3-*O*-methylfunicone, from *Penicillium pinophilum*, is a selective inhibitor of breast cancer stem cells

E. Buommino*, V. Tirino†, A. De Filippis*, F. Silvestri*, R. Nicoletti‡, M. L. Ciavatta§, G. Pirozzi† and M. A. Tufano*

*Department of Experimental Medicine, Section of Microbiology and Clinical Microbiology, SUN, Naples, Italy, †Department of Experimental Oncology, National Cancer Institute, G. Pascale, Naples, Italy, ‡Council for Research and Experimentation in Agriculture, CAT Research Unit, Scafati, Italy, and §Institute of Biomolecular Chemistry (ICB-CNR), Pozzuoli, Italy

Received 21 January 2011; revision accepted 11 March 2011

Abstract

Objectives: Cancer stem cells make up a subpopulation of cells within tumours that drive tumour initiation, growth and recurrence. They are resistant to many current types of cancer treatment, causing failure of such therapeutic approaches, including chemotherapy and radiotherapy. In the study described here, anti-proliferative effects of 3-*O*-methylfunicone (OMF), a metabolite from *Penicillium pinophilum*, were investigated on human breast cancer MCF-7 cells and cancer stem cells selected as mammospheres derived from MCF-7s.

Materials and methods: Stemness markers were analysed on isolated mammospheres showing positive expression of CD24, CD29, CD44, CD133, CD184 and CD338. Cell proliferation and apoptosis were analysed by flow cytometry and RT-PCR. Cell colony formation assays were performed to evaluate colony formation of mammospheres.

Results and conclusion: OMF treatment affected both MCF-7 and mammosphere growth, inducing apoptosis. In addition, OMF strongly reduced stemness markers and *survivin*, *hTERT* and *Nanog-1* gene expression. Growth of colonies in soft-agar was significantly affected by OMF treatment, too. Lastly, we tested ability of MCF-7 cells to form mammospheres after treatment with OMF or cisplatin, demonstrating that OMF treatment resulted in drastic

reduction in number of mammospheres. These results introduce OMF as an effective molecule in suppressing breast cancer stem cells.

Introduction

Breast cancer is the deadliest form of this disease and affects great numbers of women worldwide. Although there are effective therapies against some forms of this tumour, mortality among affected women is still quite high. Identification of cancer stem cells in breast tumours – able to give rise to cells making up the bulk of the tumour mass (termed spheroids or mammospheres) – has shifted the focus of breast cancer research (1). Cancer stem cells (CSCs) are rare subpopulations of cells in tumours, organized in a cellular hierarchy, that possess unlimited proliferation potential, self-renewal, differentiation potential, and are capable of driving tumour growth and recurrence (2–4). CSCs are resistant to many current types of cancer treatment, including chemotherapy and radiotherapy. Thus many cancer treatments, while killing the bulk of tumour cells, may ultimately fail as they do not eliminate CSCs, which survive to regenerate new tumours resulting in relapse or recurrence for the patient (5–8). Several groups have demonstrated that CD44⁺CD24^{−/low} and CD133⁺ lineage phenotypes well identify subpopulations of mammospheres of breast cancers, which have a drug-resistance phenotype and capacity to form tumours in immunodeficient mice (1,9). In addition, these mammospheres display resistance to proapoptotic agents and greater tumorigenicity in immunodeficient mice, than their parental cell line (10). The mechanism regulating drug resistance is partly conferred by expression of ATP binding cassette protein (ABC) transporters, such as ABCG2 (11). ABC transporters have the capacity to export many cytotoxic drugs (12), and are

Correspondence: M. A. Tufano, Department of Experimental Medicine, Section of Microbiology and Clinical Microbiology, Second University of Naples (SUN), Naples, via Luigi de Crecchio no. 7, 80138 Naples, Italy. Tel.: +39 081 5665667; Fax: +39 081 5665662; E-mail: mari-aan.tufano@unina2.it

up-regulated in cancer stem cells, favouring their survival from conventional therapies.

Survivin, a dual regulator of cell division and apoptosis broadly overexpressed in cancer, is a candidate effector molecule controlling stem-cell viability (13). It has been shown that survivin expression is regulated by developmental signalling pathways (such as Wnt), which are operative in stem cells, and it is possible that survivin antagonists may affect cancer stem cell populations (14). In addition, much attention has been focused on detection of telomerase activity and its essential components (hTR and hTERT) in both malignant and non-malignant tissues. Expression of hTR and hTERT is upregulated in almost all human malignant tumours, but not in benign or normal tissues, with the exception of germline cells, proliferative stem cells, activated lymphocytes, and certain benign tumour cells. Thus, telomerase has been considered a useful marker for cancer diagnosis and in some instance as a prognostic indicator of outcome (15). It has been demonstrated that inhibition of telomerase activity in human cancer cells, by using antisense against hTERT, forces them either into apoptosis or into differentiation (16).

3-*O*-methylfunicone (OMF) is a secondary metabolite produced by the soil fungus *Penicillium pinophilum* (17) that has been found to antagonize plant pathogenic fungus *Rhizoctonia solani* (18). Previous studies carried out on different tumour cell lines have demonstrated that OMF can induce the apoptotic pathway and inhibit cell motility (19–22). Results on human breast adenocarcinoma MCF-7 cells are also interesting; OMF induces reduction in proliferation and inhibition of motility in MCF-7 cells, while the same effect is not observed in normal epithelial breast cells (MCF-10). In particular, we demonstrated that OMF strongly down-regulates survivin and hTERT expression in treated MCF-7 cells, indicating that it influences the cell cycle and cell half-life.

Considering growing interest in potential for cancer stem-cell therapy to contest tumour relapse, we have tried to determine whether OMF is effective in suppressing breast cancer stem cells.

Materials and methods

OMF preparation

OMF was extracted from liquid cultures of isolate LT4 of *P. pinophilum*, as previously described (18). This strain is stored in the mycological collection of the Council for Research and Experimentation in Agriculture, Scafati, Italy. For biological assays, the compound was dissolved in absolute ethanol at a concentration of 10 mg/ml.

Cell culture, mammospheres and treatments

MCF-7 human breast carcinoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine at 37 °C, 5% CO₂. To form MCF-7 spheres, cells were plated in six-well ultra low attachment plates at density of 60 000 cells/well (Corning Inc., Corning, NY, USA) in mammary epithelial basal medium (MEBM) supplemented with mammary epithelial cell growth medium (MEGM). Fresh aliquots of EGF and βFGF were added twice weekly. After culturing for 48 h, spheres were visible by inverted phase-contrast microscopy (Nikon TS 100; Nikon, Torino, Italy), and these spheres were defined as 'mammospheres'. A preliminary time and dose-response curve was composed to determine concentration and time by which OMF produced significant effects on cell morphology. Four hundred MCF-7 cell aliquots were plated in six-well plates (35 mm diameter) with 2 ml DMEM and treated with 80 µg/ml OMF or 40 µM cisplatin (CP) for 48 h. Six hundred mammosphere aliquots at 5th and 10th passages were plated in six-well (35 mm diameter) ultra-low attachment plates with 3 ml MEBM and treated with 80 µg/ml OMF or 40 µM CP for 48 h. In addition, MCF-7 cells and mammospheres were treated or not treated with 16 µl absolute ethanol (data not shown). To test ability of OMF or CP-treated MCF-7s to form mammospheres, MCF-7s were first treated with 80 µg/ml OMF or 40 µM CP for 48 h, then trypsinized and plated in six-well ultra-low attachment plates at concentration of 6×10^5 cells per well in MEMB, and were observed daily.

Morphological analysis

Morphological features of MCF-7 cells and mammospheres, treated or not treated with OMF and CP, were defined by phase-contrast microscopy (Olympus CDK40, Milan, Italy) at 20× magnification.

Flow cytometric analysis and apoptosis

To analyse stemness characteristics, MCF-7 cells grown in adherent culture conditions, and mammospheres at 5th (P5) and 10th (P10) passage were trypsinized, counted and washed in phosphate-buffered saline. At least 1×10^5 cells were incubated with 1 µg/ml of fluorescence-labelled monoclonal antibodies or respective isotype controls, at 4 °C for 30 min in the dark. After repeated washing, labelled cells were analysed by flow cytometry using a FACS Vantage cell sorter (Becton & Dickinson, Mountain View, CA, USA). Antibodies tested

were mouse anti-human CD24 PE (BD Pharmingen, Bucinasco, Milan, Italy), mouse anti-human CD29 CY (BD Pharmingen), mouse anti-human CD44 FITC (BD Pharmingen), mouse anti-human CD133 PE, mouse anti-human CD184 CY and mouse anti-human CD338 PE (Miltenyi Biotec, Calderara di Reno, Bologna, Italy). The same procedure was also performed on mammospheres treated with OMF and CP. Data were analysed using CellQuest Software. Apoptosis induced by OMF treatment was additionally measured by fluorescence activated cell sorting (FACS) analysis. At 48 h after OMF and CP treatment, MCF-7 cells and mammospheres were trypsinized and stained using the FITC Annexin V Apoptosis Detection Kit (BD Pharmingen) according to the manufacturer's instructions. Stained cells were analysed using the FACS Vantage Cell Sorter (Becton Dickinson), and data were analysed using CellQuest Software.

Cell colony formation assay

Inhibition of mammosphere colony formation following treatment with OMF and CP was measured by soft agar assay. This was performed using 0.8% and 0.3% soft agar in DMEM at 10% FBS as base and top layers respectively. Briefly, cells at different concentrations (1000, 5000 and 10000 cells) were plated and treated with OMF and CP for 48 h. Cultures were maintained at 37 °C with 5% CO₂ atmosphere for 21 days. Cell colonies were stained with 50 mg/100 ml nitrobluetetrazolium and counted using inverted microscopy.

Real-time polymerase chain reaction

Semi-confluent MCF-7 cells or mammospheres at passage 10 were pre-treated or not with 80 µg OMF or 40 µM CP for 48 h, then, total RNA was isolated using the High Pure RNA Isolation Kit (Roche Diagnostics, Milan, Italy). One hundred nanograms of total cell RNA was reverse-transcribed (Expand Reverse Transcriptase; Roche Diagnostics) into complementary DNA

(cDNA) using random hexamer primers (random hexamers; Roche Diagnostics) at 42 °C for 45 min, according to the manufacturer's instructions. Resulting cDNA was subjected to real-time polymerase chain reaction (PCR) analysis by rapid cycling, in glass capillaries with a thermocycler (Light-Cycler; Roche Molecular Biochemicals, Milan, Italy). The reaction was performed at final volume of 20 µl with Light-Cycler-DNA Master SYBR Green I (Roche Diagnostics), which contains nucleotides, Taq DNA polymerase, buffer, SYBR Green I dye and 10 mM MgCl₂; cDNA (2 µl), primers and sterile H₂O were added. For primer sequences, PCR conditions and size of products, see Table 1. Each real-time PCR was performed three times for each sample. SYBR Green I fluorescence was monitored at the end of each cycle to assess amounts of PCR product formed. After completion of the amplification protocol, a melting curve analysis was performed to confirm specificity of amplification by cooling the sample to 65 °C at a rate of 20 °C/s, maintaining a temperature of 65 °C for 10 s and then heating to 95 °C at a rate of 0.2 °C/s, with continuous measurement of fluorescence. Cycle-to-cycle fluorescence emission readings were monitored and analysed using Light Cycler Data Software (Roche Molecular Biochemicals). Specificity of amplification products was verified by electrophoresis on a 1.4% agarose gel stained with ethidium bromide (1 µg/ml). Sequence-specific standard curves were generated using serial dilution of specific DNA standards. All quantifications were normalized to the housekeeping gene *β-actin*. Data are presented as ratios between target gene expression and gene expression in unstimulated conditions.

Statistical analysis

Each experiment was performed in triplicate and results are expressed as mean ± standard deviation (SD). Significance was assessed using Student's *t*-test, with *P* < 0.05 considered as a significant difference.

Table 1. Human sense and antisense primer sequences

Gene	Sense and antisense primers sequence	Conditions
Nanog 1	5'-TTCTTCCACCAGTCCCAAAG-3' 5-ATCTGCTGGAGGCTGAGGTA-3'	50 cycles at 95 °C for 5 s 62 °C for 4 s, 72 °C for 7 s
hTERT	5'-CGGAAGAGTGTCTGGAGCAA-3' 5'-GGATGAAGCGGAGTCTGGA-3'	45 cycles at 95 °C for 5 s 52 °C for 3 s, 72 °C for 7 s
Survivin	5'-ATGAGATACCATGGGTGCCCGACG-3' 5'-TTAAGGATCCCTGCTCGATGGCACG-3'	45 cycles at 94 °C for 5 s 53 °C for 10 s, 72 °C for 18 s
β-actin	5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3' 5'-CTAGAAGCATTGCGGTGGACGATGGAGGG-3'	

Results

Effect of OMF on MCF-7 cells and on mammospheres

We have previously shown that OMF can affect cell population growth of different tumour cell lines and induce apoptosis (20–22). In particular, we have demonstrated that MCF-7 cell motility 24 h after OMF treatment (80 µg/ml) was less, whereas human breast epithelial cells (MC-10) were not affected, thus showing selectivity of our molecule (21). In the present study, we investigated effects of OMF on population growth of MCF-7 and breast cancer stem cells. A characteristic of cancer stem cells is their ability to grow in three-dimensional structures as spherical clusters in semisolid support or in liquid culture, in the absence of attachment. We applied a previously described method to isolate mammospheres from a subpopulation of MCF-7 cells (23). Mammosphere cultures were easily obtained from MCF-7 cells, with ability of self-renewal, and to differentiate again into epithelial cell lines when grown in adherent culture conditions (data not shown). Expression of some surface markers on MCF-7 cells grown in adherent culture conditions and mammospheres at P5 and P10 were analysed by flow cytometry to better characterize the stemness phenotype. Cytometric analysis showed that MCF-7 cells grown in adherent culture conditions expressed high levels of CD24 (96%) and CD29 (100%). CD44, CD133, CD184 and CD338 positive cells were 38%, 0.78%, 1.15% and 0.90% of total cell populations respectively (Table 2). Moreover, cells which were positive for CD44 expressed CD24 and CD29 markers. In mammospheres at P5 and P10, CD24 levels remained similar to those analysed in MCF-7 cells grown in adherent culture conditions, at levels of 95% and 90% respectively. At passage 10, CD44 and CD133 stemness antigen levels increased up to 69% and 5% respectively, whereas CD29 remained 100%. Furthermore, CD184 and

CD338 markers increased by 61% and 5.6% respectively (Table 2).

Successively at passage 10, MCF-7s and mammospheres were treated with 80 µg/ml of OMF and 40 µM CP for 48 h. OMF increased MCF-7 cell detachment from the substratum, inducing modified morphology and cell rounding with typical features of apoptotic cell death (cell shrinkage, formation of blebs on the membrane, detachment from the surface and more characteristic changes) (Fig. 1b), while CP treatment resulted in reduced apoptotic effect with prevalent features of necrotic cells, as confirmed by cytometry (Fig. 1c,d). For MCF7 cells grown in adherent culture conditions, OMF treatment resulted in induction of apoptosis (70%) and reduced necrosis (10%), whereas CP treatment led to a greater necrotic effect (25%) and reduced apoptosis (59%).

This effect was absent when cells were treated with vehicle solution (ethanol) only (data not shown). Furthermore, OMF treatment induced marked reduction in mammosphere size (Fig. 2). Occurrence of apoptosis in OMF-treated mammospheres was confirmed by FACS analysis showing that OMF induced 51% of apoptosis and 13% of necrosis, compared to 40% and 8% of CP-treated mammospheres respectively (Fig. 2d). Percentage of viable mammospheres treated with OMF was 36% compared to 52% of CP-treated mammospheres.

Interestingly, OMF treatment also induced strong down-regulation of mammosphere stemness and differentiation markers compared to CP treatment. As shown in Fig. 3b, OMF-treated mammospheres at P10 exhibited very low levels of CD24, CD29, CD44, CD133, CD184, CD338 (10%, 0%, 0%, 0.36%, 7.04% and 0.20% respectively) compared to CP-treated mammospheres (72%, 80%, 24%, 3.18%, 37.85% and 3.8% respectively). Low levels of stemness and differentiation markers were also detected on mammospheres at P5 (Fig. 3a).

Lastly, we tested ability of MCF7 cells to form mammosphere after treatment with OMF or CP. OMF treatment resulted in drastic reduction in number of mammospheres and size from controls, whereas CP-treated MCF-7s conserved ability to form mammospheres, even though slightly smaller in size (Fig. 4).

Table 2. Distribution of stemness and differentiation markers on MCF7 cells and mammospheres

Markers	Mean percentage of positive MCF7 cells (%)	Mean percentage of positive mammospheres (%) at P5	Mean percentage of positive mammospheres (%) at P10
CD24	96	95	90
CD29	100	100	100
CD44	38	45	69
CD133	0.78	2	5.12
CD184	1.15	5.30	61
CD338	0.90	4.08	5.60

OMF inhibited cell colony formation of mammospheres

Mammospheres were plated on a soft agar matrix, treated with OMF and CP, and incubated at 37 °C in a 5% CO₂ incubator. After 21 days, numbers of colonies were counted. As shown in Fig. 5, OMF inhibited colony formation, modifying their size and morphology, at a percentage (43%) higher than CP (20%). This result suggests that OMF is a critical inhibitor of mammosphere cell proliferation.

Figure 1. Effect of OMF and CP treatment on cell morphology of MCF7 cells as shown by phase-contrast microscopy. (a) Untreated MCF-7 cells (48 h). (b) 80 µg/ml OMF-treated MCF-7 cells. (c) 40 µM CP-treated MCF-7 cells. Data shown are representative of three different experiments. Magnification: 20×. (d) Effect of OMF and CP treatment on cell viability and apoptosis induction of MCF-7 cells, stained with the FITC Annexin V Apoptosis Detection Kit, by cytometry. Data are presented as the means $\pm$ SD of results for three independent experiments.

Figure 2. Effect of OMF and CP treatment on cell morphology of mammospheres as shown by phase-contrast microscopy. (a) Untreated mammospheres (48 h). (b) 80 µg/ml OMF-treated mammospheres. (c) 40 µM CP-treated mammospheres. Data representative of three different experiments. Magnification: 20×. (d) Effect of OMF and CP treatment on cell viability and apoptosis induction in mammospheres, stained with the FITC Annexin V Apoptosis Detection Kit, by cytometry. Data are presented as means $\pm$ SD of results of three independent experiments.

Effect of OMF on survivin, hTERT and Nanog-1 gene expression

To better investigate molecular mechanisms by which OMF could control cancer stem cell viability, expression of survivin and hTERT were analysed in the MCF-7 cells and mammospheres, by real time PCR. As shown in Fig. 6a,d both OMF and CP were able to reduce expression of survivin and hTERT; but, the effect of OMF was stronger. Furthermore, to investigate whether OMF was able to affect cancer stem-cell multipotency, *Nanog-1* gene expression in MCF-7 cells and mammospheres treated with OMF or CP was also analysed. As shown in

Figs. 7a,b, OMF strongly reduced expression of *Nanog-1* both in MCF-7 cells and in mammospheres, while CP did not display this effect.

Discussion

Natural products can be a rich source of drugs with biomedical and industrial applications (24). As a consequence, a competitive aptitude developed in many and diverse environments, microorganisms also represent a considerable source of antitumour drugs. In particular, fungal species of the genus *Penicillium* produce a high number of biologically active extrolites that have been

Figure 3. Characterization of stemness and differentiation markers in OMF- and CP-treated mammospheres stained for CD24 PE, CD29 CY, CD44 FITC, CD133 PE, CD184 CY and CD338 PE mouse anti-human antibodies, by cytometry, at P5 (a) and P10 (b). Data presented as means $\pm$ SD of results of three independent experiments.

Figure 4. Effect of OMF and CP treatment on MCF-7 cell ability to form mammospheres, as shown by phase-contrast microscopy. (a) Mammospheres from untreated MCF-7 cells. (b) Mammospheres from 80 µg/ml OMF-treated MCF-7 cells. (c) Mammospheres from 40 µM CP-treated MCF-7 cells. Data representative of three different experiments. Magnification: 20 $\times$.

Figure 5. OMF inhibits colony formation of mammospheres on soft agar. Representative phase contrast images are shown. (a) Untreated mammospheres. (b) 80 µg/ml OMF-treated mammospheres. (c) 40 µM CP-treated mammospheres. After 21 days, number of colonies was counted. (d) graphs represent percentage of colony inhibition after OMF and CP treatments. Data presented as average number of colonies per plate, as determined in three separate experiments.

disclosed to be possible applications in cancer chemotherapy (25). We have previously demonstrated that OMF can induce growth arrest in different tumour cell lines

(19–22). In this study, we investigated the effect of OMF on breast cancer stem cells starting from the MCF-7 cell line. We purified cancer stem cells by using a

Figure 6. Real time PCR analysis using specific primers for survivin and hTERT. (a, b) Ctrl: mRNA from untreated MCF-7 cells; OMF, mRNA from MCF-7 cells treated with 80 $\mu\text{g}/\text{ml}$ OMF for 48 h; CP, mRNA from MCF-7 treated with 40 $\mu\text{g}/\text{ml}$ CP for 48 h. (c, d) Ctrl: mRNA from untreated mammospheres; OMF, mRNA from mammospheres treated with 80 $\mu\text{g}/\text{ml}$ OMF for 48 h; CP, mRNA from mammospheres treated with 40 $\mu\text{g}/\text{ml}$ CP for 48 h. Data representative of three different experiments $\pm$ SD. *Significantly different compared to control ($P < 0.01$).

Figure 7. Real time PCR analysis using specific primers for Nanog-1. (a) Ctrl: mRNA from untreated MCF-7 cells; OMF, mRNA from MCF-7 cells treated with 80 $\mu\text{g}/\text{ml}$ OMF for 48 h; CP, mRNA from MCF7 treated with 40 $\mu\text{g}/\text{ml}$ CP for 48 h. (b) Ctrl: mRNA from untreated mammospheres; OMF, mRNA from mammospheres treated with 80 $\mu\text{g}/\text{ml}$ OMF for 48 h; CP, mRNA from mammospheres treated with 40 $\mu\text{g}/\text{ml}$ CP for 48 h. Data representative of three different experiments $\pm$ SD. *Significantly different from control ($P < 0.01$).

mammosphere formation method. Before OMF/CP treatments, stemness antigens were analysed of the mammospheres obtained. CD44, CD133, CD29 and CD338 were used as main stemness markers, while CD24 was considered to be an epithelial differentiation marker. Moreover, we performed an assay for CD184, a marker involved in cell invasion and migration. At P10, mammospheres expressed high levels of CD44, CD133, CD29 and CD338 indicating their stemness phenotype, although CD24 expression levels remained similar to those obtained on corresponding cells grown in adherent conditions. Ponti *et al.* demonstrated that cancer stem cells isolated from breast cancer had CD44⁺CD24^{-/low} phenotype (23), whereas Hwang-Versluis *et al.* identified several stem/progenitor cell-like subpopulations in breast cancer with different expression of stemness markers. They reported that CD133 can identify a group of breast cancer stem cells that does not overlap with CD44⁺CD24^{-/low} cells (26). Here, we found that mammospheres not only

coexpressed both CD44 and CD24 but also CD133 and CD338. Most probably, we obtained mammospheres that were epithelial progenitors expressing stemness markers such as CD44, CD133 and CD338 and epithelial differentiation marker CD24. Interestingly, at P10, we found that CD184 is highly over-expressed on mammospheres, suggesting their capacity to invade and metastasize. The strong increase in stemness markers could be due to proliferation of progenitor cells forming mammospheres, and possibly to higher exposure of markers on their surfaces, due to their three-dimensional structures. OMF displayed stronger antiproliferative effects than CP but both MCF-7 cells, grown in adherent conditions, and mammospheres were remarkably affected by OMF treatment. This was evident in cell morphology and also from stemness markers. In particular, OMF strongly down-regulated both CD133 and CD338 expression, whereas CD44 and CD29 expression was completely abrogated. Moreover, it led to a strong decrease in CD184 and CD24 levels

demonstrating its effect on different cell subsets that constituted the mammospheres. CP treatment, however, led to weak reduction in all markers tested. Interestingly, OMF treatment displayed its effect also on viability of mammospheres, leading to more incisive apoptosis compared to CP treatment. It is noteworthy that treated cells lost their ability to form mammospheres, suggesting that OMF treatment selectively affected growth of MCF-7 subpopulations able to precede cancer stem cells. This result is interesting considering that the major cause of cancer recurrence is presence of this rare subpopulation of cells within tumours.

In addition to stem/progenitor cell properties, isolated breast cancer stem cells exhibited features related to activation of cytoprotective mechanisms and to immortalization. The antiapoptotic protein survivin and also hTERT were overexpressed both in MCF-7 adherent cells and in mammospheres, while OMF treatment strongly affected their expression. Indeed, results on MCF-7 adherent cells were expected, as we have previously demonstrated that OMF modulates survivin and hTERT in the same cell lines (21). However, the most interesting feature of the results concerns the strong down-regulation of *survivin* and *hTERT* gene expression in mammospheres. As these genes play critical roles in cancer stem-cell proliferation, OMF might be effective in reducing survival potential of cancer stem cells. The embryonic stem cell self-renewal gene product, *Nanog*, is a highly divergent homoeodomain-containing protein playing a central position in the transcriptional network of pluripotency (27). It is expressed not only in germ tumour cells but also in other tumours including breast, cervix, oral cavity, kidney and ovary carcinoma. Pluripotency does not develop without *Nanog*, and cells are trapped in a pre-pluripotent, indeterminate state that is non-viable. Our results on *Nanog*-1 are indicative of OMF's ability to limit cancer stem cells' self-renewal and differentiation potential, reducing both stemness surface markers and genes controlling pluripotency, such as *Nanog*.

These data highlight the impact of OMF on destiny of various cell subpopulations forming a tumour. In conclusion, OMF treatment results in a stronger effect in terms of apoptosis induction, reduction in mammosphere size and down-regulation of mammosphere stemness and differentiation markers compared to CP treatment. As refractory tumours may be caused by resistance of cancer stem cells to commonly used cytotoxic therapies, OMF might represent a promising chemotherapeutic agent that is able to kill both the bulk of tumour cells and any cancer stem cells, overcoming occurrence of repeat tumours and metastasis.

Further studies on the ability of OMF to inhibit cancer stem-cell population growth should be carried out, *in vivo* and *in vitro*, to more thoroughly evaluate its effect.

OMF might be an attractive drug in view of a therapeutic intervention aimed at inhibiting not only tumour-cell proliferation but also formation and spread of cancer stem cells.

Conflict of interest

The authors confirm that there are no conflicts of interest.

References

- 1 Fillmore CM, Kuperwasser C (2008) Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. *Breast Cancer Res.* **10**, R25.
- 2 Rosen JM, Jordan CT (2009) The increasing complexity of the cancer stem cell paradigm. *Science* **324**, 1670–1673.
- 3 Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Visvader DL *et al.* (2006) Cancer stem cells-perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Res.* **66**, 9339–9344.
- 4 Jordan CT, Guzman ML, Noble M (2006) Cancer stem cells. *N. Engl. J. Med.* **355**, 1253–1261.
- 5 Gupta B, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA *et al.* (2009) Identification of selective inhibitors of cancer stem cells by high-throughput screening. *Cell* **138**, 645–659.
- 6 Charafe-Jauffret E, Ginestier C, Birnbaum D (2009) Breast cancer stem cells: tools and models to rely on BMC. *Cancer* **9**, 202.
- 7 Dean M, Fojo T, Bates S (2005) Tumour stem cells and drug resistance. *Nat. Rev. Cancer* **5**, 275–284.
- 8 Costea DE, Tsinkalovsky O, Vintermyr OK, Johannessen AC, Mackenzie IC (2006) Cancer stem cells – new and potentially important targets for the therapy of oral squamous cell carcinoma. *Oral Dis.* **12**, 584.
- 9 Wright MH, Calcagno AM, Salcido CD, Carlson MD, Ambudkar SV, Varticovski L (2008) *Brcal* breast tumors contain distinct CD44+/CD24– and CD133+ cells with cancer stem cell characteristics. *Breast Cancer Res.* **10**, R10.
- 10 Cariati M, Naderi A, Brown JP, Smalley MJ, Pinder SE, Caldas C *et al.* (2008) Alpha-6 integrin is necessary for the tumorigenicity of a stem cell-like subpopulation within the MCF7 breast cancer cell line. *Int. J. Cancer* **122**, 298–304.
- 11 Doyle LS, Ross DD (2003) Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). *Oncogene* **22**, 7340–7358.
- 12 Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM (2006) Targeting multidrug resistance in cancer. *Nat. Rev. Drug Discov.* **5**, 219–234.
- 13 Altieri DC (2008) Survivin, cancer networks and pathway-directed drug discovery. *Nat. Rev. Cancer* **8**, 61–70.
- 14 Reya T, Morrison SJ, Clarke MF, Weissman IL (2001) Stem cells, cancer, and cancer stem cells. *Nature* **414**, 105–111.
- 15 Hiyama E, Hiyama K (2002) Clinical utility of telomerase in cancer. *Oncogene* **21**, 643–649.
- 16 Kraemer K, Fuessel S, Schmidt U, Kotsch M, Schwenzer B, Wirth MP *et al.* (2003) Antisense-mediated hTERT inhibition specifically reduces the growth of human bladder cancer cells. *Clin. Cancer Res.* **9**, 3794–3800.
- 17 De Stefano S, Nicoletti R, Milone A, Zambardino S (1999) 3-O-Methylfunicone, a fungitoxic metabolite produced by the fungus *Penicillium pinophilum*. *Phytochemistry* **52**, 1399–1401.

- 18 Nicoletti R, De Stefano M, De Stefano S, Trincone A, Marziano F (2004) Identification of fungitoxic metabolites produced by some *Penicillium* isolates antagonistic to *Rhizoctonia solani*. *Mycopathologia* **158**, 465–474.
- 19 Stamatii A, Nicoletti R, De Stefano S, Zampaglioni F, Zucco F (2002) Cytostatic properties of a novel compound derived from *Penicillium pinophilum*: an in vitro study. *Altern. Lab. Anim.* **30**, 69–75.
- 20 Buommino E, Nicoletti R, Gaeta GM, Orlando M, Ciavatta ML, Baroni A *et al.* (2004) 3-O-methylfunicone, a secondary metabolite produced by *Penicillium pinophilum*, induces growth arrest and apoptosis in HeLa cells. *Cell Prolif.* **37**, 413–426.
- 21 Buommino E, Boccellino M, De Filippis A, Petrazzuolo M, Cozza V, Nicoletti R *et al.* (2007) 3-O-methylfunicone by *Penicillium pinophilum* affects cell motility of breast cancer cells, down-regulating α V β 5 integrin and inhibiting MMP9 secretion. *Mol. Carcinog.* **46**, 930–940.
- 22 Baroni A, De Luca A, De Filippis A, Petrazzuolo M, Manente L, Nicoletti R *et al.* (2009) 3-O-methylfunicone, a metabolite from *Penicillium pinophilum*, inhibits proliferation of human melanoma cells by causing G₂/M arrest and inducing apoptosis. *Cell Prolif.* **42**, 541–553.
- 23 Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Corradini D *et al.* (2005) Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res.* **65**, 5506–5511.
- 24 Newman DJ, Cragg G, Snader KM (2003) Natural products as sources of new drugs over the period 1981–2002. *J. Nat. Prod.* **66**, 1022–1037.
- 25 Nicoletti R, Ciavatta L, Buommino E, Tufano MA (2008) Antitumor extrolites produced by *Penicillium* species. *Int. J. Biomed. Pharm. Sci.* **2**, 1–23.
- 26 Hwang-Verslues WW, Kuo WH, Chang PH, Pan CC, Wang HH, Tsai ST *et al.* (2009) Multiple lineages of human breast cancer stem/progenitor cells identified by profiling with stem cell markers. *PLoS One* **4**, e8377.
- 27 Jeter CR, Badeaux M, Choy G, Chandra D, Patrawala L, Liu C *et al.* (2009) Functional evidence that the self-renewal gene NANOG regulates human tumor development. *Stem Cells* **27**, 993–1005.