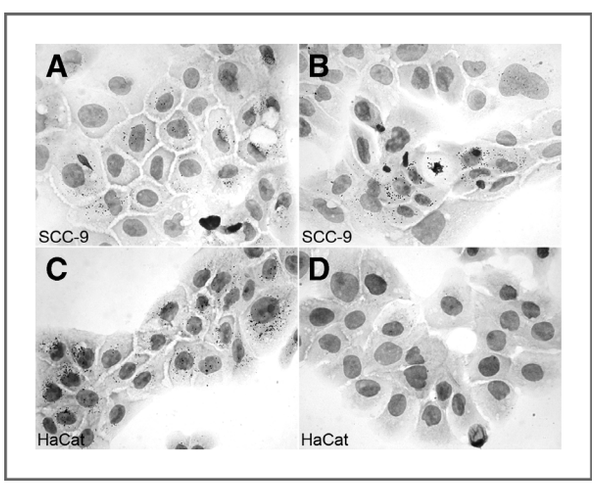


Figure 2. Endogenous lipid accumulation in SCC-9 cells. Oil red-O staining of tumorigenic SCC-9 cells and nontumorigenic HaCat cells cultured with 10% FBS (A and C) or serum-free medium (B and D) for 48 hours. Lipid droplets were clearly evidenced in SCC-9 cells cultured both in the presence (A) and absence (B) of FBS. HaCat cells were positive in the presence of 10% FBS only (C). A to D, original magnification, ×1,000.

### FASN activity is necessary for proliferation and survival of SCC-9 cells

FASN inhibition with orlistat in SCC-9 cells was verified by incorporation studies of $^{14}$C-acetate, which demonstrated a significant reduction of the total lipid production (Fig. 3A). Next, we sought to analyze the effect of orlistat on the proliferation of SCC-9 cells. A dose-dependent increase of the $G_0$–$G_1$ population associated with a clear reduction of cells in the S-phase was observed after 48 (not shown) and 72 hours of treatment (Fig. 3B). Similar effects were detected in SCC-9 cells transfected with siRNA specific for FASN (Fig. 3C). Indeed, both orlistat and siRNAs promoted accumulation of CDKN1B, a negative regulator of the $G_1$–S transition, and downregulation of SKP2, an E3 ubiquitin ligase essential for the proteasomal degradation of CDKN1B (Fig. 3D and E). Both treatments reduced ERBB2, a cell surface receptor implicated in the control of FASN expression in cancer cells. The higher FASN content observed in orlistat-treated cells (Fig. 3D) is probably a compensatory response to its chemical blockage (Fig. 3A). No differences in the apoptotic SCC-9 rates were found with orlistat in standard culture conditions, even after 72 hours (not shown). However, in low serum (2% of FBS), a significant increase in apoptosis was observed after 48 (not shown) and 72 hours (Fig. 3F), suggesting a role for FASN in SCC-9 cell survival. Necrosis levels were low and not changed within the same experimental conditions (Fig. 3F). Apoptosis was also enhanced following FASN knockdown with specific siRNAs (Fig. 3G).

### Isolation of the metastatic cell line SCC-9 ZsGreen LN-1

Parental SCC-9 cells are poorly metastatic, as few affected regional lymph nodes were found 60 days after orthotopic injection into the tongue of BALB/c nude mice and most animals were sacrificed because of the large size of the primary tumors (data not shown). Thus, to establish a SCC-9–derived cell line with increased metastatic potential, we performed *in vivo* selection. SCC-9 cells stably expressing ZsGreen (Fig. 4A) were implanted into the footpads of BALB/c nude mice (Fig. 4B) and the axillary metastatic lymph nodes (Fig. 4C) used to isolate the SCC-9 ZsGreen LN-1 cell line (Fig. 4D). The new cell line produced locally invasive primary tumors that infiltrate skeletal muscle and nerves (not shown) and spontaneously metastasize to the cervical lymph nodes in 10 to 15 days only, when injected into the tongue of BALB/c nude mice. SCC-9 ZsGreen LN-1 cells seem to be more invasive *in vitro* than the parental cells in the myoma organotypic invasion assay (Fig. 4E and F). Increased matrix metalloproteinase (MMP)-2, but not MMP-9, activity (Fig. 4G), similar FASN and CDH1 levels, an evident upregulation of CDH2, and a slight increase of ERBB2 and CTNNB1 were also observed in SCC-9 ZsGreen LN-1 cells (Fig. 4H).

Unexpectedly, the total lipid biosynthesis in SCC-9 ZsGreen LN-1 cells was lower than in the parental cells (Fig. 4I); however, FASN activity estimated by NADPH

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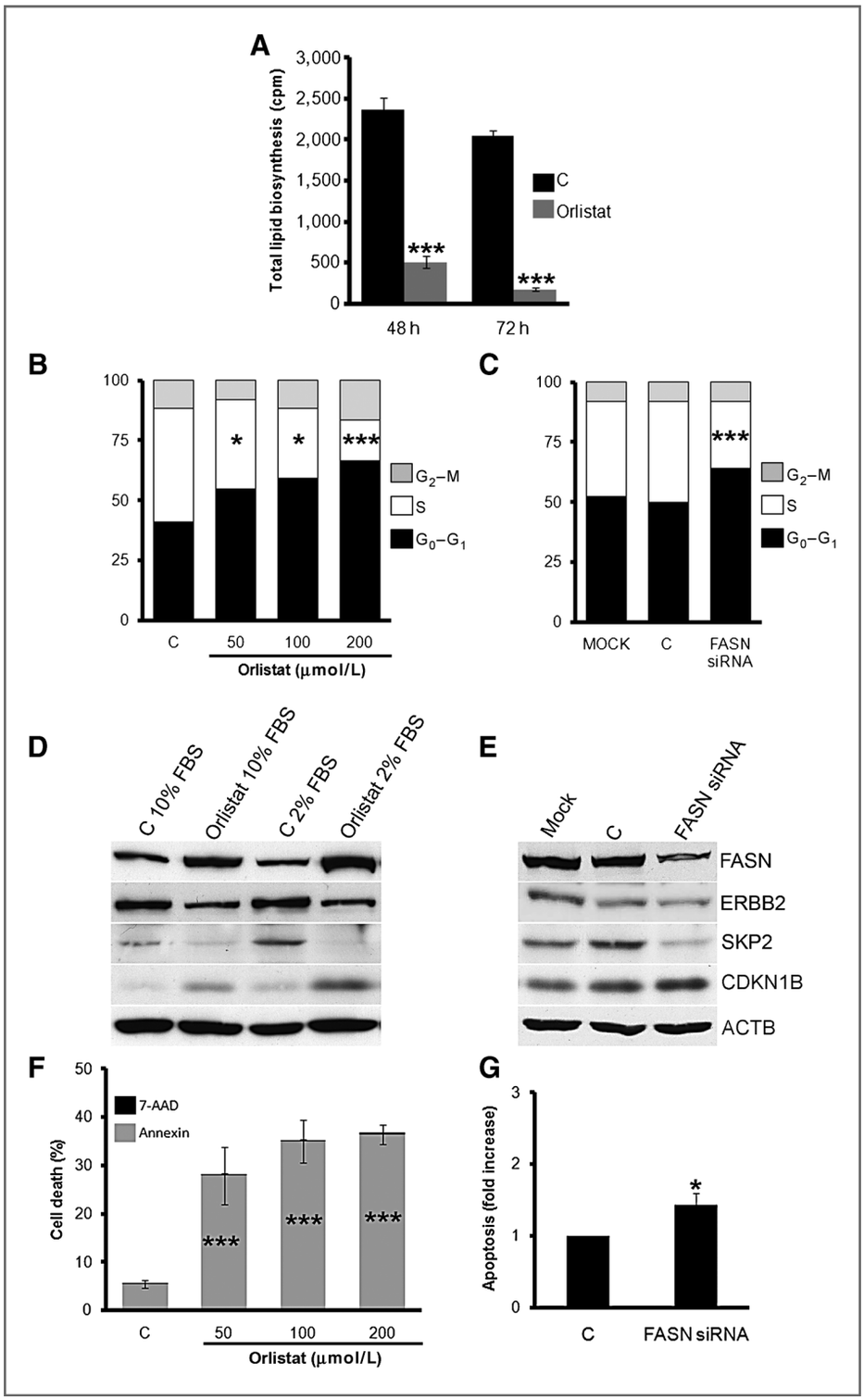


Figure 3. FASN activity is necessary for the proliferation and survival of SCC-9 cells. A, analysis of total lipid biosynthesis by the incorporation of $^{14}$C-acetate showing a significant inhibition in SCC-9 cells treated with 200 μmol/L of orlistat after both 48 and 72 hours. B, flow cytometry studies demonstrating that the incubation of SCC-9 cells with orlistat enhances the $G_0$–$G_1$ population and dramatically reduces the number of cells in the S-phase after 72 hours. C, siRNAs specific for FASN had a similar effect by promoting cell-cycle arrest in SCC-9 cells. D, Western blotting analysis of protein lysates from orlistat-treated SCC-9 cells cultured with 10% or 2% FBS indicating accumulation of CDKN1B and downregulation of SKP2 and ERBB2; similar results were found in siRNA-transfected SCC-9 cells (E). F, annexin V and 7-AAD experiments showing increase of apoptotic cell death, but not necrosis, in SCC-9 cells treated with orlistat for 72 hours in low serum condition (2%). Transfection with siRNAs specific for FASN also induces apoptosis (G). The Western blotting shown in E, top strip, demonstrates FASN knockdown for figures C and G (representative from 3 independent experiments). *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$, Student $t$ test; C, control.

oxidation was essentially the same in both cell lines (Fig. 4J). The proliferation of SCC-9 ZsGreen LN-1 cells was significantly reduced at 200 μmol/L (Fig. 5A) and similar effects detected following the transfection with siRNA specific for FASN (Fig. 5B and C). In contrast with SCC-9 cells, an increase in apoptosis was observed in SCC-9 ZsGreen LN-1 cells cultured with 10% FBS at the highest concentration of orlistat (Fig. 5D). In low serum, apoptosis was observed in all tested concentrations, as well as after FASN knockdown (Fig. 5C, E, and F). Necrosis levels were

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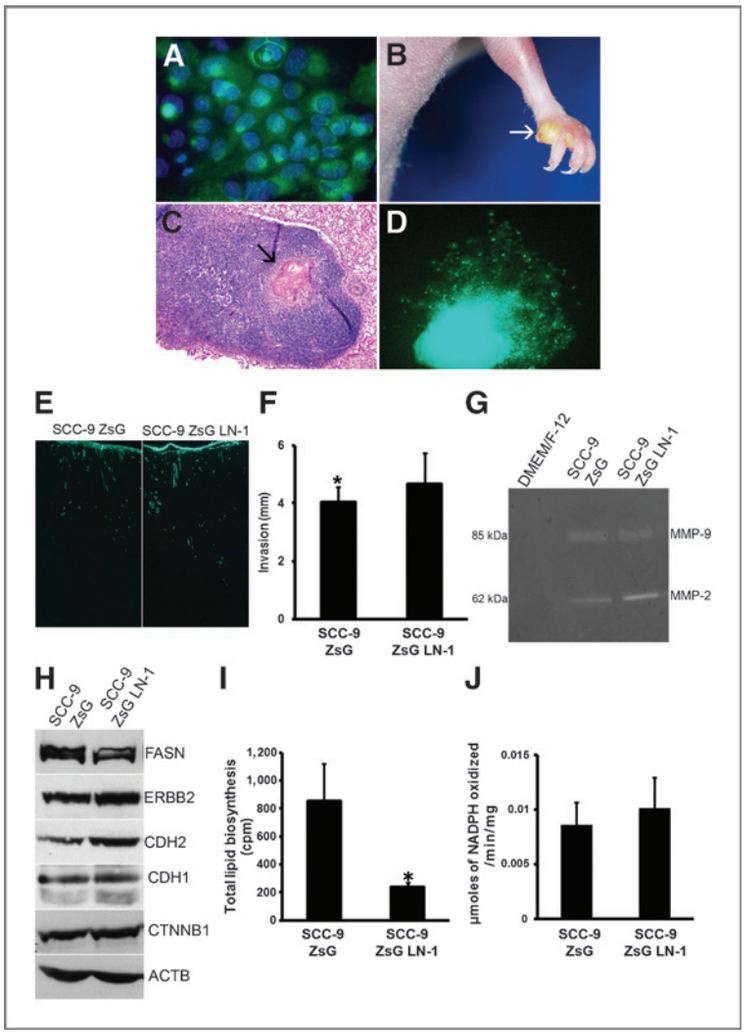


Figure 4. Isolation and characterization of the metastatic SCC-9 ZsGreen LN-1 cell line. A, SCC-9 cells stably expressing ZsGreen were obtained by the transduction with the pLNCX2-ZsGreen retroviral vector. DAPI staining shows the nuclei in blue (original magnification ×400). B, tumor in the left front limb of a BALB/c nude mouse induced by the implantation of SCC-9 ZsGreen cells. C, axillary lymph node invaded by SCC-9 ZsGreen cells (arrow; HE, original magnification ×50). D, fragment of a positive lymph node used for explant culture, from which SCC-9 ZsGreen LN-1 cells were isolated (original magnification ×25). E, cell invasion into myoma organotypic cultures, SCC-9 ZsGreen LN-1 cells were able to invade deeper than the parental cells after 10 days of culture. F, quantification of the maximal invasion depth (the distance from the lower surface of the noninvasive cell layer to the deepest invading cell). G, gelatin zymography with culture media from SCC-9 ZsGreen and SCC-9 ZsGreen LN-1 cells showing increased MMP-2, but not MMP-9, activity. H, Western blotting reactions demonstrating similar FASN and CDH1 levels, a clear upregulation of CDH2, and a slight increase of ERBB2 and CTNNB1 in SCC-9 ZsGreen LN-1 cells, in comparison with the parental cells. FASN activity was estimated by total lipid biosynthesis (I) and NADPH oxidation (J). *, $P < 0.05$; Student $t$ test.

low and not changed in the conditions described above (Fig. 5D–F). Notably, orlistat and siRNAs specific for FASN strongly increased the production of an antiangiogenic isoform of VEGFA, $VEGFA_{165b}$, by SCC-9 ZsGreen LN-1 cells (Fig. 5C, G, and H). Orlistat also reduced the migration of SCC-9 ZsGreen LN-1 cells (Fig. 5I).

### Orlistat reduces the volume of primary tumors and the number of lymph node metastasis in orthotopic OTSCC

Fifteen days after the implantation of SCC-9 ZsGreen LN-1 into the tongue of BALB/c nude mice, the animals exhibited tumors characterized by a firm mass in the middle part of the tongue (Fig. 6A). No evident side effects or body weight alterations were observed during the period of treatment with orlistat. The volumes of the primary tumors from orlistat-treated mice were significantly smaller than from the control groups (average of 0.016 $cm^3$ in the controls and 0.007 $cm^3$ in orlistat-treated mice, $P = 0.0006$; Student $t$ test; Fig. 6B and C). Of note, one animal from the orlistat-treated group did not develop primary tumor. BrdUrd incorporation studies showed a proliferation index of approximately 32% in the control tumors and 21.5% in the orlistat-treated tumors ($P < 0.05$;

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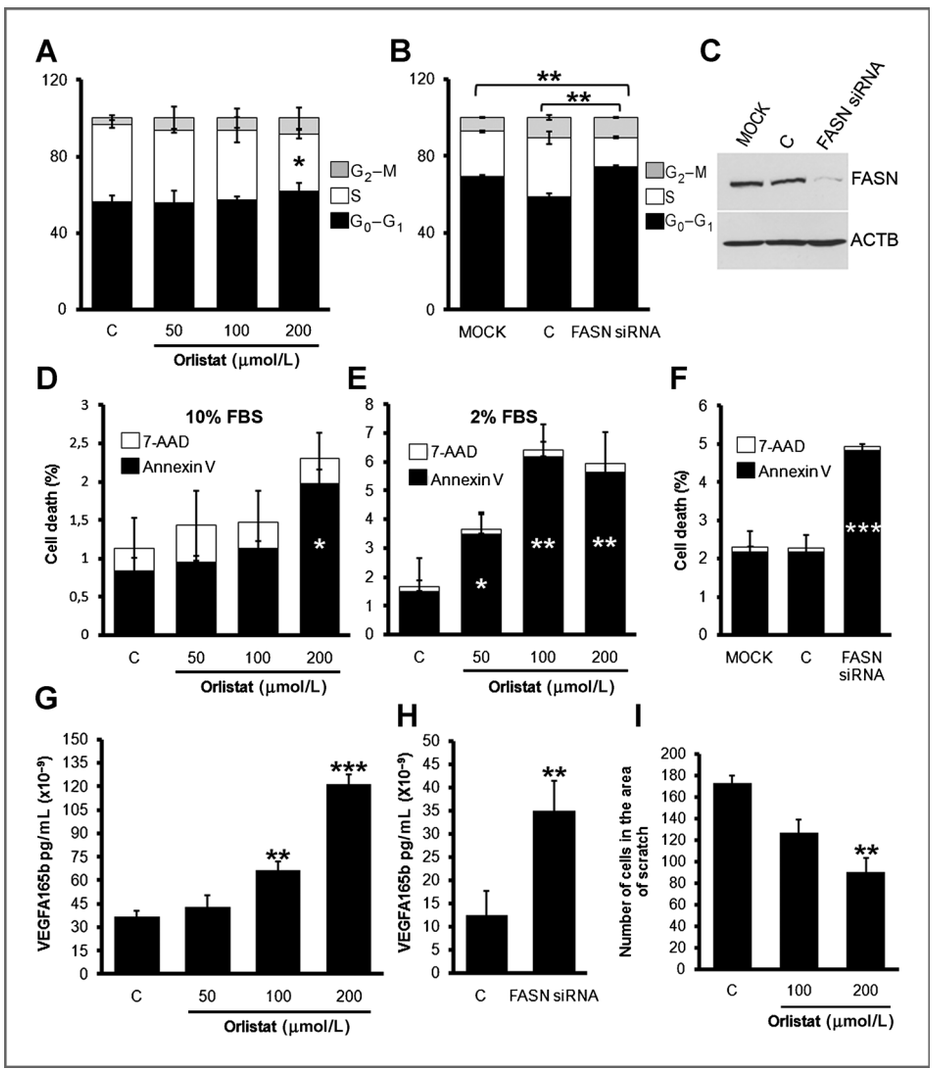


**Figure 5.** Effects of orlistat on the proliferation, apoptosis, and secretion of $VEGFA_{165b}$ by SCC-9 ZsGreen LN-1 cells. A, flow cytometry studies demonstrating that orlistat at 200 μmol/L enhances the $G_0$–$G_1$ and reduces the S-phase populations after 72 hours. B, siRNAs specific for FASN also promote a slight decrease of SCC-9 ZsGreen LN-1 cells in the S-phase. C, Western blotting showing the FASN knockdown for the experiments depicted in B, F, and H (representative from 3 independent assays). D and E, annexin V and 7-AAD experiments showing increased apoptosis in SCC-9 ZsGreen LN-1 cells treated with orlistat for 48 hours in both standard and low serum conditions (10% and 2% FBS, respectively). Necrosis levels were low and not significantly changed in the same experimental conditions. F, apoptosis in SCC-9 ZsGreen LN-1 is also induced by the FASN knockdown. G and H, orlistat for 48 hours and siRNAs specific for FASN strongly enhance the secretion of $VEGF_{165b}$ by SCC-9 ZsGreen LN-1 cells. I, orlistat reduces SCC-9 ZsGreen LN-1 migration in scratch assays. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$; Student $t$ test; C, control.

Student $t$ test; Fig. 6D and E). The systemic FASN inhibition with orlistat was confirmed by Oil red O–stained liver frozen sections (Fig. 6F), which demonstrated large cytoplasmic lipid droplets in control mice and weakly stained hepatocytes in the orlistat-treated animals. Interestingly, the positivity for FASN was slightly less intense in the treated tumors (Fig. 6G). Visible cervical lymph nodes were carefully removed and analyzed in a fluorescent microscope to investigate the presence of metastasis (Fig. 7A and B). The metastatic foci were generally smaller, although not statistically significant, in lymph nodes from the orlistat-treated animals (Fig. 7B and C). Importantly, therefore, orlistat-treated mice had 43% less metastatic cervical lymph nodes than the control animals. From a total of 217 removed lymph nodes, 106 from the control group and 111 from the orlistat-treated group, 59 metastases were found in the former and 35 in the latter. The percentages of positive lymph nodes in each animal from the control and orlistat-treated group is shown in Fig. 7D ($P = 0.007$; Student $t$ test).

## Discussion

Oral squamous cell carcinoma (OSCC) is an important cause of morbidity and mortality especially in developing countries and its prevalence may continue to rise in the near future (39). It is characterized by invasive and perineural growth, early recurrences, and frequent lymph node metastasis (5, 6, 39). The prognosis of OTSCC is significantly worse than the observed for squamous cell carcinomas from other sites of the head and neck (11).

Here we show that FASN inhibition affects proliferation and survival of both SCC-9 and SCC-9 ZsGreen LN-1 cells. Accordingly, orlistat has demonstrated potent antiproliferative and proapoptotic effects in cells derived from prostate, breast, colon, stomach and ovarian cancers, melanoma, retinoblastoma, and mantle cell lymphomas (26, 27, 29, 40–44). The cell-cycle arrest promoted by orlistat in SCC-9 cells is due to, at least in part, the modulation of SKP2 and CDKN1B. In fact, previous studies demonstrated that orlistat has strong effects on the cell cycle of other cancer cell lines by inducing $G_1$–S

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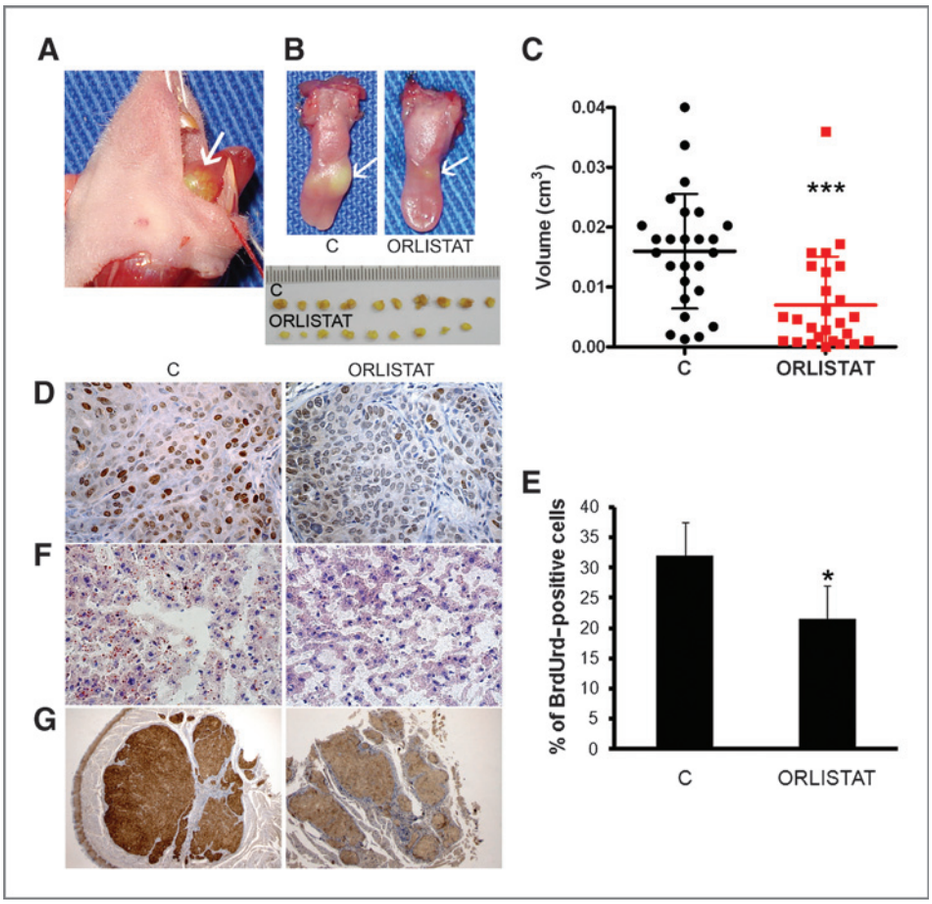


Figure 6. Orlistat reduces the volume of primary tumors in orthotopic OTSCC. A, representative primary tumor in the tongue of a BALB/c nude mouse (arrow) induced by the inoculation of SCC-9 ZsGreen LN-1 cells. B and C, the volumes of the primary tumors from orlistat-treated mice were significantly smaller (average of 0.007 $cm^3$) than the volumes of the control (C) groups (average of 0.016 $cm^3$). D and E, BrdUrd incorporation studies showing reduction of positive nuclei in the orlistat-treated tumors (original magnification, ×400). F, representative Oil Red O staining in frozen sections from the liver of control and orlistat-treated mice. Note the presence of large number of lipid droplets within the hepatocytes of the former, in contrast with the almost completely negative section from the orlistat-treated mouse (original magnification, × 400). G, the immunohistochemical positivity for FASN was slightly less intense in the tumors from orlistat-treated mice (original magnification × 25). *, $P < 0.05$; ***, $P < 0.001$; Student *t* test.

arrest (29, 35). Here, the proapoptotic effect of orlistat was observed mainly in a low serum condition. Similar findings were described by Dowling and colleagues (42) in gastric cancer cells and attributed to the excess of available fatty acids from the serum. We recently described that FASN inhibition with orlistat in B16-F10 mouse melanoma cells promotes oxidative stress by increasing ROS levels and activates the intrinsic pathway of apoptosis, while significantly changes the mitochondrial fatty acid composition (30, 31). Moreover, FASN overexpression decreased adriamycin- and mitoxantrone-induced apoptosis in breast cancer cells and orlistat was able to sensitize these cells to the same drugs (28).

Orthotopic xenograft models mimic both local tumor growth and pathways of metastasis. Our present study showed that orlistat-treated mice have significantly smaller primary tumors and 43% less metastatic cervical lymph nodes. Indeed, FASN inhibitors were shown to limit the growth of ovarian, breast, prostate, mesothelioma, kidney, thyroid, and colorectal xenografts, as well as orthotopic lung cancers (15). Orlistat showed antitumor activity in prostate cancer xenografts (26) and increased the survival rates of transgenic mice that spontaneously develop gastrointestinal cancer (42). In our hands, orlistat was shown to reduce spontaneous metastasis by 52% from B16-F10 intraperitoneal melanomas to the mediastinal lymph nodes (29). Moreover, FASN inhibition with cerulenin or specific shRNAs suppressed liver metastasis of colorectal cancer in mice (45, 46). The biologic mechanisms that link FASN activity with metastatic spread are largely unknown and it is possible to speculate that the decreased number of metastatic lymph nodes here observed with orlistat is consequence of the cell growth/migration inhibition and enhanced apoptosis. It was also recently shown that the inhibition of lipogenesis reduces invadopodia formation and gelatinolytic activity of Src-transformed 3T3 cells (47) and that orlistat prevents pseudopodia formation and inhibits human prostate cancer cell migration and invasion (48).

Kuemmerle and colleagues (49) recently demonstrated that in addition to *de novo* lipogenesis, cancer cells can use the lipoprotein lipase (LPL) and CD36 to acquire fatty acids from the circulation by lipolysis. These authors demonstrated LPL expression by immunohistochemistry in the majority of the studied breast, liposarcoma, and prostate tumor tissues. Although the expression of LPL in human OSCC is still unknown, it is possible to speculate that, in addition to FASN, orlistat also affects LPL activity in our model. Here, orlistat had opposite effects on the FASN protein production in cultured cells and tumor tissues. The biologic mechanisms responsible for these findings are unknown; however, orlistat may affect FASN

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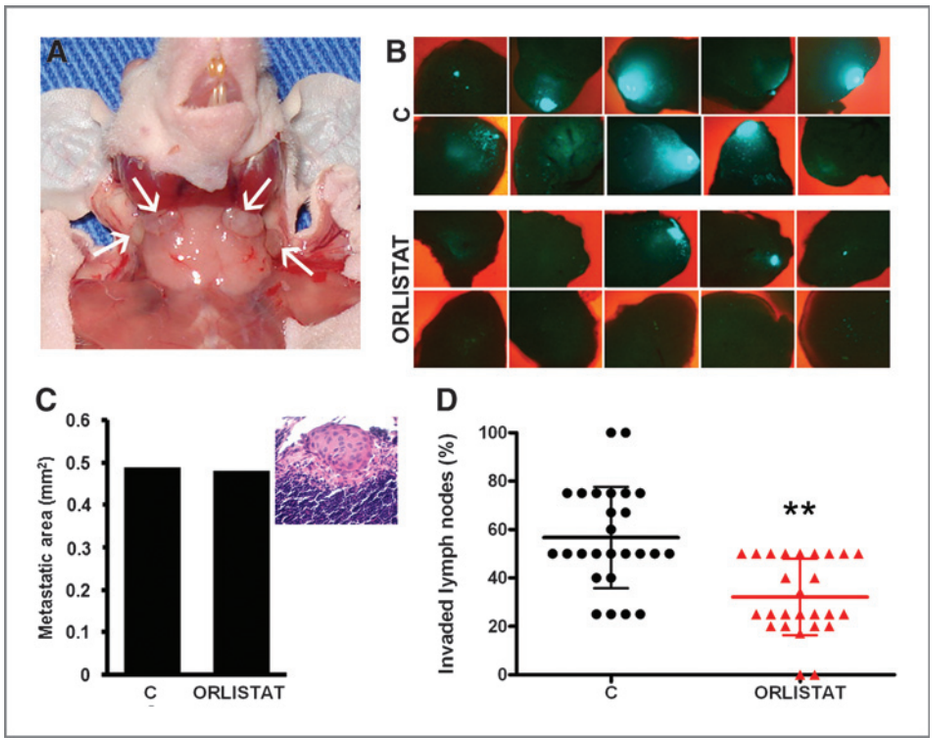


**Figure 7.** The number of cervical metastatic lymph nodes is reduced in orlistat-treated mice. A, representative cervical lymph nodes (arrows) of a BALB/c nude mouse with a primary tumor in the tongue induced by the inoculation of SCC-9 ZsGreen LN-1 cells. An average of four lymph nodes was collected from each animal for observation in a fluorescence microscope. B, representative lymph nodes from each group showing that the metastatic foci were generally smaller in the orlistat-treated animals than in the controls (C). C, mean metastatic area of the lymph nodes from control (C) and orlistat-treated animals. The insert shows a metastatic island within the lymph node subcapsular sinus from an animal of the control group (HE, original magnification, ×200). D, percentages of invaded lymph nodes for each control (C) and orlistat-treated mouse. Treated mice had 43% less metastatic cervical lymph nodes than the controls. **, $P = 0.007$; Student *t* test.

stability, which is regulated by the the deubiquitinase Usp2a in prostate cancer cells (50). Of note, Usp2a is overexpressed in OSCC (21). The lower total lipid biosynthesis of SCC-9 ZsGreen LN-1 in comparison with SCC-9 cells apparently minimizes the role of the *de novo* fatty acid synthesis in OTSCC metastatic dissemination. However, FASN activity determined by the NADPH oxidation levels was quite similar in both cell lines. Total lipid biosynthesis was measured by $^{14}$C-acetate incorporation and although tumor cells incorporate $^{14}$C-acetate preferentially into lipids than into amino acids or $CO_2$ (51), it is possible to speculate that in SCC-9 ZsGreen LN-1 cells part of $^{14}$C-acetate is converted to $CO_2$ via the tricarboxylic acid (TCA) cycle. Thus, distinct metabolic destinations for the FASN end-product could explain, at least in part, the less intense induction of apoptosis and cell-cycle inhibition found in SCC-9 ZsGreen LN-1 cells. Despite the fact that similar FASN protein bands were found in SCC-9 and SCC-9 ZsGreen LN-1 cells, variations in $^{14}$C-acetate incorporation were recently shown in prostate cancer cell lines (48). These authors also demonstrated that FASN expression is proportional to the loss of cell viability and xenograft shrinkage induced by orlistat. Of note, both the volumes of the SCC-9 ZsGreen LN-1–induced primary tongue tumors as well as their BrdUrd positivity were significantly reduced by the orlistat treatment.

Here we also show that the production of an antiangiogenic VEGFA variant, $VEGFA_{165b}$, by SCC-9 ZsGreen LN-1 cells was strongly increased by orlistat. It was previously reported that orlistat has antiangiogenic properties by inhibiting proliferation and promoting apoptosis in VEGF-A–stimulated human blood vessel endothelial cells (52). Indeed, we very recently demonstrated that orlistat reduces peritumoral angiogenesis in experimental melanomas and that culture medium conditioned by orlistat-treated SCC-9 cells has antiangiogenic abilities (53). Furthermore, we have also observed that cerulenin differentially regulates the production of VEGF-C and -D in SCC-9 cells, which might affect lymphangiogenesis. A remarkable network of intratumoral lymphatics was recently reported after implantation of UMSCC2 cells, derived from a squamous cell carcinoma of the alveolar ridge, in the tongue of nude mice (54).

The antitumor and antimetastatic properties of orlistat in our OTSCC model may also be associated with the PI3K/AKT signaling pathway or expression of CD44, as both were previously shown to be downregulated after FASN inhibition (15, 46). It was recently demonstrated that orlistat-treated T lymphoma cells show altered ROS generation, a shift in the cytokine balance, and modulation of cell survival molecules like HSP70, Bcl2, p53, PUMA, Caspase-3, and CAD (55). Importantly, FASN support membrane phospholipid synthesis in prostate cancer cells, which may be implicated in signal transduction, intracellular trafficking, cell polarization, and migration (56). Finally, FASN inhibition increases polyunsaturation and decreases monounsaturation levels leading to lipid peroxidation, oxidative stress, and apoptosis (57, 58).

In summary, the findings presented here show that FASN inhibition with orlistat restrains OTSCC growth

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and metastatic spread to regional lymph nodes. Therefore, FASN may be a potential target for OTSCC chemotherapy and considered in combination with the drugs already in clinical use.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** M. Agostini, F. Seguin, K.G. Zecchin, H.F. Raposo, H.C.F. Oliveira, N.D. Amoêdo
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** M. Agostini, D.C. Bastos, R.M. Ortega, F. Santos Moreira, F. Seguin, K.G. Zecchin, H.F. Raposo, H.C.F. Oliveira, N.D. Amoêdo, E. Graner
**Writing, review, and/or revision of the manuscript:** M. Agostini, K.G. Zecchin, N.D. Amoêdo, T. Salo, E. Graner
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** E. Graner
**Study supervision:** E. Graner

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