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 VEGFA 121, 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.

Keywords: fatty acid synthase; melanoma; metastasis; angiogenesis; vascular endothelial growth factor A (VEGFA); orlistat

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; Alô 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; Alô 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; Ali 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 neuovascularisation in an ex vivo assay, suggesting an antiangiogenic ability for this drug (Browne et al., 2006).

Orlistat is a pancreatic lipase inhibitor developed as an 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 intraocular 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-1α accumulation in Her2/Neu-overexpressing breast and ovarian cancer cells (Menendez et al., 2005b).
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, 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 maintained in RPMI 10% FBS. Human umbilical vein endothelial cells (HUVECs) were cultured in HAM-F12 (Invitrogen) containing 10% FBS. Human oral squamous carcinoma cells (SCC-9, ATCC, Manassas, VA, USA) were maintained in RPMI (Invitrogen) containing 10% FBS and 400 ng/ml 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 μl 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 paraaffin 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 triypsinised 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 × 10^5) 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 × 10^5) 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 × 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 VEGF165a, in endothelial proliferation, monoclonal antibodies (10 μg/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 μl 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 TTT T-3′ | NM_00798 |
| FASN mouse | 5′-TGG GAA GAC CCG AAC TCC AAG TTA T-3′ | NM_00798 |
| FASN mouse | 5′-CCT CTG GGC ATG GCT ATC TTC TTT A-3′ | NM_00798 |
| FASN human | 5′-CAG AGU CGG AGA ACU UGC AGU U-3′ | NM_004104 |

Abbreviation: FASN = fatty acid synthase.
**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 metastasis 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 33.4% 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 cerulенин 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, cerulенин 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 cerulенин or orlistat (Figures 3A and B). Total human VEGFA, VEGFA121b, 165b, 189, and 165, were also increased by both drugs in SK-MEL-25 and SCC-9 cells (Figures 3C–F). Vascular endothelial growth factor A189b 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 VEGFA165b by SK-MEL-25 cells was not statistically different from control (data not shown). Therefore, conditioned media from orlistat-treated cancer cells do not stimulate or inhibit endothelial cell growth.

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

| Experiment | Number of mice | Colonies (average per animal) |
|------------|----------------|------------------------------|
| Control    | 1 9            | 1019 (13.32)                 |
|            | 2 13           | 2204 (169.53)                |
|            | 3 12           | 1998 (166.5)                 |
| Total      | 34             | 5221 (149.75)                |
| Orlistat   | 1 8            | 608 (76)                     |
|            | 2 13           | 1038 (79.84)                 |
|            | 3 13           | 773 (50.83)                  |
| Total      | 34             | 2419 (68.89)*                |

*P<0.01, t-test.

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

| Quantitative RT–PCR | Sequence | Genbank accession |
|---------------------|----------|-------------------|
| Mouse total VEGFA forward | 5'-TGGTTGACATCCAGGATG-3' | NM_95200 |
| Mouse total VEGFA reverse | 5'-GGCGCACAGTCAGATCCA-3' | NM_008042 |
| Mouse GAPDH forward | 5'-CATGGCTTCCGTGTCCTCA-3' | NM_002046.3 |
| Mouse GAPDH reverse | 5'-CGAGGGGCTGGACTGTT-3' | NM_001025366 |
| Human total VEGFA forward | 5'-CGGAGGCGATGAGG-3' | NM_001033756 |
| Human total VEGFA reverse | 5'-GCATATAATCGATGAGCC-3' | NM_001033756 |
| Human VEGFA121b forward | 5'-CGAAGAAAGGAAAGGAC-3' | NM_001033756 |
| Human VEGFA121b reverse | 5'-CCACACACGAGAGGCAC-3' | NM_001033756 |
| Human VEGFA165b forward | 5'-CCAATGCAGATTATGCGATC-3' | NM_001033756 |
| Human VEGFA165b reverse | 5'-GACGGGCCGCCAGGAATTTC-3' | NM_001033756 |
| Human VEGFA189b forward | 5'-ATGGCAGATAGGGCCATCAAAC-3' | NM_001033756 |
| Human VEGFA189b reverse | 5'-CCGGGCTTGCACATTTCGTC-3' | NM_001033756 |
| Human VEGFA165b forward | 5'-CAAGAGAGGGGAGATGTTG-3' | NM_001033756 |
| Human VEGFA165b reverse | 5'-TGGTGGACATCTTCCAGGA-3' | NM_001033756 |

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: ×25 and D: ×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: ×8, F and H: ×20).
**Figure 2**  FASN is essential for rabbit aortic endothelial cell (RAEC) growth and survival. (A and B) 0.75 μg 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; - ▲), in comparison with the respective controls (- ■; *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 μg 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 (■ G0/G1; □ S; △ 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 μg 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: × 40).
significantly decrease the length of capillary-like structures
However, conditioned media from orlistat-treated cancer cells
matrigel were not affected by cerulenin or orlistat (Figures 6A–D).
viability and length of the capillary-like structures formed in
for the orlistat-induced secretion of VEGF165b by tumour cells.
VEGFA165b antibodies, strongly suggesting an anti-angiogenic role
medium from orlistat-treated SK-MEL-25 cells containing anti-
Figure 5J, the proliferation of HUVECs was restored in conditioned
antibodies were used to neutralise its activity. Indeed, as depicted in
with VEGFA 165b, the only inhibitory isoform detected, specific
induced by the treatment of cancer cells with orlistat was associated
bones) significantly inhibited the proliferation of HUVEC
conditioned medium from control cells. Interestingly, conditioned
media from orlistat-treated SK-MEL-25 (Figure 5I) or SCC-9 cells
growth was not changed by the cell culture medium from orlistat-
treated B16-F10 cells, although cell death occurred after 48 h with the
conditioned medium from orlistat-treated cancer cells. As depicted in Figure 5D, RAEC cell
HUVEC cells with culture medium previously conditioned by
shown). Next, we sought to verify whether the orlistat-induced
(Figure 5E–F), further suggesting that this drug induces an anti-angiogenic phenotype.
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
HUVeca cells with culture medium previously conditioned by
orlistat-treated cancer cells. As depicted in Figure 5D, RAEC cell
growth was not changed by the cell culture medium from 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 VEGFA165b, 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-
VEGFA165b antibodies, strongly suggesting an anti-angiogenic role
for the orlistat-induced secretion of VEGFA165b 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
synthesize fatty acids de novo, despite nutritional supply
(Okkhtens 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 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, VEGFA 189, 165, 121, and 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).
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 mitocondrial 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 (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 (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).

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 VEGFA120 seems to be involved in peritumoral vascularity due to its high diffusibility (Grunstein et al, 2000). This isoform is also associated with vasodilatation and increased permeability of peritumoral vessels (Küsters et al, 2003). Our orlistat-treated melanomas also showed enhanced expression of VEGFA189 (data not shown). Vascular endothelial growth factor (VEGFA189), its human counterpart, when overexpressed in Mel57 melanoma cells promotes less pronounced vasodilatation than VEGFA165 and VEGFA123 (Küsters et al, 2003) and is non-tumorigenic 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 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).
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 μM 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 μg ml⁻¹ of neutralising anti-VEGFA_{165b} monoclonal antibodies. C: ethanol or nonspecific siRNA oligos.
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. Importantly, the endothelial cell growth inhibition promoted by human cancer cell lines was reversed by anti-VEGF165b neutralising antibodies, indicating a major role for this factor as an orlistat-induced gene product. In fact, VEGFA165b is down-regulated in metastatic melanomas and seems to predict their metastatic spread (Pritchard-Jones et al., 2007). 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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