The fatty acid synthase inhibitor orlistat reduces experimental metastases and angiogenesis in B16-F10 melanomas

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BACKGROUND: Fatty acid synthase (FASN) is overexpressed and associated with poor prognosis in several human cancers. Here, we investigate the effect of FASN inhibitors on the metastatic spread and angiogenesis in experimental melanomas and cultured melanoma cells.

METHODS: The lung colonisation assay and cutaneous melanomas were performed by the inoculation of mouse melanoma B16-F10 cells in C57BL6 mice. Blood vessel endothelial cells (RAEC and HUVEC) were applied to determine cell proliferation, apoptosis, and the formation of capillary-like structures. Vascular endothelial growth factor A (VEGFA) expression was evaluated by quantitative RT-PCR and ELISA in B16-F10, human melanoma (SK-MEL-25), and human oral squamous carcinoma (SCC-9) cells. Conditioned media from these cancer cell lines were used to study the effects of FASN inhibitors on endothelial cells.

RESULTS: B16-F10 melanoma-induced metastases and angiogenesis were significantly reduced in orlistat-treated mice. Fatty acid synthase inhibitors reduced the viability, proliferation, and the formation of capillary-like structures by RAEC cells, as well as the tumour cell-mediated formation of HUVEC capillary-like structures. Cerulenin and orlistat stimulated the production of total VEGFA in B16-F10, SK-MEL-25, and SCC-9 cells. Both drugs also enhanced VEGFA₁₂₁, ₁₆₅, ₁₈₉, and _{165b} in SK-MEL-25 and SCC-9 cells.

CONCLUSION: FASN inhibitors reduce metastasis and tumour-induced angiogenesis in experimental melanomas, and differentially modulate VEGFA expression in B16-F10 cells.

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Fatty acid synthase (FASN) (EC2.3.1.85) is responsible for the endogenous fatty acid production from acetyl-CoA and malonyl-CoA (Menendez and Lupu, 2007). Its expression and activity is downregulated in normal cells, except in the liver, lactating breast, foetal lung, and adipose tissue (Weiss *et al*, 1986; Kuhajda, 2000). Conversely, in several human malignancies, such as those of prostate, breast, ovary, oral cavity, melanoma, and soft tissue sarcomas, FASN is overexpressed (Pizer *et al*, 1996a, b; Gansler *et al*, 1997; Alò *et al*, 2000; Swinnen *et al*, 2002; Innocenzi *et al*, 2003; Rossi *et al*, 2003; Takahiro *et al*, 2003; Visca *et al*, 2004; Kapur *et al*, 2005; Van de Sande *et al*, 2005; Rossi *et al*, 2006; Silva *et al*, 2008; Ogino *et al*, 2008; Dowling *et al*, 2009; da Silva *et al*, 2009; Walter *et al*, 2009; Ueda *et al*, 2010) and associated with poor prognosis (Pizer *et al*, 1996b; Gansler *et al*, 1997; Alò *et al*, 2000; Swinnen *et al*, 2002; Innocenzi *et al*, 2003; Rossi *et al*, 2003; Takahiro *et al*, 2003; Visca *et al*, 2004; Kapur *et al*, 2005; Van de Sande *et al*, 2005; Rossi *et al*, 2006; Ogino *et al*, 2008; da Silva *et al*, 2009; Walter *et al*, 2009; Ueda *et al*, 2010). Fatty acid synthase inhibition reduces cell proliferation, enhances apoptosis, decrease the size of prostate, ovarian, and breast cancer xenografts, and is

chemopreventive in the Neu-N mouse model for breast cancer (Pizer *et al*, 1996a, b; Furuya *et al*, 1997; Pizer *et al*, 1998; Li *et al*, 2001; Kridel *et al*, 2004; Alli *et al*, 2005; Zhou *et al*, 2007). Importantly, FASN was recently described as a metabolic oncogene in prostate cancer, as its forced expression transforms and confer tumorigenicity to immortalised prostate epithelial cells over-expressing androgen receptor (Migita *et al*, 2009). Despite the marginal role of FASN in normal cells, orlistat reduces endothelial cell proliferation and neovascularisation in an *ex vivo* assay, suggesting an antiangiogenic ability for this drug (Browne *et al*, 2006).

Orlistat is a pancreatic lipase inhibitor developed as anti-obesity drug, which also acts as an irreversible FASN inhibitor with antitumour properties (Kridel *et al*, 2004). Our prior studies show that orlistat reduces the proliferation and promotes apoptosis in the mouse melanoma cell line B16-F10 (Carvalho *et al*, 2008; Zecchin *et al*, 2011). These observations explain, at least in part, the significant decrease in the metastatic spread of the same cells in orlistat-treated mice with intraperitoneal melanomas (Carvalho *et al*, 2008). In addition, orlistat blocks cell cycle progression and promotes apoptosis through a PEA-3-mediated transcriptional repression of the Her2/Neu oncogene in Her2/Neu-overexpressing breast cancer cells (Menendez *et al*, 2005a). Moreover, FASN inhibition with C75, a synthetic analogue of cerulenin, upregulates vascular endothelial growth factor A (VEGFA) production, MAPK activation, and HIF-1 α accumulation in Her2/Neu-overexpressing breast and ovarian cancer cells (Menendez *et al*, 2005b).

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Vascular endothelial growth factor A is the main regulator of angiogenesis, generated as multiple isoforms grouped in pro- and anti-angiogenic families, both functioning mainly through the vascular endothelial cell receptor-2 (VEGFR-2; Harper and Bates, 2008). Vascular endothelial growth factor A production is low in benign nevi and progressively increased from dysplastic nevi to melanoma (Einspahr *et al*, 2007). Accordingly, the transition from radial to vertical growth phase in human melanomas is also characterised by increased VEGFA expression (Erhard *et al*, 1997). In a recent tissue microarray study including benign nevi, primary and metastatic melanomas, VEGFA, VEGFR-1, and -2 were found to be upregulated in the malignant tumours whereas VEGFR-2 positivity was higher in metastases than in the primary tumours (Mehnert *et al*, 2010). On the other hand, the anti-angiogenic VEGFA_{165b} is downregulated in metastatic melanomas and seems to predict their metastatic spread (Pritchard-Jones *et al*, 2007). Here, we report that orlistat significantly reduces experimental metastases, clearly demonstrating a cytotoxic effect against melanoma cells. Moreover, this drug inhibits angiogenesis associated with B16-F10 tumours and modulates VEGFA production.

MATERIALS AND METHODS

Cell culture

B16-F10 mouse melanoma and SK-MEL-25 human melanoma cells (ATCC, Manassas, VA, USA) were maintained in RPMI (Invitrogen, Camarillo, CA, USA) with 10% FBS (Cultilab, Campinas, Brazil). Human oral squamous carcinoma cells (SCC-9, ATCC) were grown in DMEM/F-12 (Invitrogen) with 10% FBS and 400 ng ml⁻¹ hydrocortisone. Endothelial cells from the rabbit aorta (RAEC, kindly provided by Dr Helena B Nader, UNIFESP, Brazil) were cultured in HAM-F12 (Invitrogen) containing 10% FBS. Human umbilical vein endothelial cells (HUVECs) were maintained in RPMI 10% FBS. Orlistat (Xenical, Roche, Basel, Switzerland), prepared as described by Knowles *et al* (2004), or cerulenin (Sigma-Aldrich, St Louis, MO, USA) were used to inhibit FASN.

In vivo studies

The animal experiments were performed according to the Animal Ethics Committee in Animal Research of UNICAMP. For the lung metastases assay, 8-week-old male C57BL6 mice (68) were inoculated at the tail vein with 2×10^5 B16-F10 cells suspended in 100 μ l of PBS. After 24 h, the animals received daily intraperitoneal injections of orlistat or its vehicle (Kridel *et al*, 2004) for 19 days, when were killed and dissected. After fixation in Bouin solution, lung colonies were counted in a dissection microscope and embedded in paraffin for H&E staining. Angiogenesis was analysed in 6-week-old male C57BL6 mice (78) by injecting B16-F10 cells (10^6) intradermally at the ventral skin. Simultaneously, animals started to be treated as described above and were killed 10 days latter. The skin with the tumour was removed and photographed in a dissection microscope. The length and area of tumour-directed vessels were analysed with the Scion Image software (Scion Corporation, Frederick, MD, USA).

Cell proliferation, apoptosis, and viability

For the proliferation curves, RAEC cells were seeded in 24-well plates (8×10^3 per well) and after 24 h serum-starved for the same period of time. After that, complete medium plus FASN inhibitors or their vehicles was added, and cells from triplicate wells trypsinised and counted in a Neubauer chamber. Flow cytometry experiments were performed as described by Zecchin *et al* (2011). Cell viability was determined by plating RAEC (3×10^4) or HUVEC (8×10^4) cells in 6-well culture plates with 3 (4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (Sigma) according to the manufacturer's instructions. All experiments were repeated at least three times independently.

Capillary-like assay

The formation of capillary-like structures by RAECs and HUVECs (3×10^4) was evaluated as described elsewhere (Pyriochou *et al*, 2007). The extension of vessel-like structures developed after 16 h in the presence of FASN inhibitors or conditioned media was assessed with the ImageJ software (Scion Corporation).

Conditioned media and ELISA

Conditioned media were obtained by plating 3×10^5 B16-F10, 6×10^5 SK-MEL-25 and SCC-9 cells in T-75 culture flasks. After 24 h, cells were washed with pre-warmed PBS and cultured in RPMI 10% FBS for 48 h. Medium was removed, centrifuged at $1100 \times g$ for 3 min, and used to incubate endothelial cells (80% conditioned medium diluted with fresh complete medium) previously seeded in 6-well plates (3×10^4 RAECs and 8×10^4 HUVECs) and serum starved for 24 h. To verify the role of VEGF_{165b} in endothelial proliferation, monoclonal antibodies (10 μ g ml⁻¹, clone 56-1, R&D Systems, Minneapolis, MN, USA) were added to the conditioned medium obtained from SK-MEL-25 cells. After trypsinisation (from triplicate wells), endothelial cells were counted in a Neubauer chamber after 24 and 48 h. The concentration of VEGFA was determined with VEGFA enzyme-linked immunosorbent assays (VEGF Quantikine ELISA and VEGF DuoSet ELISA, R&D Systems) following the manufacturer's instructions and normalised by the number of cells after the treatments.

siRNA

siRNAs (Table 1) were synthesised, annealed, and purified by the manufacturer (Stealth RNAi, Invitrogen). B16-F10 cells were transfected as described earlier Carvalho *et al* (2008). SK-MEL-25 and SCC-9 cells were transfected with 50 nm of the siRNAs by using jetPRIME (2 μ l ml⁻¹, Polyplus Transfection, Illkirch, France). As negative controls, cells were transfected with equimolar concentration of a nonspecific control oligo (Stealth RNAi Negative Control Duplexes, Medium GC, Invitrogen). Fatty acid synthase knockdown was assessed by western blotting 48 h after transfections (Carvalho *et al*, 2008).

Table 1 siRNA sequences for the knockdown of mouse and human FASN

Description	Sequence	Genbank accession
FASN mouse	5'-CAA TGA TGG CCA ACC GGC TCT CTT T-3'	NM_00798
FASN mouse	5'-TGG GAA GAC CCG AAC TCC AAG TTA T-3'	
FASN mouse	5'-CCT CTG GGC ATG GCT ATC TTC TTG A-3'	
FASN human	5'-CAG AGU CGG AGA ACU UGC AGG AGU U-3'	NM_004104

Abbreviation: FASN = fatty acid synthase.

RNA isolation and quantitative RT-PCR

Total RNA was obtained with the Trizol reagent (Invitrogen) according to the manufacturer's instructions. cDNAs were synthesised from 2 µg of total RNA using the First-Strand cDNA Synthesis SuperScript II RT (Invitrogen). Primer sequences and GenBank accession numbers are listed in Table 2. Quantitative RT-PCR was conducted using SYBR Green Real-time PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The reaction conditions were 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 57 °C for 1 min in a StepOne Plus Real-Time PCR System (Applied Biosystems). Relative gene expression was determined by the $2^{-\Delta\Delta CT}$ or standard curve methods in a SDS software version 2.0 (Applied Biosystems).

RESULTS

Orlistat inhibits lung colonisation by B16-F10 cells and reduces blood vessels at the periphery of experimental melanomas

To evaluate the antimetastatic effects of orlistat in melanoma cells, we exploited the well-described lung colonisation assay, which represents a significant challenge for potential anticancer drugs. Twenty days after the inoculation of B16-F10 cells at the tail vein, the number of macroscopic lung colonies was decreased by 53.4% in orlistat-treated mice (Table 3 and Figures 1A–D). In addition, MMP-2 and -9 activities were not modified by the drug in cultured B16-F10 cells (data not shown). Ten days after the intradermal inoculation of B16-F10 cells, all mice showed well-developed tumours at the ventral skin, which were variable in size and shape. Both the length and area of peritumoral blood vessels were significantly reduced by the treatment with orlistat, in comparison with the controls (Figures 1E–J).

Effects of FASN inhibitors on RAEC endothelial cells

The growth curves shown in Figures 2A and B show an important inhibitory effect of both cerulenin and orlistat on RAEC cell proliferation. Accordingly, these drugs promoted an increase of the G0–G1 and a clear reduction of the S phase, in comparison with the untreated controls (Figures 2C and D). In addition, cerulenin and orlistat reduced the viability of RAEC cells in a dose–response manner, enhanced apoptosis, and inhibited the formation of capillary-like structures in matrigel (Figures 2E–K).

Effects of orlistat on VEGFA expression

Differences in the expression of total mouse VEGFA within the experimental melanoma tissues were not detected after the treatment with orlistat (data not shown). Therefore, we next investigated the expression of VEGFA in melanoma cell lines. Total VEGFA was upregulated in B16-F10 cells treated with cerulenin or orlistat (Figures 3A and B). Total human VEGFA, VEGFA₁₂₁, ₁₆₅, ₁₈₉, and _{165b} were also increased by both drugs in SK-MEL-25 and SCC-9 cells (Figures 3C–F). Vascular endothelial growth factor A_{189b} and _{121b} were not expressed by these cell lines.

Conditioned media from orlistat-treated cancer cells do not stimulate or inhibit endothelial cell growth

To check whether VEGFA(s) released by B16-F10 cells stimulate the proliferation of RAEC endothelial cells, we first estimated their concentration in the cell culture medium. As expected, B16-F10 cells produced more total VEGFA than RAEC cells, and the conditioned medium from the former stimulates the proliferation of the latter (Figures 4A and B). Similarly, conditioned media from SK-MEL-25 or SCC-9 cells increase the growth rate of HUVEC cells (Figures 4C and D). Orlistat stimulates the secretion of total VEGFA, which was confirmed by the FASN knockdown with specific siRNAs in both B16-F10 and SK-MEL-25 cells (Figures 5A–C and E–G). The production of VEGFA_{165b} by SK-MEL-25 cells was

Table 3 Lung colonisation by B16-F10 cells in orlistat-treated and control mice

Experiment	Number of mice	Colonies (average per animal)
<i>Control</i>		
1	9	1019 (113.22)
2	13	2204 (169.53)
3	12	1998 (166.5)
Total	34	5221 (149.75)
<i>Orlistat</i>		
1	8	608 (76)
2	13	1038 (79.84)
3	13	773 (50.83)
Total	34	2419 (68.89)*

* $P < 0.01$, t-test.

Table 2 Primer sequences for the amplification of mouse and human VEGFA

Quantitative RT-PCR	Sequence	Genbank accession
Mouse total VEGFA forward	5'-TG GTGGACATCTTCCAGGAGTA-3'	NM_95200
Mouse total VEGFA reverse	5'-GCACACAGGACGGCTTGAA-3'	
Mouse GAPDH forward	5'-CATGGCCTTCCGTGTTCCCTA-3'	NM_008084.2
Mouse GAPDH reverse	5'-GCGGCACGTCAGATCCA-3'	
Human total VEGFA forward	5'-CGAGGGCCTGGAGTGTGT-3'	NM_001025366
Human total VEGFA reverse	5'-CGCATAATCTGCATGGTGATG-3'	
Human VEGFA ₁₈₉ forward	5'-CGAGGAAAGGGAAGGGGC-3'	NM_003376
Human VEGFA ₁₈₉ reverse	5'-CCCACAGGGAACGCTCCAG-3'	
Human VEGFA ₁₆₅ forward	5'-CCAATGCAGATTATGCGGATC-3'	NM_001025368
Human VEGFA ₁₆₅ reverse	5'-CAAGGCCACAGGGATTTTC-3'	
Human VEGFA ₁₂₁ forward	5'-ATGCAGATTATGCGGATCAAACC-3'	NM_001025370
Human VEGFA ₁₂₁ reverse	5'-CGGCTTGTCACATTTTCTTGTC-3'	
Human VEGFA _{165b} forward	5'-CAAGAAATCCCTGTGGGCC-3'	NM_001033756
Human VEGFA _{165b} reverse	5'-TGAGAGATCTGCAAGTACGTTTCG-3'	
Human GAPDH forward	5'-ATGGAAATCCCATCACCATTCTT-3'	NM_002046.3
Human GAPDH reverse	5'-CGCCCCACTTGATTTTGG-3'	

Abbreviations: GAPDH = glyceraldehyde-3-phosphate dehydrogenase; RT-PCR = reverse transcription-PCR; VEGFA = vascular endothelial growth factor A.

Figure 1 Orlistat inhibits lung colonisation by B16-F10 cells and peritumoral blood vessels in experimental melanomas. **(A)** Macroscopic aspects of the lungs from control mice, with many black B16-F10 colonies at the surface, which were clearly reduced in orlistat-treated animals **(B)**. **(C and D)** Histological sections showing B16-F10 metastatic colonies at the periphery and central portion of the lung (arrows). Representative images from three independent experiments (original magnification: **C**: $\times 25$ and **D**: $\times 100$, H&E staining). **(E–H)** Representative images of the mouse ventral skin showing blood vessels at the periphery of the tumour. **(E)** Blood vessels directed to the tumour of a control animal (arrows) and **(F)** detail of the small peritumoral blood vessels of the same animal shown in **E**. **(G)** Animal treated with orlistat and **(H)** detail of **G**. Orlistat-treated mice showed a significant reduction in both length **(I)** and area **(J)** of peritumoral blood vessels in comparison with the controls treated with the vehicle ethanol. The total length of the more calibrous blood vessels **(E and G)** and the area of small blood vessels (represented by the dashed lines in **F and H**) were estimated with the aid of a computer programme. All measurements were normalised by the area of the tumour (**C**: ethanol control; orl: orlistat; * $P=0.024$; ** $P=0.002$, Mann–Whitney test; original magnification: **E and G**: $\times 8$, **F and H**: $\times 20$).

Figure 2 FASN is essential for rabbit aortic endothelial cell (RAEC) growth and survival. (**A** and **B**) 0.75 $\mu\text{g ml}^{-1}$ of cerulenin (**A**) or 100 μM of orlistat (**B**) strongly reduced the proliferation of RAEC cells cultured in standard conditions (10% of FBS; $\blacktriangle$), in comparison with the respective controls ($\blacksquare$; $*P < 0.001$, Mann–Whitney test). (**C** and **D**) Cell cycle analysis by flow cytometry demonstrated that the incubation of RAEC cells with 0.75 $\mu\text{g ml}^{-1}$ of cerulenin for 48 h or 100 μM of orlistat for 96 h enhances the G0–G1 population and reduces the number of cells in the S phase ($\blacksquare$ G0/G1; $\square$ S; $\blacksquare$ G2/M; $*P < 0.05$, t-test). (**E** and **F**) 3 (4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide experiments showing that the viability of RAEC cells is reduced by cerulenin or orlistat in a dose-response manner ($*P < 0.05$, t-test). (**G** and **H**) The percentage of B16-F10 cells in apoptosis was significantly enhanced following the treatment with 0.75 $\mu\text{g ml}^{-1}$ of cerulenin or 100 μM of orlistat for 48 h ($*P < 0.05$, t-test). (**I**–**L**) The length of capillary-like structures formed by RAEC cells in matrigel was significantly reduced by both drugs (**C**: control with dimethyl sulfoxide for cerulenin or ethanol for orlistat; cer: cerulenin; orl: orlistat; original magnification in **K**: $\times 40$).

Figure 3 FASN inhibitors enhance VEGFA expression. Quantitative RT-PCR analysis of total mouse VEGFA in B16-F10 cells (**A** and **B**) and total human VEGFA, VEGFA₁₈₉, VEGFA₁₆₅, VEGFA₁₂₁, and VEGFA_{165b} transcripts in SK-MEL-25 (**C** and **D**) and SCC-9 cells (**E** and **F**) after 24 h of treatment with cerulenin (cer) or orlistat (orl). Fatty acid synthase inhibitors at different concentrations increased both total VEGFA and VEGFA isoforms, in comparison with the respective controls (**C**: control with dimethyl sulfoxide for cerulenin or ethanol for orlistat; expression levels in control cells = 1; **P* < 0.05, *t*-test).

also significantly increased after the incubation with the drug (Figure 5H). Similar results were found in SCC-9 cells (data not shown). Next, we sought to verify whether the orlistat-induced VEGFA(s) are pro- or anti-angiogenic by incubating RAEC or HUVEC cells with culture medium previously conditioned by orlistat-treated B16-F10 cells, although cell death occurred after 48 h with the conditioned medium from control cells. Interestingly, conditioned media from orlistat-treated SK-MEL-25 (Figure 5I) or SCC-9 cells (data not shown) significantly inhibited the proliferation of HUVEC cells. To verify whether the endothelial cell growth inhibition induced by the treatment of cancer cells with orlistat was associated with VEGFA_{165b}, the only inhibitory isoform detected, specific antibodies were used to neutralise its activity. Indeed, as depicted in Figure 5J, the proliferation of HUVECs was restored in conditioned medium from orlistat-treated SK-MEL-25 cells containing anti-VEGFA_{165b} antibodies, strongly suggesting an anti-angiogenic role for the orlistat-induced secretion of VEGF_{165b} by tumour cells.

In contrast with RAEC cells (Figures 2E and F), HUVEC cell viability and length of the capillary-like structures formed in matrigel were not affected by cerulenin or orlistat (Figures 6A–D). However, conditioned media from orlistat-treated cancer cells significantly decrease the length of capillary-like structures

developed by these cells (Figures 6E–H), further suggesting that this drug induces an anti-angiogenic phenotype.

DISCUSSION

The association between FASN expression and activity with tumour growth and metastasis has been clearly demonstrated in several types of human malignancies. In fact, tumour cells synthesise fatty acids *de novo*, despite nutritional supply (Ookhtens *et al*, 1984; Weiss *et al*, 1986).

Here, we show that FASN is critical for the proliferation and survival of RAEC cells, as orlistat or cerulenin significantly reduced their growth, promoted apoptosis, and impaired the formation of capillary-like structures *in vitro*. Curiously, the viability of HUVECs was not affected by FASN inhibitors, possibly due to their lower proliferation rates in comparison with the immortalised RAECs. Despite the fact that FASN pharmacological blockage or knockdown inhibits cell cycle progression and cause apoptosis in many cancer cell lines (Pizer *et al*, 1998; Li *et al*, 2001; Kridel *et al*, 2004; Zhou *et al*, 2007; Migita *et al*, 2009), the role of FASN in nonmalignant cells is still uncertain. Indeed, we observed that the growth of gingival fibroblasts in primary cultures is reduced by cerulenin (Almeida *et al*, 2005). Furthermore, Browne

Figure 4 (A) Total VEGFA protein levels in B16-F10-conditioned medium is higher than in RAEC-conditioned medium after 24 and 48 h. The growth of RAEC (B) and HUVEC (C and D) cells is stimulated by the incubation with culture medium previously conditioned by B16-F10 (CM-B16-F10), SK-MEL-25 (CM-SK-MEL-25), or SCC-9 (CM-SCC-9) cells, in contrast with medium conditioned by RAEC (CM-RAEC) or HUVEC (CM-HUVEC) cells (* $P < 0.05$, t -test).

et al (2006) described that orlistat inhibits the proliferation and promotes apoptosis in VEGFA-stimulated HUVECs.

We previously demonstrated that orlistat reduces proliferation and promotes apoptosis in B16-F10 cells (Carvalho *et al*, 2008; Zecchin *et al*, 2011). Both orlistat- and cerulenin-induced apoptotic cell death in this cell line occur through the intrinsic pathway, as demonstrated by the cytochrome *c* release and caspase-9 and -3 activation, independent of p53 activation or mitochondrial permeability transition (Zecchin *et al*, 2011). Importantly, we found that orlistat reduces B16-F10 cell metastatic spread from the peritoneal cavity to the mediastinal lymph nodes in a mouse model for melanoma spontaneous metastasis (Carvalho *et al*, 2008). This drug also inhibits metastasis of oral squamous cell carcinoma to the cervical lymph nodes in an orthotopic mouse model (Agostini M, unpublished results). Here, we observed a remarkable reduction of lung colonies in the treated animals (50%), which demonstrates the cytotoxic effect of orlistat on B16-F10 cells and further suggests that FASN activity may be targeted in melanoma chemotherapy.

This study shows for the first time that FASN modulates tumour angiogenesis through differential expression of VEGFA isoforms. The biological mechanisms responsible for this phenomenon are unknown. However, preliminary results from our laboratory show that the *in vitro* proteasomal degradation of HIF-1 α by B16-F10 protein lysates is accelerated by orlistat (Agostini M, unpublished results), suggesting that the downregulation of this transcription factor contributes for the anti-angiogenic phenotype. Vascular endothelial growth factor A, a potent growth factor for blood vessel endothelial cells, is also known to regulate vascular permeability (Dvorak *et al*, 1995) then contributing for the metastatic spread (Karamysheva *et al*, 2008). The gene encoding human VEGFA is formed by eight exons (Tischer *et al*, 1991) from which seven isoforms are produced through alternative splicing: VEGFA₁₂₁, ₁₄₅, ₁₄₈, ₁₆₅, ₁₈₉, ₁₈₃, and ₂₀₆ (Harper and Bates, 2008). Mouse transcripts contain one amino acid less in each isoform (Shima *et al*, 1996; Catena *et al*, 2007). An additional splice site in exon 8 generates the

VEGFA_{xxx} family of anti-angiogenic peptides, that differ from their angiogenic counterparts in the last six residues (Bates *et al*, 2002; Woolard *et al*, 2004; Perrin *et al*, 2005; Cbe Suarez *et al*, 2006) and are downregulated in renal, colorectal, and prostate carcinomas (Bates *et al*, 2002; Daz *et al*, 2008; Varey *et al*, 2008), metastatic melanomas (Pritchard-Jones *et al*, 2007), and diabetic retinopathies (Perrin *et al*, 2005). However, the anti-angiogenic activity of these factors is not completely characterised, as VEGFA_{121b} and VEGFA_{165b} were recently shown to be weakly angiogenic (Catena *et al*, 2010). In agreement with Menendez *et al* (2005b), which observed increased VEGFA in Her-2/Neu-over-expressing breast cancer cells following FASN inhibition with C75, we observed that orlistat and FASN knockdown raise the production of VEGFA(s) in B16-F10, SK-MEL-25, and SCC-9 cells. In this study, we found that VEGFA(s) produced by B16-F10 in the presence of orlistat do not increase the proliferation of RAEC endothelial cells. On the other hand, conditioned media from orlistat-treated human cancer cells (SK-MEL-25 and SCC-9) decreased the proliferation of HUVEC cells as well as the length of capillary-like structures in matrigel.

The expression of VEGFA₁₂₀ in our mouse melanoma specimens (data not shown) is consistent with previous findings in human melanomas (Potgens *et al*, 1995; Redondo *et al*, 2000; Yu *et al*, 2002), in which VEGFA₁₂₁ seems to be involved in peritumoral vascularisation due to its high diffusibility (Grunstein *et al*, 2000). This isoform was also associated with vasodilation and increased permeability of peritumoral vessels (Ksters *et al*, 2003). Our orlistat-treated melanomas also showed enhanced expression of VEGFA₁₈₈ (data not shown). Vascular endothelial growth factor (VEGFA₁₈₉), its human counterpart, when overexpressed in Mel57 melanoma cells promotes less pronounced vasodilatation than VEGFA₁₆₅ and VEGFA₁₂₁ (Ksters *et al*, 2003) and is nontumorigenic in WM1341B early-stage melanoma cells (Yu *et al*, 2002). Thus, as the complete organisation of the murine VEGFA gene is not still available, we searched for these factors in SK-MEL-25 human melanoma cells and found that FASN inhibitors

Figure 5 Total VEGFA production by B16-F10 and SK-MEL-25 melanoma cells is stimulated by the treatment with orlistat (orl) for 48 h (**A** and **E**) or FASN knockdown with specific siRNAs (**C** and **G**). Western blotting reactions confirming FASN knockdown in the siRNAs transfected B16-F10 (**B**) and SK-MEL-25 (**F**) cell lysates. (**H**) The incubation with 300 μ M of orlistat for 48 h enhances the secretion of VEGF_{165b} by SK-MEL-25 cells. (**D**) Conditioned medium from orlistat-treated B16-F10 cells does not affect the growth of RAEC cells after 24 h. At 48 h, RAEC cells were well preserved in the presence of medium from orlistat-treated B16-F10 cells, while cell death was observed with control medium. (**I**) Conditioned medium from orlistat-treated SK-MEL-25 cells significantly decreased the growth of HUVEC cells after 48 h (* P < 0.05, t -test). (**J**) The proliferation of HUVEC cells in conditioned medium from orlistat-treated SK-MEL-25 cells is restored in the presence of 10 μ g ml⁻¹ of neutralising anti-VEGFA_{165b} monoclonal antibodies. **C**: ethanol or nonspecific siRNA oligos.

Figure 6 Effect of orlistat on the formation of capillary-like structures by HUVEC cells. (**A** and **B**) 3 (4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide experiments showing that the viability of HUVEC cells is not affected by the presence of cerulenin (cer) or orlistat (orl). (**C** and **D**) Orlistat alone ($300 \mu\text{M}$) does not change the formation of capillary-like structures by HUVECs in matrigel. However, conditioned media (CM) from orlistat-treated ($300 \mu\text{M}$) SK-MEL-25 or SCC-9 cells significantly decrease the length of capillary-like structures, in comparison with the respective controls (**E–H**). **C**: ethanol control.

significantly stimulate VEGFAs₁₂₁, ₁₆₅, ₁₈₉, and _{165b}. Therefore, it is possible to hypothesise that overexpression of a particular sub-set of VEGFA isoforms have, at least in part, a role in the reduction of melanoma peritumoral angiogenesis that follows orlistat treatment. Importantly, the endothelial cell growth inhibition promoted by human cancer cell lines was reversed by anti-VEGF_{165b} neutralising antibodies, indicating a major role for this factor as an orlistat-induced gene product. In fact, VEGFA_{165b} is down-regulated in metastatic melanomas and seems to predict their metastatic spread (Pritchard-Jones *et al*, 2007).

In summary, here we show that FASN activity reduces both metastases and peritumoral angiogenesis in experimental melanomas. In addition, the reduction of endothelial cell growth and organisation of capillary-like structures *in vitro* further indicate a VEGFA_{165b}-mediated anti-angiogenic effect of orlistat. Taken

together, these observations suggest that FASN inhibition with orlistat may help to restrain melanoma metastatic dissemination.

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Conflict of interest

The authors declare no conflict of interest.

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