The present invention relates to the field of biomedicine and in particular to the treatment of cancer and preventing and/or treating resistance to cancer drugs and therapies, and delaying or preventing tumor recurrence. Specifically, it relates to secoiridoid polyphenol compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, for use in a method of treating a subject suffering from cancer by inhibiting, or reducing the frequency of, cancer stem cells (CSCs). It further relates to pharmaceutical compositions, kits, methods of treatment and combination therapies using thereof.
FIELD OF THE INVENTION

The present invention relates to the field of biomedicine and in particular to the treatment of cancer and preventing and/or treating resistance to cancer drugs and therapies, and delaying or preventing tumor recurrence. Specifically, it relates to secoiridoid polyphenols for use in the treatment of cancer by inhibiting cancer stem cells. It further relates to pharmaceutical compositions, kits, methods of treatment and combination therapies using thereof.

STATE OF THE ART

Resistance to cancer drugs remains a key obstacle to successful cancer therapy. A proposed mechanism explaining primary and acquired resistance invokes the existence of a specialized subset of cancer cells defined as cancer stem cells (CSCs). CSCs have the capacity to self-renew and to generate new tumors that consist entirely of clonally derived cell types present in the parental tumor. CSCs are resistant to many conventional cancer therapies and they can survive treatment in spite of dramatic shrinkage of the tumor. Residual CSCs can then eventually regrow, which results in disease relapse. CSCs have also been related with the propensity of a tumor to form metastasis (Wei and Lewis, Endocr Relat Cancer. 2015, 22(3): R135-R155; Pattabiraman and Weinberg, Nat Rev Cancer. 2014, 13(7): 497-512).

The inherent drug resistance of CSCs, together with their ability to regenera the primary tumor and their life-threatening ability to metastasize to other sites in the body, are seen as the ultimate root causes of the lack of curative options in many types of malignancies. Recently, it has been discovered the potential of non-CSCs to transit a two-way street to become CSCs, and vice versa. This implies that cancer therapies do not necessarily enrich cancer tissues with pre-existing, genetically defined populations of treatment-refractory CSCs, as previously thought; rather, these may accelerate the de novo production of CSC-like states from the residual cancer tissue which may easily repopulate the tumor while the older CSCs die (Visvader and Lindeman, Nat Rev Cancer. 2008, 8(10): 755-768; Magee et al., Cancer Cell. 2012, 21(3): 283-296; Marjanovic et al., Clin Chem. 2013, 59(1):1, 168-179; Gupta et al., Cell. 2011, 146(4): 633-644).

A complex circuit of metabolo-epigenetic mechanisms has been proposed by the inventors as the underlying mechanism that generates, maintains and perpetuates CSC states, which, in turn, dynamically repopulate the tumor tissue in response to any kind of therapy (Menendez et al., Front Oncol. 2014, 4:262; Menendez et al., Oncoscience. 2014, 1(12), 803-806; Menendez JA, Curr Pharm Des. 2015, 21(25):3644-3653). The fact that aberrant sternness is an acquired trait provides the challenge of finding a drug targeting the epigenetic programs that dynamically regulate the plastic interchange between non-CSC and CSC states.

Plant polyphenols have been identified as potentially useful in the inhibition of cancer stem cells. Notably, Kawasaki et al. (Mol Interv. 2008, 8(4): 174-184), referred to epigallocatechin gallate (EGCG) from green
tea and curcumin present in turmeric, as promising drugs for targeting cancer stem cells. More specifically, fluorine-substituted analogs of curcumin have demonstrated their potential as therapeutic agents for eliminating CSCs in highly aggressive pancreatic tumors (US 2014/03031 09 A1).

Besides, the cytotoxic and anti-proliferative activities of tyrosol, hydroxytyrosol and oleuropein, the major phenolic compounds present in olive oil, have previously been described in human breast cancer and leukemia (WO2014/085613 A1; Samet et al., Oxid Med Cell Longev. 2014, 2014:927619; Han et al., Cytotechnology. 2009, 59(1), 45-53). Gulcin Tezcan et al., Am J Cancer Res, 1 January 2014, discloses the use of an extract of the leaves of Olea europaea (OLE), which contains oleuropein, in the treatment of cancer stem cell positive glioblastoma (GBM) tumors. More specifically, Gulcin Tezcan et al., discloses the anti-proliferative activity of OLE in various GBM cell lines based on the results in the WST-1 assay. It is silent however on the ability of these compounds to inhibit the self-renewal and tumor initiating capacity of cancer stem cells (CSCs), let alone the ability to do it selectively in the absence of cytotoxic effects.

Furthermore, the inventors have previously tested different extra virgin olive oil (EVOO) varieties and related a higher presence of secoiridoid polyphenols in the phenolic extracts with higher cytotoxic effects in human breast cancer cell lines (Lozano-Sanchez et al., J Agric Food Chem. 2010, 58(18), 9942-9955; Garcia-Villalba et al., J Pharm Biomed Anal. 2010, 51(2): 416-429). In addition, secoiridoid-rich EVOO phenolic extracts (PE) have been reported for the use in the therapeutic treatment of highly aggressive HER-2 positive breast tumors resistant to trastuzumab and lapatinib based on the cell growth inhibitory effects of EVOO-PE observed on JIMT-1 cell line which is intrinsically resistant to trastuzumab and other HER-2 inhibiting drugs (Oliveras-Ferraros et al., Int J Oncol. 2011, 38(6): 1533-1547). In particular, oleuropein aglycone was said to increase the anti-proliferative effects of trastuzumab, the observed effects being associated to the induced downregulation of HER2 expression (Menendez et al., BMC Cancer. 2007, 9, 7:80).

EVOO secoiridoids were also reported to act as chemopreventing, anticancer and/or antiangiogenic agents by their ability to prevent fibrogenic and oncogenic epithelial to mesenchymal transition (EMT) (Vazquez-Martín et al., Rejuvenation Res. 2012, 15(1), 3-21). Vazquez-Martín et al. mentions that EVOO phenolic extracts "impede" or "prevent" the acquisition of stem cell features through EMT induction but does not directly and unambiguously disclose the effect of EVOO secoiridoids in "reversing" an EMT-induced de novo cancer stem cell states, let alone their ability to inhibit the sternness features of any stem cell states already existing within a tumor. In addition, EVOO polyphenols were reported to activate adenosine monophosphate-activated protein kinase (AMPK); and said to reduce the expression of crucial genes involved in cell metabolism (i.e., Warburg effect) and in the self-renewal capacity of CSCs (Menendez et al., Cell Cycle. 2013, 12(4), 555-578; Coraminas-Faja et al., Aging. 2014, 6(9), 731-741). ALDH1A3 gene (a biomarker of breast CSCs) is one of the many genes which were disclosed to be downregulated in Menendez et al., (Cell Cycle, 2013, 12(4), 555-578). This document also mentions that it was under investigation whether treatment with EVOO secoiridoids impedes the propensity of CSCs to form multicellular microtumors under non-adherent and non-differentiating conditions. It does not disclose however that an effect in sphere inhibition was actually obtained, let alone that CSCs inhibition was selective.
Accordingly, there is an on-going need for new treatments to successfully address the heterogeneity within cancer cell populations. In particular, new treatments are needed to regulate the plastic interchange between non-CSC and CSC states, and successfully inhibit CSCs.

SUMMARY OF THE INVENTION

The present invention establishes that the compounds of formula (I), notably oleuropein aglycone (OA) and decarboxymethyl oleuropein aglycone (DOA), inhibit cancer stem cells (CSCs) within a tumor. In particular, DOA has been shown to regulate the non-CSC to CSC conversion by interfering in metabolic pathways (i.e., mTOR inhibitor) and epigenetic mechanisms (e.g., DNMT inhibitor). Moreover, DOA has been found to interact with various epigenetic enzymes. Accordingly, the present invention provides novel multiple epigenetic modulators and dual metabolo-epigenetic inhibitors (e.g., mTOR and DNMT inhibitor) that are useful for the treatment of cancer and/or cancer resistance, specifically through the inhibition, or reduction of the frequency of, CSCs within a tumor.

The inventors have shown for the first time that a compound of formula I has the ability of suppressing the CSC state characterizing features of self-renewal ability and tumor initiating capacity within a cancer cell population. In particular, it has been shown in Examples 2 and 3 for DOA and in Example 5 for OA, that these compounds prevent CSC-containing tumor cell lines from developing spheres when cultured under non-adherent and non-differentiating conditions, which is commonly regarded as an in vitro model of the self-renewal and tumor initiating capacities of CSCs.

Moreover, Example 7 shows that DOA significantly reduces the ability of a cancer cell line containing cancer stem cells (i.e., SUM-1 59 cells) to reproduce a tumor when injected into an immunocompromised animal, the in vivo assay being the ultimate evidence of inhibition of stem cell tumor initiating capacity.

The fact that these compounds are shown to inhibit various cancer cell lines tumor initiating capacity, evidences the suppression of pre-existing CSC states within a tumor and the prevention of the de novo generation of cells with stem cell properties, for instance by preventing a epithelial to mesenchymal (EMT) transition.

Two interchangeable populations of epithelial-like (aldehyde dehydrogenase expressing, ALDhT) and mesenchymal-like (CD44+CD24~*~W) CSCs can be found in significantly different proportions across all intrinsic breast cancer subtypes. Whereas the HER2-enriched breast cancer subtype exhibits a high identity with ALDhT CSCs, the claudin-low subtype (e.g., SUM-1 59 cell line) exhibits a high preponderance of CD44+CD24~*~W CSCs. Without willing to be bound by theory, the observed results in inhibiting tumor initiating properties of the compounds of formula I of the invention are found to be based on their ability to inhibit both preexisting epithelial-like (ALDhT) and mesenchymal-like (CD44+CD24~*~W) CSCs (see Brooks et al., Cell Stem Cell 2015, 17(3),260-71; and Martin-Castillo et al., Oncotarget 2015, 6(32), 3231 7-38).
The ability of DOA to inhibit the formation of spheres was shown in Example 3 of the earlier application for various ALDH+ human-derived breast cancer cell lines. Moreover, in Example 2 DOA was shown to reverse the newly acquired CSC status by the human mammary epithelial cell line (HMLER) immortalized further to an EMT induction by E-cadherin knock-down (HMLERshEcad). Notably, the ability of the HMLERshEcad cell line to form spheres was reduced by DOA in more than 90% (Fig. 3A & B), this cell line being characterized by having a 90% of CD44+/CD24low cells (see Gupta et al., Cell 2009, 138, 645-659).

Therefore, the observed effect of CSC properties inhibition is not limited to cell lines presenting a particular cell marker phenotype, but has shown to be effective in cells expressing mesenchymal and/or epithelial stem cell markers.

Thus, this invention is directed to a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof for use in the treatment of cancer by inhibiting, or reducing the frequency of, CSCs. It further relates to pharmaceutical compositions, kits, methods of treatment and combination therapies using thereof.

In accordance with one aspect of the invention, the invention relates to a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, for use in a method of treating a subject suffering from cancer or suspected of having cancer by inhibiting, or reducing the frequency of, CSCs. In a related aspect, it refers to a dual mTOR and DNMT inhibitor, preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, for use in a method for inhibiting, or reducing the frequency of, cancer stem cell cells (CSCs), preferably by at least 50%. Said method for inhibiting, or reducing the frequency of, CSCs is also encompassed within the general term “method of treatment of the invention”.

In addition, the present invention provides a method of treating cancer comprising administering to a subject in need of such treatment a therapeutically effective amount of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, for inhibiting, or reducing the frequency of, CSCs, preferably by at least about 50%.

It further provides a method of inhibiting, or reducing the frequency of, cancer stem cells in a subject in need thereof, comprising administering to said subject a therapeutically effective amount of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, wherein said amount reduces the frequency of cancer stem cells, preferably by at least about 50%.
The present invention also provides a method of inhibiting, or reducing the frequency of, cancer stem cells in a subject having an increased risk of developing cancer, comprising administering to said subject an effective amount of a dual mTOR and DNMT inhibitor, preferably a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, wherein said subject has one or more risk factors selected from the group consisting of:

- a preliminary diagnosis of cancer but no confirmatory test,
- a preliminary diagnosis of cancer but no confirmatory test about the stage of cancer, and
- a cancer in remission.

It further provides the use of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, in the manufacture of a medicament for the treatment of cancer by inhibiting, or reducing the frequency of, CSCs.

The invention also relates to a pharmaceutical composition comprising a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, and a pharmaceutically acceptable excipient for use in the treatment of cancer by inhibiting, or reducing the frequency of, CSCs.

In a related aspect, the invention provides a pharmaceutical composition, comprising a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, in an amount effective to inhibit, or reduce the frequency of, cancer stem cells in a subject in need thereof, preferably by at least about 50%, and a pharmaceutically acceptable excipient.

The invention also provides a dosage form, comprising an effective amount of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I) or, pharmaceutically acceptable salt or prodrug thereof, wherein said dosage form is suitable for administration said compound in an amount effective to inhibit, or reduce the frequency of, cancer stem cells, preferably by at least about 50%.

The invention further provides that said CSCs inhibition is selective. Selective action in CSCs is shown by the fact that OA and DOA at the concentrations used in the sphere formation and ALDH cell marker expression tests do not decrease the viability of said tumor cells cultured in adherence as a monolayer (i.e., absence of CSCs is not due to a non-specific cytotoxic effect against the entire population of cancer cells). See for instance Example 2, Figure 3C and Example 3, Figures 4C and 5C.

Accordingly, preferably the inhibition, or reduction of the frequency, of CSCs is selective, wherein selective inhibition refers to the inhibition of the self-renewal and tumor initiating capacity of CSCs in the absence of a significant reduction in cell viability.
In accordance with another aspect of the invention, we provide combination therapies for the treatment of cancer, based on a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), a pharmaceutically acceptable salt or prodrug thereof and another anticancer drug. In a preferred embodiment, the other anticancer drug is a cytotoxic or anti-proliferative drug.

Thus, the invention provides a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, for use in a method of treating a subject suffering from cancer in combination therapy with another anticancer drug.

It further provides, a method of treating cancer comprising administering to a subject in need of such treatment a therapeutically effective amount of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, and a therapeutically effective amount of another anticancer drug, administered prior, concomitantly or after its administration. The two drugs may form part of the same composition, or be provided as a separate composition for administration at the same time or at a different time.

In another aspect, the invention also relates to a method of increasing or potentiating the therapeutic efficacy of an anticancer drug in the treatment of cancer, which comprises administering to a patient in need thereof a therapeutically effective amount of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof.

The invention also relates to the use of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, for the manufacture of a medicament for use in combination therapy with another anticancer drug in the treatment of cancer.

The invention also provides a pharmaceutical composition comprising a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, another anticancer drug, and a pharmaceutically acceptable excipient for use in combination therapy for the treatment of cancer.

The invention further provides a kit for use in the treatment of cancer which comprises a dosage form of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof; and/or a dosage form of another anticancer drug; and instructions for the use of both drugs in a combination treatment.
In a preferred aspect, the present invention is concerned with synergistic combinations of a compound, pharmaceutical composition or kit as described herein, and another anticancer drug.

In a further aspect, the present invention also provides a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, pharmaceutical composition or kit, for use in a method of preventing or treating a disease or disorder mediated by increased DNA methyltransferases activity wherein said disease is not a solid tumor or an age-related disease.

In addition, the present invention provides a method for preventing or treating a disease or disorder mediated by increased DNA methyltransferases activity wherein said disease is not a solid tumor or an age-related disease, said method comprising administering to a subject in need of such treatment a therapeutically effective amount of a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof.

It further provides the use of a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, in the manufacture of a medicament for the treatment or prevention of a disease or disorder mediated by increased DNA methyltransferases activity wherein said disease is not a solid tumor or an age-related disease.

**BRIEF DESCRIPTION OF THE FIGURES**

The following symbols are used in the figures with the following meaning:

- * means that statistical significance is $p \leq 0.05$
- ** means that statistical significance is $p \leq 0.01$
- n.s. stands for "non-significant" and means that $p > 0.05$

**FIG. 1.** Isolation, purification and characterization of DOA from an extra virgin olive oil phenolic extract (EVOO-PE). (A) Base peak chromatogram (BPC) of EVOO-PE obtained by semi-preparative HPLC-DAD-ESI-TOF-MS, wherein DOA peak is highlighted; (B and C) HPLC-UV chromatograms of DOA isolated fraction detected at 240 nm and 280 nm, respectively; (D) Accumulated mass spectrum (MS) over 27 min of DOA isolated fraction; (E) BPC of DOA isolated fraction by HPLC-ESI-TOF (peak with experimental $m/z$ 319 was characterized as DOA).

**FIG. 2.** Isolation, purification and characterization of OA from an EVOO-PE. (A) BPC chromatogram of EVOO-PE obtained by semi-preparative HPLC-DAD-ESI-TOF-MS, wherein OA peak is highlighted; (B and C) HPLC-UV chromatograms of OA isolated fraction detected at 240 nm and 280 nm, respectively (Peak 1: OA or isomer; Peak 2: Ligstroside Aglycone (LA); (D) Accumulated MS over 27 min of OA isolated fraction; (E) BPC of OA isolated fraction by HPLC-ESI-TOF (peaks with experimental $m/z$ 377, 285, and 361 were characterized as OA or isomer, luteoline (LU) and LA, respectively.)
FIG. 3. (A) Morphology (10X magnification) of HMLER\textsuperscript{shECad} cells growing in suspension culture under non-differentiating conditions in the absence (untreated control) or presence of 10 \( \mu \text{gol} / \text{L} \) DOA and 20 \( \mu \text{gol} / \text{L} \) DOA for 7 days (with DOA re-feeding every 3 days); (B) Mammosphere forming efficiency (MSFE, %) of untreated (control) HMLER\textsuperscript{shECad} cells and HMLER\textsuperscript{shECad} cells treated with 10 \( \mu \text{gol} / \text{L} \) DOA and 20 \( \mu \text{gol} / \text{L} \) DOA; (C) Cell viability determined by the MTT assay for untreated (control) HMLER\textsuperscript{shECad} cells untreated and HMLER\textsuperscript{shECad} cells treated with 10 \( \mu \text{gol} / \text{L} \) and 20 \( \mu \text{gol} / \text{L} \) DOA.

FIG. 4. (A) Morphology (10X magnification) of MCF-7 cells growing in suspension culture under non-differentiating conditions in the absence (control) or presence of 20 \( \mu \text{gol} / \text{L} \) DOA for 7 days (with DOA re-feeding every 3 days); (B) MSFE (%) of untreated (control) MCF-7 cells and MCF-7 cells treated with 20 \( \mu \text{gol} / \text{L} \) DOA; (C) Cell viability determined by the MTT assay for untreated (control) MCF-7 cells and MCF-7 cells treated with 20 \( \mu \text{gol} / \text{L} \) DOA.

FIG. 5. (A) Morphology (10X magnification) of MCF-7/HER-2 cells growing in suspension culture under non-differentiating conditions in the absence (control) or presence of 20 \( \mu \text{gol} / \text{L} \) DOA for 7 days (with DOA re-feeding every 3 days); (B) MSFE (%) of untreated (control) MCF-7/HER-2 cells and MCF-7/HER-2 cells treated with 20 \( \mu \text{gol} / \text{L} \) DOA; (C) Cell viability determined by the MTT assay for untreated (control) MCF-7/HER-2 cells and MCF-7/HER-2 cells treated with 20 \( \mu \text{gol} / \text{L} \) DOA; (D) MSFE (%) of various breast cancer cell lines (from left to right): DCIS.com, T47D, ZR-75-1, and SUM-159.

FIG. 6. Fluorescence-activated cell sorting (FACS) dot-plot of untreated/DOA-treated SUM-159 breast cancer cells with aldehyde dehydrogenase (ALDH) marker (ALDEFLUOR\textsuperscript{TM}) and with/without DEAB (DEAB inhibits ALDH activity and is used as a negative control for the ALDEFLUOR\textsuperscript{TM} assay). In the Y-axis it is represented the side scatter (SSC) and in the X-axis it is represented the FL1 (ALDEFLUOR\textsuperscript{TM} Fluorescence). The cells without the DEAB inhibitor shifted to the right were considered ALDH-positive cells. The percentage of the ALDH-positive sub-population is indicated.

FIG. 7. Mammosphere forming efficiency (MSFE%) of (A) MCF-7 and (B) ZR-75-1 breast cancer cells untreated and treated with 1 \( \mu \text{gol} / \text{L} \); 2 \( \mu \text{gol} / \text{L} \); 5 \( \mu \text{gol} / \text{L} \); 10 \( \mu \text{gol} / \text{L} \); 15 \( \mu \text{gol} / \text{L} \); and 20 \( \mu \text{gol} / \text{L} \) of OA.

FIG. 8. Fluorescence-activated cell sorting (FACS) dot-plot of untreated/OA-treated SUM-159 breast cancer cells with aldehyde dehydrogenase (ALDH) marker (ALDEFLUOR\textsuperscript{TM}); and with/without DEAB (DEAB inhibits ALDH activity and is used as a negative control for the ALDEFLUOR\textsuperscript{TM} assay). In the Y-axis it is represented the side scatter (SSC) and in the X-axis it is represented the FL1 (ALDEFLUOR\textsuperscript{TM} Fluorescence). The cells without the DEAB inhibitor shifted to the right were considered ALDH-positive cells. The percentage of the ALDH-positive sub-population is indicated.

FIG. 9. (A) Schematic representation of SUM-159 breast cancer cells pre-treatment with 50 \( \mu \text{gol} / \text{L} \) DOA (3 day treatment with daily re-feeding of DOA) prior to injection into mice (top); graphic representation of the tumor volume (mm\(^3\)) increase along time in mice injected with untreated (vehicle pre-treated)/DOA pretreated SUM-159 cells (down); (B) Photographs of tumors isolated from mice injected with untreated
(top) and DOA-treated (down) cells 6 weeks after injection; Kaplan-Meier plots of tumor-free survival (%) for untreated and treated with 50 μM/L mice which developed tumors with a volume of (C) ≥ 50 mm³ and (D) ≥ 10 mm³.

FIG. 10. (A) Schematic representation of SUM-159 breast cancer cells enrichment of CSC-like cellular states by culture under non-adherent and non-differentiating conditions for 48 hours and the subsequent pre-treatment with 5 μM/L, 10 μM/L or 20 μM/L DOA 2 hours before injection into the mammary fat pads of SCID beige mice; Kaplan-Meier plots of tumor-free survival (%) for mice (B) untreated and treated with 5 μM/L DOA and 10 μM/L DOA; and (C) untreated and treated with 20 μM/L DOA.

FIG. 11. (A) Semi-synthesis of DOA using Krapcho decarboalkoxylation; (B) Representative microphotograph of phenotype microarrays used to simultaneously evaluate the ability of semi-synthetic DOA to interact with 92 different anti-cancer drugs using CSCs-enriched SUM-159 breast ability cancer cells; (C) Synergy analysis using the fractional effect (FE) method of the interaction of semi-synthetic DOA with graded concentrations of the mTOR inhibitor rapamycin, the DNMT1 inhibitor 5-Azacitidine (5-Aza), and the chemotherapeutic agent doxorubicin (D: Dose; D 1 < D 2 < D 3 < D 4).

FIG. 12. (A) Representative immunoblot (n = 3) showing the expression levels of PP-p70S6K1 at Thrreonine 389 (which is used as a readout of mTOR activity), total p70S6K1 and β-actin in SUM-159 cells treated with 20 μM/L DOA or 100 nmol/L of rapamycin (a well-known mTOR inhibitor) at different time points (top); and expression levels of PP-p70S6K1 and β-actin in cells untreated and treated with graded concentrations of DOA (5, 10, 20 μmol/L)(down). (B) Schematic representation of DOA binding to the catalytic domain of mTOR. (Top) molecular modeling of the interaction between DOA and the ATP-binding pocket of mTOR. The protein is depicted in ribbon representation and colored by secondary structures (i.e., helix, strand, and loop);(down) a close-up view of the consensus orientation of DOA within the ATP-binding pocket of mTOR.

FIG. 13. (A) Graphical representation showing the percentage of DNMT activity inhibition obtained in purified cell nuclei directly incubated in the absence or presence of 5-AZA or DOA. (B) Schematic representation of DOA proposed mechanisms of action for inhibiting DNMT activity (top) directly by binding the DNMT active site, and (bottom) indirectly by inhibiting S-adenosyl methionine (SAM) dependent DNMT-catalyzed DNA methylation (wherein SAM acts as the methyl donor) (COMT: catechol-O-methyltransferase).

FIG. 14. Graphical representation showing the size (μm) of the microspheres as determined by the Cell2Sphere™ kit assay in MDA-MB-436 and BT-474 cells using different concentrations of DOA.

FIG. 15. Graphical representation showing the size (μm) of the microspheres as determined by the Cell2Sphere™ kit assay in MDA-MB-436 cells after treatment with DOA in combination with the following drugs: PTX: Paclitaxel; DOX: Doxorubicin; RAPA: Rapamycin; 5-AZA: 5-azacytidine; MET: Metformin; PEM: Pemtrexed; OLAP: Olaparib; T-DM1: Trastuzumab emtansine. Drug combinations were added to quintuplicate sets of wells on days 1 and 4 without removing the old media. Image J software was used to
quantify the size of 6 days-old mammospheres. Centre lines indicate mean values. Data are representative of two experiments performed in quintuplicate. *P<0.05. (Ct. Control).

FIG. 16. Graphical representation showing the size (μm) of the microspheres as determined by the Cell2Sphere™ kit assay in BT-474 cells after treatment with DOA in combination with the following drugs:PTX: Paclitaxel; DOX: Doxorubicin; RAPA: Rapamycin; 5-AZA: 5-azacytidine; MET: Metformin; PEM: Pemetrexed; OLAP: Olaparib; T-DM 1: Trastuzumab emtansine. Drug combinations were added to quintuplicate sets of wells on days 1 and 4 without removing the old media. Image J software was used to quantify the size of 6 days-old mammospheres. Centre lines indicate mean values. Data are representative of two experiments performed in quintuplicate. *P<0.05. (Ct. Control).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term “multiple epigenetic modulator” refers to the ability to modulate the biological activity of more than one epigenetic enzymes. Preferably, these epigenetic targets are those indicated in Table 6. In a particular embodiment, said epigenetic enzyme is a methyltransferase (MT). Examples of methyltransferases are DNA methyl transferases (DNMTs) (e.g., DNMT1), catechol-O-methyltransferases (COMT), protein arginine methyltransferases (PRMTs), histone methyl transferases (e.g., KMT2A); Histone deacetylases (e.g., HDAC); Protein deacetylases or sirtuins (e.g., SIRT1), and histone acetyl transfers (ie. p300 HAT).

Methyltransferases have previously been reported to be involved in cancer and in other diseases as follows:

- COMT: Estrogen-related cancers. Other diseases include CNS-related disorders: PArkinson's, Schizophrenia, hungtington's
In a particular embodiment, said epigenetic modulator is an inhibitor or down-regulator of one of more of said epigenetic enzymes, preferably of one or more of said methyltransferases. In a preferred embodiment, said multiple epigenetic modulator is at least a DNMT inhibitor (e.g., a DNMT1 inhibitor).

The term "cancer" as used herein is meant to include tumors, neoplasias, and any other malignant disease having as cause malignant tissue or cells. There are several types of cancer. A complete list of cancer types can be found in the website of the National Cancer Institute (NCI): http://www.cancer.gov/types. The international classification of diseases for oncology (ICD-O) published by the World Health Organization (WHO) provides internationally recognized histopathological and clinical diagnostic criteria (Fritz A et al (eds), 2000, ICD-0 International classification of diseases for oncology. World Health Organization, Geneva).

Cancers are typically classified in two ways: by the type of tissue in which the cancer originates (histological type) and by primary site, or the location in the body where the cancer first developed. From a histological standpoint there are many different cancers, which are grouped by the NCI into the six major categories referred below.

- **Carcinoma**: a malignant neoplasm of epithelial origin or cancer of the internal or external lining of the body. Carcinomas are divided into two major subtypes: adenocarcinoma, which develops in an organ or gland, and squamous cell carcinoma, which originates in the squamous epithelium.
- **Sarcoma**: a cancer arising from supportive and connective tissues such as cells in bone, tendons, cartilage, fat, muscle, or blood vessels;
- **Myeloma**: a cancer that originates in the plasma cells of bone marrow;
- **Leukemia**: a cancer that arises in blood-forming tissue such as the bone marrow, and causes large numbers of abnormal blood cells to be produced and enter the bloodstream. Examples of leukemia include: Myelogenous or granulocytic leukemia; Lymphatic, lymphocytic, or lymphoblastic leukemia; and polycythemia vera;
- **Lymphoma**: a cancer developing in the glands or nodes of the lymphatic system. The lymphomas are subclassified into two categories: Hodgkin lymphoma and Non-Hodgkin lymphoma;
- **Mixed types**: a cancer composed by cells of different histological origins, such as adenosquamous carcinoma, mixed mesodermal tumor, carcinosarcoma and teratocarcinoma.

The term "hematologic cancer" as used herein refers to leukemia, lymphoma, and multiple myeloma.

The term "treating", as used herein, unless otherwise indicated, includes the amelioration, cure, and/or maintenance of a cure (i.e., the prevention or delay of relapse) of a disease or disorder. Treatment after a disorder has started aims to reduce, alleviate, ameliorate or altogether eliminate the disorder, and/or its associated symptoms, to prevent it from becoming worse, to slow the rate of progression, or to prevent the disorder from re-occurring once it has been initially eliminated (i.e., to prevent a relapse).
The term "treatment", as used herein, unless otherwise indicated, refers to the act of "treating" defined immediately above.

The term "therapeutically effective amount" as used herein refers to an amount that is effective, upon single or multiple dose administration to a subject (such as a human patient) in the prophylactic or therapeutic treatment of a disease, disorder or pathological condition as defined herein. For instance, said prophylactic or therapeutic effect is comparable to that of imatinib (Gleevec®) in a variety of leukemias and gastrointestinal stromal tumors, trastuzumab (Herceptin®) in HER2-positive breast cancer, rituximab (Rituxan®/MabThera®) in non-Hodgkin's lymphoma and chronic lymphocytic leukemia, abiraterone (Zytiga®) in prostate cancer, or cetuximab (Erbitux®) in colorectal cancer.

The term "combination" or "combination therapy" as used throughout the specification, is meant to encompass the administration of the referred therapeutic agents to a subject suffering from cancer, in the same or separate pharmaceutical formulations, and at the same time or at different times. If the therapeutic agents are administered at different times they should be administered sufficiently close in time to provide for the potentiating or synergistic response to occur. In such instances, it is contemplated that one would typically administer both therapeutic agents within about 12-24 hours of each other and, more preferably, within about 6-12 hours of each other. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations. In other situations, it might be desireable to reduce the time between administration, administering both therapeutic agents within seconds or minutes to hours, preferably within about 6 hours from each other, more prefereably within about 1 or 3 hours.

The term "cancer stem cells" or "CSCs" as used herein refers to cells (or to a cell state or phenotype) with stem cell properties in a cancer cell population. CSCs are also referred throughout the specification as CSC states, CSC-like cells or stem-like cells. The term CSCs encompasses normal tumor cells that have undergone a transformation process, such as EMT for epithelial cells (see definition below). CSCs are tumors cells that have the principal properties of self-renewal, clonal tumor initiation capacity and clonal long-term repopulation capacity. CSCs are an underlying cause of tumor recurrence and metastasis. Common CSC characterizing properties are: the ability of growing in suspension under non-differentiating conditions (i.e., "sphere-forming" assay) in vitro; in vivo giving rise to new tumors and recapitulate tumor heterogeneity of original tumors when xenografted at low numbers into immunodeficient mice; the expression of stem cell markers, such as CD133, CD44 or aldehyde dehydrogenase (ALDH) and/or being capable of surviving diverse treatments including hormones, radiation, chemotherapeutic agents, and molecularly targeted drugs.

The term "epithelial-to-mesenchymal transition (EMT)" as used herein, refers to an aberrant activation of a latent embryonic program that can confer cancer cells with the migratory and invasive capabilities associated with metastatic competence. Normal and tumor cells exist in various states of differentiation in vitro and in vivo. These differentiation states are regulated through the integration of complex signals.
arising at least in part from the tissue microenvironment in which a cell resides. Cells can be induced to undergo an EMT through a number of genetic perturbations, e.g. via the overexpression of particular factors (e.g., Twist, Snail, TGFβ, or MMPs), or by the inhibition of adherence junction proteins such as E-Cadherin (Alison et al., J Pathol. 201 1, 223, 147-1 61; Pattabiraman and Weinberg, Nat Rev Cancer. 2014, 13(7): 497-512). The induction of EMT typically entails the loss of epithelial characteristics and the de novo acquisition of a mesenchymal phenotype. In breast cancer, the EMT state has been associated with cancer stem cell properties including expression of the stem cell associated CD44+/CD24−/low antigenic profile, self-renewal capabilities and resistance to conventional therapies. Thus, in addition to conferring migratory and invasive potential, induction of EMT in immortalized and transformed human mammary epithelial cells significantly enhances their self-renewal and tumor-initiating capabilities and leads to the expression of stem-cell markers, typically associated with breast CSCs. The fact that EMT can be sporadically triggered by extracellular stimuli and microenvironmental factors, provides a plausible explanation for the de novo generation of CSCs from differentiated tumor cells. The passage through EMT having been suggested as an alternative and/or additional driving force in tumorigenesis (May et al., Breast Cancer Res. 201 1, 13(1):202).

The term "inhibition of cancer stem cells" as used herein refers to both i) reducing or suppressing CSCs already present within a tumor at the time of treatment. i) preventing the appearance of de novo generated CSC states (e.g., following induction of EMT-like or other dedifferentiating phenomena in non-CSC epithelial cells) in response to microenvironmental or therapeutic stimuli.

The term "resistance" as used herein refers to lack of response to a cancer treatment. It can be either "primary (de novo)" when resistance occurs because of traits that a tumor has before treatment (i.e., some inherent characteristics of the cancer cells prevent the treatment's effectiveness), or "secondary (acquired)" which occurs when tumors become resistant during treatment because of traits that tumor cells gain in response to the treatment. Typically said treatment is an anticancer drug treatment.

The term "subject" as used herein refers to a mammalian subject having or suspected of having cancer. Preferably, it is selected from a human, companion animal, non-domestic livestock or zoo animal. For example, the subject may be selected from a human, dog, cat, cow, pig, sheep, horse, bear, and so on. In a preferred embodiment, said mammalian subject is a human subject. The presence of CSC markers may be evaluated in a clinical sample of said subject (e.g., a tumor biopsy or cells isolated therefrom). Preferably, a subject where a CSC marker is detected is treated with the compounds as described herein. Cancer stem cell markers are as described below and include ALDH1+, CD44+/CD24−/low, CD133, CD44+, etc.

The term "subject suspected of having cancer" as used herein, refers to a subject that presents one or more signs or symptoms indicative of a cancer or is being screened for a cancer. A subject suspected of having cancer may also have one or more risk factors. A subject suspected of having cancer encompasses an individual who has received a preliminary diagnosis (e.g., an X-ray computed tomography scan showing a mass) but for whom a confirmatory test (e.g., biopsy and/or histology) has
not been done or for whom the stage of cancer is not known. The term further includes individuals in remission.

The term "pharmaceutically acceptable salt" as used herein refers to the relatively non-toxic, inorganic and organic acid addition salts of compounds as described herein. These salts can be prepared in situ during the final isolation and purification of the compounds or by separately reacting the purified compound in its free base form with a suitable organic or inorganic acid and isolating the salt thus formed. Representative salts include the hydrobromide, hydrochloride, sulfate, bisulfate, nitrate, acetate, oxalate, valerate, oleate, palmitate, stearate, laurate, borate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, naphthylate mesylate, glucoheptonate, lactobsonate, and laurylsulphonate salts, and the like. These may include cations based on the alkali and alkaline earth metals, such as sodium, lithium, potassium, calcium, magnesium, and the like, as well as non-toxic ammonium, quaternary ammonium, and amine cations including, but not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and the like (see, for example, Berge S.M, et al, "Pharmaceutical Salts," J.Pharm.Sci., 1977;66: 1-19 which is incorporated herein by reference.)

The term "prodrug" as used herein refers to inactive, bioreversible derivatives of the compounds as described herein. Generally, these must undergo an enzymatic and/or chemical transformation in vivo to release the active parent compound, which can then elicit its desired pharmacological effect in the body. Prodrugs are typically designed to overcome formulation, delivery, and toxicity problems. A thorough discussion of prodrugs is provided in T. Higuchi and V. Stella, "Pro-drugs as Novel Delivery Systems," Vol. 14 of the A.C.S. Symposium Series; in Bioreversible Carriers in Drug Design, ed. Edward B. Roche, American Pharmaceutical Association and Pergamon Press, 1987; and in Rautio et al., Nature Reviews Drug Discovery 2008, 7, 255-270.

Suitable protecting groups are well known to the skilled person in the art. A general review of protecting groups in organic chemistry is provided in Greene's Protective Groups in Organic Synthesis, Fourth Edition, John Wiley & Sons, Inc; and by Kocienski P.J. in Protecting Groups, Third Ed. Georg Thieme Verlag. These references provide sections on protecting groups for -OH, amino, and -SH groups. All these references are incorporated by reference in their entirety.

The term "a protecting group for -OH" as used herein refers to the O-bonded moiety resulting from the protection of the -OH group through the formation of a suitable protected -OH group. Examples of such protected -OH groups include ethers, silyl ethers, esters, sulfonates, sulfoxides, sulfones, carbonates, and carbamates.

The term "dosage form" refers to a pharmaceutical composition devised to enable administration of a drug medication in the prescribed dosage amounts. Depending on the method/route of administration different dosage forms will be used. Oral dosage forms comprise liquids (i.e., solutions, suspensions, and emulsions), semi-solids (i.e., pastes), and solids (i.e., tablets, capsules, powders, granules, premixes, and medicated blocks), these may be immediate release or modified release forms. Parenteral dosage forms and delivery systems include injectables (i.e., solutions, suspensions, emulsions, and dry powders for
reconstitution), intramammary infusions, intravaginal delivery systems, and implants. Topical dosage forms include solids (i.e., dusting powders), semi-solids (i.e., creams, ointments, and pastes), and liquids (i.e., solutions, suspension concentrates, suspemulsions, and emulsifiable concentrates). It further includes transdermal delivery systems.

The term "radiation therapy" or "radiotherapy" as used herein refers to a cancer treatment that uses high doses of radiation to kill cancer cells and shrink tumors. It includes external beam radiation therapy and internal radiation therapy.

The term "chemotherapy" as used herein refers to drugs which stop or reduce the growth of cancer cells which grow and divide quickly. Examples of chemotherapeutic drugs are platinum coordination complex compounds, antimetabolite compounds, mitotic inhibitors, anthracycline antibiotics and topoisomerase I and/or II inhibitors.

The term "immunotherapy" as used herein includes monoclonal antibodies (may also be considered "targeted therapy"), vaccines, cytokines, and adoptive cell transfer.

The term "hormone therapy" as used herein refers to compounds which block hormone production or prevent these to produce their biological effect. Hormone therapy is useful for those cancers that are hormone sensitive or hormone dependent (e.g., prostate or breast cancer).

The term "targeted therapy" as used herein refers to compounds that block the growth and spread of cancer by interfering with specific molecules ("molecular targets") that are involved in the growth, progression, and spread of cancer. Targeted therapies differ from standard chemotherapy in several ways, including that they have been designed for acting on specific molecular targets that are associated with cancer and often produce a cytostatic effect (i.e., block tumor cell proliferation), whereas most standard chemotherapies act on all rapidly dividing normal and cancerous cells and often have a cytotoxic effect.

The term "tumor free-survival", "disease free survival", "progression free survival" or PFS as used herein, is defined as the interval of time from start of treatment to the first measurement of cancer growth. The term "overall survival" or OS as used herein, is defined as the interval of time from the start of treatment to death from any cause.

**Detailed description**

**A COMPOUND OF THE INVENTION FOR USE IN THE TREATMENT OF CANCER BY INHIBITING CSCs**

In accordance with one aspect of the invention, the invention relates to a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, for use in
a method of treating a subject suffering from cancer or suspected of having cancer by inhibiting, or reducing the frequency of, cancer stem cells (CSCs). In a related aspect, it refers to a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, for use in a method for inhibiting, or reducing the frequency of, cancer stem cell cells (CSCs). Said method for inhibiting, or reducing the frequency of, CSCs is also encompassed within the general term "method of treatment of the invention".

It further provides a method of inhibiting, or reducing the frequency of, cancer stem cells in a subject in need thereof, comprising administering to said subject a therapeutically effective amount of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, wherein said amount inhibits, or reduces the frequency of, cancer stem cells, preferably by at least about 50%.

In addition, the present invention provides a method of treating cancer, preferably a solid tumor, comprising administering to a subject in need of such treatment a therapeutically effective amount of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, for inhibiting, or reducing the frequency of, CSCs, preferably by at least 50%.

The present invention also provides a method of inhibiting, or reducing the frequency of, cancer stem cells in a subject having an increased risk of developing cancer, comprising administering to said subject an effective amount of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, wherein said subject has one or more risk factors selected from the group consisting of:

- a preliminary diagnosis of cancer but no confirmatory test,
- a preliminary diagnosis of cancer but no confirmatory test about the stage of cancer, and
- a cancer in remission.

It further provides the use of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, in the manufacture of a medicament for the treatment of cancer by inhibiting, or reducing the frequency of, CSCs.

In a particular embodiment, said dual mTOR inhibitor and multiple epigenetic modulator is a dual mTOR and DNMT inhibitor, preferably a mTOR and DNMT1 inhibitor.

Methods for determining mTOR inhibition and DNMT inhibition are well known in the art.
Methods for determining mTOR inhibition are typically based on measuring phosphorylated mTOR (pSer2448) or quantifying the degree of phosphorylation of p70S6K1 at Thr389 directly in the cells. This may be done for instance by Western blot analysis with antibodies against phospho-p70S6K1 (Thr389), as described in Example 1.

Said effect of reduction of DNA methyltransferase activity can be direct (i.e., by direct inhibition of the enzyme) or indirect (e.g., by decreasing the availability of methyl groups). Three active DNA methyltransferases (DNMTs) have been identified in mammals. They are named DNMT1, DNMT3A, and DNMT3B. Methods for determining DNMT inhibition are typically based on quantifying the degree of methylation of DNA. This determination can be performed, for example, by determining the methyl CpG binding domain (MBD) protein, which has a high binding affinity towards methylated DNA, in order to detect DNA methyltransferase activity on a provided CpG-enriched DNA substrate. MBD protein levels can be assessed by an appropriate method known to one of ordinary skill in the art, such as immunoassays, including ELISA (enzyme linked immunosorbent assay) or radioimmunoassays. Preferably, said method is an ELISA-based assay. More preferably, said method is the DNMT activity/inhibition assay (Active Motif, Carlsbad, CA, USA) used in Example 1.

In particular, the present invention provides compounds of formula (I):

![Chemical Structure](image)

wherein A is selected from the group consisting of:

![Chemical Structures](image)
wherein

R¹ and R² are independently selected from hydrogen, C₁-C₈ alkyl, C₂-C₈ alkenyl, C₂-C₈ alkynyl and -OR⁷;

R³ is selected from hydrogen, C₁-C₈ alkyl, C₂-C₈ alkenyl or C₂-C₈ alkynyl, -OR⁸, -COR⁸, -COOR⁸, and -OCOR⁸;

R⁴ is selected from hydrogen, C₁-C₈ alkyl, C₂-C₈ alkenyl, C₂-C₈ alkynyl and -COOR⁹;

R⁵ is selected from C₁-C₈ alkyl, C₂-C₈ alkenyl or C₂-C₈ alkynyl, -OR¹⁰, -COR¹⁰, -COOR¹⁰, and -OCOR¹⁰;

R⁶ is selected from hydrogen, C₁-C₈ alkyl, C₂-C₈ alkenyl, C₂-C₈ alkynyl, -COR¹¹, and -COOR¹¹;

each of R⁷, R⁸ R⁹, R¹⁰, and R¹¹ is independently selected from hydrogen, saturated or unsaturated C₅-C₇ cycloalkyl, saturated or unsaturated C₅-C₇ heterocycloalkyl, C₅-C₇ aryl, C₅-C₇ heteroaryl, C₁-C₈ alkyl, C₂-C₈ alkenyl or C₂-C₈ alkynyl, and a protecting group for -OH;

wherein the C₅-C₇ cycloalkyl, C₅-C₇ heterocycloalkyl, C₅-C₇ aryl, C₅-C₇ heteroaryl, C₁-C₈ alkyl, C₂-C₈ alkenyl or C₂-C₈ alkynyl group is unsubstituted or substituted with one or more of a -OR¹², -SR¹², -NHCOR¹², -CONHR¹², -COR¹², -COOR¹², -OCOR¹², -NR¹²R¹³, -S₀₂R¹², -S₀₂NR¹²R¹³, -CF₃, -OCF₃ or -CN, wherein R¹² and R¹³ are independently of each other hydrogen, C₁-C₄ alkyl, C₂-C₄ alkenyl, C₂-C₄ alkynyl, and a protecting group of -SH, -OH or amino.

Alkyl groups may be branched or unbranched, and preferably have from 1 to about 8 carbon atoms. One more preferred class of alkyl groups has from 1 to about 6 carbon atoms. Even more preferred are alkyl groups having 1, 2, 3 or 4 carbon atoms. Methyl, ethyl, n-propyl, isopropyl and butyl, including n-butyl, tert-butyl, sec-butyl and isobutyl are particularly preferred alkyl groups.

Preferred alkenyl and alkynyl groups in the compounds of the present invention may be branched or unbranched, have one or more unsaturated linkages and from 2 to about 8 carbon atoms. One more preferred class of alkenyl and alkynyl groups has from 2 to about 6 carbon atoms. Even more preferred are alkenyl and alkynyl groups having 2, 3 or 4 carbon atoms.

Suitable aryl groups include single and multiple ring compounds, including multiple ring compounds that contain separate and/or fused aryl groups. Typical aryl groups contain from 5 to about 18 carbon ring atoms, preferably from to about 14 carbon ring atoms. Specially preferred aryl groups include substituted or unsubstituted phenyl, substituted or unsubstituted naphthyl, substituted or unsubstituted biphenyl, substituted or unsubstituted phenanthryl and substituted or unsubstituted anthrly. Preferably aryl groups contain from 5, 6 or 7 carbon ring atoms. The most preferred aryl group is substituted or unsubstituted phenyl.
Suitable heterocyclic groups include heteroaromatic and heteroalicyclic groups containing from 1 to 3 separated or fused rings and from 5 to about 18 ring atoms. Preferably heteroaromatic and heteroalicyclic groups contain from 5 to about 10 ring atoms, more preferably 5, 6 or 7 ring atoms. Suitable heteroaromatic groups in the compounds of the present invention contain one, two or three heteroatoms selected from N, O or S atoms.

In certain embodiments, said compound of formula (I) is present as a pure enantiomer or as a mixture of enantiomers. In preferred embodiments, the compound of formula (I) is in one or more of the following enantiomeric forms or tautomeric forms thereof:

\[
\begin{align*}
R^1 & - \text{Ar} - \text{O} - \text{A} \quad (I) \\
R^2 & \end{align*}
\]

wherein

\[
\begin{align*}
A = & \quad R^3 \quad \text{OR}^6, \quad R^3 \quad \text{OR}^{10}, \quad R^4 \quad \text{OR}^6, \quad R^4 \quad \text{OR}^{10}, \\
& \quad R^5 \quad \text{OR}^{10} \quad \text{OR}^{10}, \quad R^6 \quad \text{OR}^{10}, \quad R^5 \quad \text{OR}^6 \quad \text{OR}^6
\end{align*}
\]

In more preferred embodiments, the compound of formula (I) is in one or more of the following enantiomeric forms or tautomeric forms thereof:

\[
\begin{align*}
R^1 & - \text{Ar} - \text{O} - \text{A} \quad (I) \\
R^2 & \end{align*}
\]

wherein
Preferred substituents of the compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, are defined as follows.

R³ is preferably selected from hydrogen, C1-C4 alkyl, and -OH; R⁴ is selected from -CH₃, and -OH; R⁵ is selected from hydrogen and -COOCH₃; and

More preferably, R³ is hydrogen and R⁴-OH. In a preferred embodiment R³ and R⁴ are -OH.

R⁵ is preferably selected from hydrogen, C1-C4 alkyl, C2-C4 alkenyl, and -OR₈, R⁶ being preferably selected from hydrogen or C1-C4 alkyl. More preferably, R⁵ is selected from -CH₃, =CH₂, and -OH.

R⁶ is preferably selected from hydrogen, C1-C4 alkyl and -COOR⁹, R⁹ being preferably selected from hydrogen or C1-C4 alkyl. More preferably, R⁶ is selected from hydrogen and -COOCH₃.

R³, R⁷, R⁸, R⁹, R¹₀, R¹₁, R¹² and R¹³ are preferably independently selected from hydrogen, saturated or unsaturated C5-C6 cycloalkyl, saturated or unsaturated C5-C6 heterocycloalkyl, C5-C6 aryl, C5-C6 heteroaryl, C1-C4 alkyl, C2-C4 alkenyl or C2-C4 alkynyl, and a protecting group for OH;.

More preferably, R⁶ and R¹₀ are independently selected from hydrogen, C1-C4 alkyl, and a saturated or unsaturated C5-C6 heterocycloalkyl.

A C5-C6 heterocycloalkyl is preferably a C5-C6 O-cycloalkyl, more preferably a hydroxy substituted C5-C6 O-cycloalkyl. Said hydroxy substituted C5-C6 O-cycloalkyl may be a pentose monosaccharide, such as ribose, arabinose, xylose or lyxose; or an hexose monosaccharide, such as allose, altrose, glucose, mannose, gulose, iodose, galactose or talose. D-forms of pentose or hexose monosaccharides are preferred. In preferred embodiments, R¹⁰ is D-glucose.

In preferred embodiments, R⁶ and R¹⁰ are hydrogen. The compounds of the invention wherein R⁶ and R¹⁰ are hydrogen are also referred as aglycon forms.

In particularly preferred embodiments, R¹ and R² are independently selected from hydrogen and -OH; R³ is selected from -CH₃, and -OH; R⁴ is selected from hydrogen and -COOCH₃; and
$R^0$ and $R^6$ are independently selected from hydrogen, -CH₃ and D-glucose.

In further particularly preferred embodiments, $R^1$ and $R^2$ are -OH;
$R^3$ is -CH₃;
$R^4$ is selected from hydrogen and -COOCH₃; and
$R^5$ and $R^6$ are hydrogen.

Preferred compounds of formula (I) are selected from one or more of the group consisting of the following compounds:

Oleuropein aglycone (homoacetalic cyclic form)

Oleuropein aglycone (dialdehydic open form)

Oleuropein aglycone (monomethylaldehydic forms)

Oleuropein aglycone (isomer)
Oleuropein aglycone (isomer)

Oleuropein aglycone (isomer)

Oleuropein aglycone (isomer)

Oleuropein aglycone (isomer)

Oleuropein aglycone (isomer)
Oleuropein aglycone (isomer)

Hydrated dialdehydic form of oleuropein aglycone
Decarboxymethyl Oleuropein aglycone (homoacetalic cyclic form)

Decarboxymethyl Oleuropein aglycone (dialdehydic open form)

Decarboxymethyl Oleuropein aglycone (isomer)

Decarboxymethyl Oleuropein aglycone (homoacetalic cyclic form)

3,4-DHPEA-EDA
Decarboxymethyl Oleuropein aglycone (dialdehydic open form); 3,4-DHPEA-EDA

Decarboxymethyl Oleuropein aglycone (isomer)

Decarboxymethyl Oleuropein aglycone (isomer)

Decarboxymethyl Oleuropein aglycone (isomer)
Decarboxymethyl Oleuropein aglycone (isomer)

5

Decarboxymethyl Oleuropein aglycone (isomer)

10

Decarboxymethyl Oleuropein aglycone (isomer)

15

Decarboxymethyl Oleuropein aglycone (isomer)

20
Decarboxymethyl Oleuropein aglycone (isomer)

Ligstroside aglycone (homoacetalic cyclic form)

Ligstroside aglycone (dialdehydic open form)

Ligstroside aglycone (isomer)
Ligstroside aglycone (isomer)

5

Ligstroside aglycone (isomer)

10

Ligstroside aglycone (isomer)

15

Ligstroside aglycone (isomer)

20
Ligstroside aglycone (isomer)

Decarboxymethyl Ligstroside aglycone (homoacetalic cyclic form)

Decarboxymethyl Ligstroside aglycone (dialdehydic open form)
Decarboxymethyl Ligstroside aglycone (isomer)

Oleuropein

Ligstroside

hydroxy oleuropein aglycone
Particularly preferred compounds of formula (I) are DOA and OA which have been shown by the inventors to present CSC inhibition activity in vitro and/or in vivo (Examples 2 to 9).

DOA is the most preferred compound. It has also been shown by the inventors that it presents mTOR inhibitor activity and DNA methyltransferase (DNMT) inhibitor activity (see Examples 11 and 12, respectively). Accordingly, in a preferred embodiment, the compound of formula (I), a pharmaceutical salt or prodrug thereof for use in a method of treatment of the invention, is a mTOR inhibitor and/or a DNA methyltransferase (DNMT) inhibitor, preferably a dual mTOR-DNMT inhibitor.

The present invention further provides the use of a combination of compounds of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, described herein, including a combination of two or more of the preferred compounds of formula (I) referred above, in particular combinations of OA and DOA.

The terms pharmaceutically acceptable salt and prodrug are described above. In addition, any compound referred to herein may be in crystalline form either as free base or as solvates (e.g. hydrates) and it is intended that both forms are within the scope of the present invention. Methods of solvation are generally known within the art.
The term "cancer stem cells" and "inhibition of cancer stem cells (CSCs)" have been defined above. In a particular embodiment, the inhibition, or reduction of the frequency of, CSCs is characterized by the suppression or reduction of one or more, preferably all, of the following:

i. the expression of stem cell marker(s) by cancer cells;
ii. the ability of cancer cells to form spheres when grown in suspension (also referred to as "sphere formation" assays);
iii. the ability of cancer cells to reproduce a tumor in vivo when injected into an immunocompromised animal.

In a particular embodiment, optionally in combination with one or more of the features or embodiments described above or below, inhibition of CSCs refers to the inhibition of the self-renewal and tumor initiating capacity of CSCs and is characterized by the suppression or significant reduction of one or more of the following:

i. the ability of CSCs to form spheres when grown in suspension under non-differentiating conditions; and
ii. the ability of CSCs to reproduce a tumor in vivo when injected into an immunocompromised animal.

The suppression, inhibition, or reduction may be at least about, preferably more than, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, or 99% of a reference level (e.g., a control level). In a preferred embodiment, suppression, inhibition or reduction is of at least 50%, preferably of at least 55%, more preferably of at least 65%, even more preferably of at least 90%.

In some cases the level of suppression, inhibition, or reduction compared with a control level is statistically significant. As used herein, "statistically significant" refers to a p-value of less than 0.05, e.g., a p-value of less than 0.025, a p-value of less than 0.01 or a p-value of less than 0.005, using an appropriate statistical test. A person skilled in the art will know appropriate statistical tests to be used, such as ANOVA or t-test.

Typically said reference or control level is obtained from a vehicle treated or untreated biological sample. Said biological sample is a sample containing tumor cells. Said biological sample may be cells from an established cancer cell line, e.g., obtained from a cell culture collection. It may also be obtained from a cancer patient. Tumors or portions thereof may be surgically resected from the patient or obtained by routine biopsy. Preferably, a tumor sample is obtained from the primary tumor. These types of samples are used routinely used in the clinical practice and a person skilled in the art will know how to identify the most appropriate means for their obtaining and preservation. Once a sample has been obtained, it may be used fresh, it may be frozen or preserved using appropriate means (e.g., as a formalin-fixed, paraffin-embedded tissue sample). Such biological samples can be taken around the time of diagnosis, before, during or after treatment are preferably taken around the time of diagnosis and before treatment.
Markers for CSCs have been described which are commonly used for the identification, quantification and/or isolation of CSCs (e.g., by fluorescence-activated cell sorting (FACS)). CSCs markers which may be used are cell surface molecules, such as cell adhesion molecules, or drug-efflux pumps (e.g. ABC transporters) and intracellular enzymes, such as aldehyde dehydrogenase (ALDH). Different CSC markers and combinations thereof may be used to determine CSCs inhibition in the method of treating of the invention. In one embodiment, said stem cell markers comprise or consist of ALDH. ALDH enzymatic activity may be determined by any suitable method for determination of this enzymatic activity, e.g., by the ALDEFLUOR™ assay (Stem Cell Technologies Inc.). In another embodiment, one or more CSC markers are selected from the group consisting of: ALDH1, Bmi1, CD24, CD26, CD29 (β1-integrin), CD44, CD90 (Thy1), CD105, CD117 (c-kit), CD123, CD133 (prominin-1), CD166 (ALCAM), Oct4+, Sox2+, Nanog+, nestin+, CD166, EpCAM (ESA), Lineage-, Wnt pathway activity, β-catenin, Hoechst Side Population (i.e., Hoechst 33342 dye exclusion), CD15, CD20, CD34, CD38, CD45, CD105, ESA, SCA1, ABCG2+, ABCB5+, LGR-5, Pecam, and Stro1. In a preferred embodiment, combinations of cell surface markers and non-cell surface markers are used, such as ALDH+ CD44+CD24-αW profile.

The best marker or combination thereof will often depend on the particular type or subtype of cancer. Examples of CSCs marker combinations for particular types of cancer are provided in the tables below.

<table>
<thead>
<tr>
<th>Tumour</th>
<th>Marker</th>
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<tbody>
<tr>
<td>Acute childhood acute B lymphoblastic leukaemia</td>
<td>CD1 33/CD1 97CD38 -</td>
</tr>
<tr>
<td>Acute myeloid leukaemia</td>
<td>CD34/CD38</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute T lymphoblastic leukaemia</td>
<td>SSc10/ALDH bri</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>ALDH bri</td>
</tr>
<tr>
<td>Bladder carcinoma</td>
<td>CD90/CD1 10 (c-Mpl)</td>
</tr>
<tr>
<td>Colorectal carcinoma</td>
<td>CD44/CD247ALDH</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometrial carcinoma</td>
<td>ALDH b</td>
</tr>
<tr>
<td>Ewing's sarcoma</td>
<td>CD133</td>
</tr>
<tr>
<td>Gastric carcinoma</td>
<td>CD133</td>
</tr>
<tr>
<td>Head and neck SCC</td>
<td>CD133</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatocellular carcinoma (HCC)</td>
<td>CD133</td>
</tr>
<tr>
<td>HCC (cell lines)</td>
<td>CD133</td>
</tr>
<tr>
<td>Hodgkin lymphoma</td>
<td>CD133</td>
</tr>
<tr>
<td>Lung carcinoma (NSCLC)</td>
<td>CD133</td>
</tr>
<tr>
<td>Lung carcinoma (SCLC)</td>
<td>CD133</td>
</tr>
<tr>
<td>Malignant melanoma</td>
<td>CD133/ASCL/ALDH1</td>
</tr>
<tr>
<td>Medulloblastoma, glioma</td>
<td>CS271</td>
</tr>
<tr>
<td></td>
<td>JARID1 B</td>
</tr>
</tbody>
</table>
ALDH = aldehyde dehydrogenase; AML = acute myeloid leukaemia; ASCL1 = achaete-scute complex homologue 1; EpCAM = epithelial cell adhesion molecule; HCC = hepatocellular carcinoma; MyD88 = myeloid differentiation factor 88; NSCLC = non-small cell lung cancer; SCC = squamous cell carcinoma; SCLC = small cell lung cancer; SSC = side scatter.

Table 2. CSCs markers in solid tumors (Visvader and Lindeman, Nat Rev Cancer. 2008, 8(10), 755-768).

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>CSC marker</th>
<th>Tumour cells expressing CSC marker, %</th>
<th>Minimal number of cells expressing CSC markers for tumour formation</th>
<th>Injected in Matrigel</th>
<th>Transplantation site</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>CD44⁺/CD24⁺</td>
<td>11-35</td>
<td>200</td>
<td>+</td>
<td>Mammary fat pad</td>
<td>NOD-SCID</td>
</tr>
<tr>
<td>Breast</td>
<td>CD44⁺/CD24⁻</td>
<td>ND</td>
<td>2 x 10⁴</td>
<td>-</td>
<td>Mammary fat pad</td>
<td>NOD-SCID</td>
</tr>
<tr>
<td>Brain</td>
<td>CD133⁺ (GBM)</td>
<td>19-29</td>
<td>100</td>
<td>-</td>
<td>Brain</td>
<td>NOD-SCID</td>
</tr>
<tr>
<td>Brain</td>
<td>CD133⁺ (MB)</td>
<td>6-21</td>
<td>100</td>
<td>-</td>
<td>Brain</td>
<td>NOD-SCID</td>
</tr>
<tr>
<td>Brain</td>
<td>CD133⁺</td>
<td>2-3</td>
<td>500</td>
<td>-</td>
<td>Brain</td>
<td>nu/nu</td>
</tr>
<tr>
<td>Colon</td>
<td>CD133⁺</td>
<td>1.8-25</td>
<td>200</td>
<td>+</td>
<td>Kidney capsule</td>
<td>NOD-SCID</td>
</tr>
<tr>
<td>Colon</td>
<td>CD133⁺</td>
<td>0.7-6</td>
<td>3 x 10³</td>
<td>-</td>
<td>Subcutaneous</td>
<td>SCID</td>
</tr>
<tr>
<td>Colon</td>
<td>EpCAM⁺/CD44⁺</td>
<td>0.03-38</td>
<td>200</td>
<td>+</td>
<td>Subcutaneous</td>
<td>NOD-SCID</td>
</tr>
<tr>
<td>Head and neck</td>
<td>CD44⁺</td>
<td>0.1-42</td>
<td>5x10³</td>
<td>+</td>
<td>Subcutaneous</td>
<td>Rag2/y⁻ DKO, NOD-SCID</td>
</tr>
<tr>
<td>Pancreas</td>
<td>CD44⁺/CD24⁺/ESA⁺</td>
<td>0.2-0.8</td>
<td>100</td>
<td>+</td>
<td>Pancreas</td>
<td>NOD-SCID</td>
</tr>
<tr>
<td>Pancreas</td>
<td>CD133⁺</td>
<td>1-3</td>
<td>500</td>
<td>-</td>
<td>Pancreas</td>
<td>NMRI-nu/nu</td>
</tr>
<tr>
<td>Lung</td>
<td>CD133⁺</td>
<td>0.32-22</td>
<td>10⁴</td>
<td>-</td>
<td>Subcutaneous</td>
<td>SCID</td>
</tr>
<tr>
<td>Liver</td>
<td>CD90⁺</td>
<td>0.03-6</td>
<td>5 x 10³</td>
<td>-</td>
<td>Liver</td>
<td>SCID/Beige</td>
</tr>
<tr>
<td>Melanoma</td>
<td>ABCB5⁺</td>
<td>1.6-20</td>
<td>10⁶</td>
<td>-</td>
<td>Subcutaneous</td>
<td>NOD-SCID</td>
</tr>
<tr>
<td>Mesenchymal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Side population (Hoechst dye)</td>
<td>0.07-10</td>
<td>100</td>
<td>-</td>
<td>Subcutaneous</td>
<td>NOD-SCID</td>
</tr>
</tbody>
</table>
Table 3: CSC markers for breast tumors subtypes (Wei and Lewis, Endocr Relat Cancer. 2015, 22(3):R135-R155).

<table>
<thead>
<tr>
<th>TIC markers</th>
<th>Tumor subtypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human breast TIC markers</td>
<td></td>
</tr>
<tr>
<td>CD44+ / CD24low</td>
<td>NR</td>
</tr>
<tr>
<td>ALDH</td>
<td>Basal-like</td>
</tr>
<tr>
<td>STAT3-GFP</td>
<td>Claudin-low (cell line xenografts)</td>
</tr>
<tr>
<td>EpCAM / CD49f</td>
<td>Triple-negative</td>
</tr>
<tr>
<td>Mouse mammary TIC markers</td>
<td></td>
</tr>
<tr>
<td>Sca1 / CD24</td>
<td>Luminal (MMTV-Neu)</td>
</tr>
<tr>
<td>CD49f / CD61c</td>
<td>Luminal (MMTV-Neu)</td>
</tr>
<tr>
<td>CD24+ / CD49c</td>
<td>Luminal (MMTV-PyMT)</td>
</tr>
<tr>
<td>CD29+ / CD24+ / CD61+</td>
<td>Basal-like (MMTV-Wnt1)</td>
</tr>
<tr>
<td>Thy1+ / CD24</td>
<td>Basal-like (MMTV-Wnt1)</td>
</tr>
<tr>
<td>EpCAM / CD49f</td>
<td>Basal-like (MMTV-Wnt1)</td>
</tr>
<tr>
<td>CD24+ / CD49f</td>
<td>Basal-like (MMTV-Wnt1)</td>
</tr>
<tr>
<td>CD29+ / CD24</td>
<td>Basal-like (p53-null)</td>
</tr>
<tr>
<td>TOP-GFP</td>
<td>Basal-like (p53-null)</td>
</tr>
<tr>
<td>CD24+ / CD29</td>
<td>Basal-like (Brca1-deficient)</td>
</tr>
</tbody>
</table>

Methods to evaluate the ability of cancer cells (e.g., tumor cells) to form spheres when grown in suspension (also referred as "sphere" or "tumorsphere" formation assays) are well known in the art. Cells can be seeded at clonal density in non-adherent conditions, and the percentage of cells generating 'spheres' (e.g., neurospheres, colonospheres or mammospheres) can be measured. Commonly, the sphere-forming efficiency (SFE) is calculated, which is determined as the number of sphere-like structures (diameter >50 µm) divided by the original number of cells seeded. In breast cancer, sphere-forming efficiency is referred as mammosphere-forming efficiency (MSFE).

Preferably, culturing is carried out under non-differentiating conditions, i.e. in the presence of growth factors enabling CSCs growing in an undifferentiated status. These are well known by a person skilled in the art and include, but are not limited to, combinations of one or more of the following: leukemia inhibitory factor (LIF), interleukin 11 (IL11), interleukin 6 (IL6), interleukin 6 receptor (IL6R), ciliary neurotrophic factor (CNTF), oncostatin , cardiotrophin, epidermal growth factor (EGF), stem cell factor (SCF), insulin growth factor 1 (IGF-1) and fibroblast growth factor (including basic FGF (bFGF) or human FGF (hFGF)). For example, cells can be cultured with F-12/DMEM medium containing 1% L-glutamine, 1% penicillin/streptomycin, 30% F12 (Sigma), 2% B27 (Invitrogen, Carlsbad, CA), 20 ng/ml EGF (Sigma, St. Louis, MO) and 20 ng/ml FGFB (Invitrogen, Carlsbad, CA). The medium may be made semi-solid by the addition of 0.5% methylcellulose (R&D Systems, Minneapolis, MN) to prevent cell aggregation.

Cancer cells for use in the sphere formation assay can be cancer cells isolated from a cancer patient, cancer cell lines (as commercially available or genetically modified), cells that have undergone EMT, and cancer stem cells identified or generated using any suitable method. These can be from any cancer type as described below. It should be noted however that all cancer cells do not have the capacity of forming spheres. It has been described by Juan Manuel Iglesias et al. (PLOS One 2013; 8:e77281) that mammosphere formation in breast carcinoma cell lines depends upon expression of E-cadherin. Thus, breast cancer cell lines not expressing E-cadherin such as SKBR3 (ATCC® HTB-30™) will not be suitable for testing the inhibition of CSCs by the compounds described herein. A person skilled in the art
will know which cells are appropriate for performing the sphere formation assay. For example suitable commercially available human breast cancer cell lines are MCF7 (ATCC® HTB-22™), AU565 (ATCC® CRL-2351™), BT474 (ATCC® HTB-20™), T47D (ATCC® HTB-133™), ZR-75-1 (ATCC® CRL-1500™), DCIS.com (Asterand, Detroit, MI), MDA-MB-468 (ATCC® HTB-132™), JIMT1 (ACC-589 - DSMZ) and SUM159 (Asterand, Detroit, MI).

Cancer cells will be cultured in a culture medium appropriate for their growth and proliferation. The terms "cell growth medium", "cell culture medium" or "culture media" are used interchangeably and refer herein to a nutritive solution for culturing or growing cells. The ingredients that compose such media may vary depending on the type of cell to be cultured. In addition to nutrient composition, osmolarity and pH are considered important parameters of culture media. The cell growth medium comprises a number of ingredients well known by the person skilled in the art, including amino acids, vitamins, organic and inorganic salts, sources of carbohydrate, lipids, trace elements (CuSO4, FeSO4, Fe(N03)3, ZnSO4...), each ingredient being present in an amount which supports the cultivation of a cell in vitro (i.e., survival and growth of cells). Ingredients may also include different auxiliary substances, such as buffer substances (like sodium bicarbonate, Hepes, Tris...), oxidation stabilizers, stabilizers to counteract mechanical stress, protease inhibitors, animal growth factors, plant hydrolyzates, anti-clumping agents, anti-foaming agents. Characteristics and compositions of the cell growth media vary depending on the particular cellular requirements. Examples of commercially available cell growth media are: MEM (Minimum Essential Medium), BME (Basal Medium Eagle) DMEM (Dulbecco's modified Eagle's Medium), Iscoves DMEM (Iscove's modification of Dulbecco's Medium), GMEM, RPMI 1640, Leibovitz L-15, CHO, McCoy's, Medium 199, HEK293, Ham (Ham's Media) F10 and derivatives, Ham F12, DMEM/F12, etc.

Preferably, CSCs inhibition is determined by measuring the inhibition of the sphere formation efficiency. In a particular embodiment, optionally in combination with one or more of the features or embodiments described above or below, the compound for use in a method of treatment of the invention reduces sphere formation efficiency at least 50%, preferably at least 55%, more preferably at least 65%, even more preferably at least 90%.

The compounds of the invention have shown to result in the inhibition of CSCs sternness properties irrespectively of the initial cells surface marker phenotype. Notably, these have been shown to be effective in inhibiting CSCs properties in cells expressing mesenchymal and/or epithelial stem cell markers. Accordingly, in a preferred embodiment, prior to CSCs inhibition said CSCs are characterized by a stem cell marker profile ALDH+, CD44+/CD24low/ or ALDH+ and CD44+/CD24low/.

Methods to evaluate the ability of a compound to inhibit cancer cells tumor initiating capacity in an immunocompromised animal are well known in the art. For instance, it may be evaluated the animal median tumor-free survival or overall survival. In a particular embodiment, said dual mTOR and DNMT inhibitor, preferably a compound of formula (I), or pharmaceutically acceptable drug or prodruk thereof, increases the immunocompromised median-tumor free survival by at least 2 fold, preferably by at least 3 fold with respect to an untreated or vehicle treated animal.
Cells can be xenotransplanted into an immunocompromised animal orthotopically (e.g., into the mammary fat pads for breast cancer) and also ectopically (e.g., subcutaneously for breast or ovarian cancer). The term "immunocompromised" as used herein refers to a recipient animal in which the immune system has been partly or completely suppressed in order to allow engrafted foreign cells or tissue to grow with minimal chance of rejection by the recipient animal. Particularly, the term immunocompromised is used to describe an animal that is partially or completely immunosuppressed by biological or chemical means. Chemical immunosuppressive agents are well known in the art (e.g., cyclosporine). The term immunocompromised also includes animals that are partially or completely immunodeficient. These animals comprise rodents, such as mice or rats, having no functional T-cell, and/or B cell and/or NK cell immunity. Non-limiting examples of such rodents are: athymic nude mice, severe combined immunodeficient (SCID) mice, SCID/beige mice, non-obese diabetic/severe combined immunodeficient (NOD/SCID), Rag2-/- vc-/-, and NOD/SCID/IL-2rv- mice or NIH-nu-bg-xid mice. Preferably, said immunocompromised animal is immunodeficient. In a preferred embodiment, said animal is an immunodeficient mouse.

Tumor cells used for injection into the immunosuppressed animal (e.g., mice) can be as described above for the sphere formation assay. It has been shown that cells isolated based on the expression of cancer stem cell markers (CSC-enriched tumor cells) would be more tumorigenic that an unsorted tumor population when xenografted into an immunocompromised mouse. Accordingly, preferably, CSC-enriched tumor cells are used to evaluate the ability of the compounds described herein to inhibit cells tumor initiating capacity. CSC-enriched tumor cells may have been isolated by the presence of stem cell markers or by culturing under non-adherent conditions. It is also preferred that tumor cells are xenotransplanted in small numbers (e.g., 10, 10², 10³, 10⁴, or 10⁵).

In a preferred embodiment, said compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or pharmaceutically acceptable drug or prodrug thereof, is capable of achieving selective inhibition of CSCs, wherein selective inhibition is herein understood as inhibition of the ability to form spheres when grown in suspension culture, generally under non-differentiating conditions, in the absence of a significant reduction of cell viability, i.e., in the absence of a significant inhibition of cell growth and/or survival in a monolayer adherent culture. Preferably, the monolayer adherent cell culture is performed under differentiating conditions. A person skilled in the art will know the appropriate cell culture conditions for a given cancer cell type monolayer adherent culture. For example, for the culturing of breast cancer cells in monolayer adherent culture under differentiating conditions, one or more growth factors, hormones and/or cytokines may be added to the culture medium such as EGF, insulin, IGF-1, IL-6, IL-1β, etc. Also, typically human cell lines are cultured at 37°C and with 5% CO₂.

In a particular embodiment, optionally in combination with one or more of the features or embodiments described above or below, cell viability is reduced less than 20%, preferably less than 15%, more preferably less than 10%, even more preferably less than 5%.
Preferably, the compound of formula (I) is characterized by presenting an EC50 of less than 50 µM, preferably less than 20 µM, 15 µM, 10 µM, even more preferably less than 5 µM in the sphere-forming efficiency assay.

Methods for the determination of cell growth and/or survival are well known in the art. The cell growth and/or survival may be determined by an assay selected from: a cell counting assay, a replication labelling assay, a cell membrane integrity assay, a cellular ATP-based viability assay, a mitochondrial reductase activity assay, a caspase activity assay, an Annexin V staining assay, a DNA content assay, a DNA degradation assay, and a nuclear fragmentation assay. Other exemplary assays include BrdU, EdU, or H3-Thymidine incorporation assays; DNA content assays using a nucleic acid dye, such as Hoechst Dye, DAPI, Actinomycin D, 7-aminoactinomycin D or Propidium Iodide; Cellular metabolism assays such as AlamarBlue, MTT, MTS, XTT, WST-1 and CellTitre Glo®; Nuclear Fragmentation Assays; Cytoplasmic Histone Associated DNA Fragmentation Assay; PARP Cleavage Assay; TUNEL staining; and Annexin staining. In a preferred embodiment, cell viability under monolayer adherent conditions is determined by an assay based on tetrazolium salts reduction.

A variety of tetrazolium reduction assays have been used to detect viable cells. The most commonly used compounds include MTT, MTS, XTT and WST-1. These compounds fall into two basic categories: 1) MTT which is positively charged and readily penetrates viable eukaryotic cells and 2) those such as MTS, XTT and WST-1 which are negatively charged and do not readily penetrate eukaryotic cells. The MTT assay, is a well-known colorimetric assay for assessing cell viability based on NAD(P)H-dependent cellular oxidoreductase enzymes ability to reduce the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) to formazan, which has a purple color. The later class, are typically used with an intermediate electron acceptor that can transfer electrons from the cytoplasm or plasma membrane to facilitate the reduction of the tetrazolium into the coloured formazan product. Preferably, cell viability is determined by the MTT assay.

The cancer to be treated may be any type of cancer. In a particular embodiment, said cancer is a solid tumor. Different types of solid tumors are named for the type of cells that form them. Examples of solid tumors are lung cancer, sarcoma, malignant melanoma, mesothelioma, bladder carcinoma, prostate cancer, pancreas carcinoma, gastric carcinoma, ovarian cancer, hepatoma, breast cancer, colorectal cancer, kidney cancer, esophageal cancer, suprarenal cancer, parotid gland cancer, head and neck carcinoma, cervix cancer, mesothelioma and lymphoma. In a preferred embodiment, said tumor is not a hematologic tumor.

In another preferred embodiment, said tumor is a tumor of the nervous system. These include, according to their tissue of origin: tumors of neuroepithelial tissue; tumors of peripheral nerves; tumors of meninges; tumors of the sellar region; germ cell tumors; lymphomas and metastatic tumors. Preferably, said nervous system tumors are tumors from the neuroepithelial tissue. In a preferred embodiment, said nervous system tumor is a glioma. The term "glioma" as used herein refers to a type of brain tumor that grows from glial cells and encompasses different types of gliomas, such as astrocytoma, oligodendroglioma, and glioblastoma.
In another preferred embodiment, said solid tumor is a carcinoma. Carcinoma can be classified according to the site of origin or cell type and include *inter alia:* breast carcinoma (e.g., ductal carcinoma in situ, invasive (or infiltrating) ductal carcinoma, or invasive (or infiltrating) ductal carcinoma); ovarian carcinoma; cervix carcinoma; gastric carcinoma; non-small cell lung carcinoma; small cell lung carcinoma; pancreatic carcinoma; prostate carcinoma; colon carcinoma; liver carcinoma; renal carcinoma; bladder carcinoma; prostate carcinoma; head and neck carcinoma; squamous cell carcinoma; epidermoid carcinoma; choriocarcinoma; seminoma; embryonal cell carcinoma; and mesothelioma. In a preferred embodiment, said carcinoma is breast or ovarian carcinoma. In a more preferred embodiment, said carcinoma is breast carcinoma. Breast carcinoma can be subclassified according to molecular subtypes, such as, luminal A; luminal B; human epidermal growth factor receptor 2 (HER2)-enriched; basal-like; claudin-low; triple negative (estrogen receptor (ER), progesterone receptor (PR) and HER2 negative) and normal-like.

Said cancer may be resistant to an anticancer drug or therapy. In a particular embodiment, the subject is previously untreated. In another embodiment, the subject has previously been treated with another anticancer drug or therapy and has developed resistance to it, in other words, the subject to be treated is suffering from a cancer resistant to an anticancer drug or therapy. Said anticancer therapy is not particularly limited and can be any anticancer therapy, preferably those mentioned herein. The acquisition of resistances to several anticancer therapies, such as chemotherapy (e.g. doxorubicin or paclitaxel), radiotherapy, hormone therapy (e.g. tamoxifen and aromatase inhibitors) or anti-HER2 therapy (e.g. trastuzumab) has been associated with the presence of CSCs. This has been particularly documented in solid tumors, such as breast and ovarian cancers (Wei and Lewis, Endocrine-Related Cancer 2015, 22(3):R135-R155; Gupta et al., Cell 2009, 138, 645-659; Kurrey et al., Stem Cells 2009, 27, 2059-2068). Said cancer, or cancer resistant to an anticancer drug or therapy, is preferably a relapsing, recurrent and/or metastatic cancer.

The invention also provides a method of treating a cancer resistant to an anticancer drug or therapy, comprising administering to a subject suffering from said cancer resistant to an anticancer drug or therapy an effective amount of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, wherein said amount inhibits, or reduces the frequency of, cancer stem cells by at least about 50%.

The invention further relates to a method of treating solid tumors resistant to anticancer drug or therapy as described herein, comprising administering to a subject suffering from said cancer resistant to an anticancer drug or therapy a therapeutically effective amount of compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, wherein said amount inhibits, or reduces the frequency of, cancer stem cells by at least about 50%.

In addition it discloses a method of reducing the frequency of cancer stem cells (CSCs) in a subject having a solid tumor with at least a 1% of CSCs, comprising administering to a subject in need thereof a
therapeutically effective amount of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, to inhibit, or reduce the frequency of, CSCs by at least about 50%. The % of CSCs may be determined according to the number of cells within said tumor presenting cancer stem cell markers as defined herein, such as ALDH^+ and/or CD44^+CD24^-low. Preferably, said tumor comprises at least 5%, preferably at least 10%, 15%, 20% or more than 20% of CSCs.

In the context of the present invention, the clinical outcome of a subject, may be expressed as overall survival or progression-free survival. Survival of cancer patients is generally suitably expressed by Kaplan-Meier curves, named after Edward L. Kaplan and Paul Meier who first described it (Kaplan, Meier: Amer. Statist. Assn. 53:457-481). The Kaplan-Meier estimator is also known as the product limit estimator. It serves for estimating the survival function from life-time data. A plot of the Kaplan-Meier estimate of the survival function is a series of horizontal steps of declining magnitude which, when a large enough sample is taken, approaches the true survival function for that population. The value of the survival function between successive distinct sampled observations is assumed to be constant. With respect to the present invention, the Kaplan-Meier estimator may be used to measure the fraction of patients living for a certain amount of time after beginning a therapy. The clinical outcome predicted may be the (overall/progression-free) survival in months/years from the time point of taking the sample.

In a particular embodiment, optionally in combination with one or more of the embodiments and/or features described herein, the overall survival or progression free survival of the subject treated with a compound of the invention is of at least 2 fold, preferably of at least 3 fold with respect to an untreated or vehicle treated subject. It may be survival for a certain period from taking the sample, such as of six months or more, one year or more, two years or more, three years or more, four years or more, five years or more, six years or more. In each case, "survival" may refer to "overall survival" or "progression-free-survival".

A pharmaceutical composition for use in the method of treatment of the invention

The present invention also provides a pharmaceutical composition comprising a compound as defined above, and a pharmaceutically acceptable excipient, for use in a method of treatment as defined herein.

In a related aspect, the invention provides a pharmaceutical composition, comprising a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, in an amount effective to inhibit, or reduce the frequency of, cancer stem cells in a subject in need thereof by at least about 50%, and a pharmaceutically acceptable excipient.

The invention also provides a dosage form, comprising an effective amount of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, wherein
said dosage form is suitable for administration said compound in an amount effective to inhibit, or reduce
the frequency of, cancer stem cells by at least about 50%.

Pharmaceutically acceptable excipients include, but are not limited to a carrier or diluent, such as a gum,
a starch (e.g. corn starch, pregelatinized starch), a sugar (e.g. lactose, mannitol, sucrose, dextrose), a
cellulosic material (e.g. microcrystalline cellulose), an acrylate (e.g. polymethylacrylate), calcium
carbonate, magnesium oxide, talc, or mixtures thereof; a binder (e.g. acacia, cornstarch, gelatin,
carbomer, ethyl cellulose, guar gum, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, povidone);
a disintegrating agent (e.g. cornstarch, potato starch, alginic acid, silicon dioxide, croscarmelose sodium,
crospovidone, guar gum, sodium starch glycolate), a buffer (e.g. Tris-HCl, acetate, phosphate) of various
pH and ionic strength; and additive such as albumin or gelatin to prevent absorption to surfaces; a
detergent (e.g. Tween 20, Tween 80, Pluronic F68, bile acid salts); a protease inhibitor; a surfactant (e.g.
sodium lauryl sulfate); a permeation enhancer; a solubilizing agent (e.g. glycerol, polyethylene glycerol);
an antioxidants (e.g. ascorbic acid, sodium metabisulfite, butylated hydroxyanisole); a stabilizer (e.g.
hydroxypropyl cellulose, hydroxypropylmethyl cellulose); a viscosity increasing agent (e.g. carbomer,
colloidal silicon dioxide, ethyl cellulose, guar gum); a sweetener (e.g. aspartame, citric acid); a
preservative (e.g. thimerosal, benzyl alcohol, parabens); a lubricant (e.g. stearic acid, magnesium
stearate, polyethylene glycol, sodium lauryl sulfate); a flow-aid (e.g. colloidal silicon dioxide), a plasticizer
(e.g. diethyl phthalate, triethyl citrate); an emulsifier (e.g. carbomer, hydroxypropyl cellulose, sodium
lauryl sulfate); a polymer coating (e.g. poloxamers or poloxamines); a coating and film forming agent (e.g.
ethyl cellulose, acrylates, polymethacrylates); a pharmaceutically acceptable carrier for liquid
formulations, such as an aqueous (water, alcoholic/aqueous solution, emulsion or suspension, including
saline and buffered media) or non-aqueous (e.g., propylene glycol, polyethylene glycol, and injectable
organic esters, such as ethyl oleate) solution, suspension, emulsion or oil; and a parenteral vehicle (e.g.,
for subcutaneous, intravenous, intraarterial, or intramuscular injection), including but not limited to, water,
oils, saline solution, Ringer's dextrose, aqueous dextrose and other sugar solutions. A pharmaceutically
acceptable excipient also includes excipients for nanoencapsulation purposes, such as a cationic
polyelectrolyte (e.g. gelatin and an anionic polyelectrolyte (e.g. arabic gum).

A pharmaceutical composition for use in a method of treatment of the invention is formulated to be
compatible with its intended route of administration. Methods to accomplish the administration are known
to those of ordinary skill in the art. This includes, for example, injections, by parenteral routes such as
intravenous, intravascular, intraarterial, subcutaneous, intramuscular, intraperitoneal, intraventricular,
intraepidural, or others as well as oral, nasal, ophthalmic, rectal or topical. Sustained release
administration is also specifically contemplated, e.g., as depot injections or erodible implants. Localized
delivery is particularly contemplated, e.g., as delivery via a catheter to one or more arteries, such as the
renal artery or a vessel supplying a localized site of interest. Local administration on the tumor surface
(e.g., in liquid form for rinsing or lavage) during surgery or biopsy sampling may also be contemplated. In
one embodiment, said pharmaceutical composition is for oral administration. In another embodiment, said
pharmaceutical composition is for intravenous, intramuscular or subcutaneous infusion or injection.
A compound or a pharmaceutical composition comprising thereof for use according to the invention can be administered a single time. It may also be administered regularly throughout the course of the method of treatment, for example, one, two, three, four, or more times a day, weekly, bi-weekly, every three weeks or monthly. This compound or pharmaceutical composition may also be administered continuously to the subject (e.g., intravenously or by release from an implant, pump, sustained release formulation, etc.). The dosage to be administered can depend on multiple factors, including the type and severity of the cancer and/or on the characteristics of the subject, such as general health, age, sex, body weight and tolerance to drugs and should be adjusted, as needed, according to individual need and professional judgment. The dosage may also vary depending upon factors, such as route of administration, target site, or other therapies administered. The skilled artisan will be able to determine appropriate doses depending on these and other factors. A therapeutically effective amount may include, but is not limited to, dosage ranges of about 0.01 mg/kg to about 200 mg/kg, 0.05 mg/kg to about 150 mg/kg, about 0.1 mg/kg to about 100 mg/kg; about 0.25 mg/kg to about 10 mg/kg; about 0.5 mg/kg to 75 mg/kg; 1 mg/kg to about 50 mg/kg; 1 mg/kg to about 10 mg/kg; about 0.5 mg/kg to about 25 mg/kg; or about 1 mg/kg to about 5 mg/kg. In acute toxicity studies no lethality or adverse effects were observed in mice even when oleuropein was administered at doses as high as 1000 mg/kg. Hamdi HK, Castellon R. Biochem Biophys Res Commun. , 2005, 334(3):769-78.

Further details of said multiple epigenetic modulator compound, the compound of formula (I), the methods of treatment, as well as preferred features and embodiments thereof, are as described above for the methods of treatment of the invention.

Combination therapies for use in the method of treatment of the invention

The invention also provides the compound or the pharmaceutical composition for use in a method of treatment of the invention wherein their administration is performed in combination with another anticancer therapy. The term "anticancer therapy" includes surgery, radiation therapy, chemotherapy, immunotherapy, hormone therapy, targeted therapy, and stem cell transplant. Preferably said anticancer therapy is selected from the group consisting of surgery, radiation therapy, chemotherapy, immunotherapy, hormone therapy, and targeted therapy. One, two, three or more of said anticancer therapies may be combined with the compounds and pharmaceutical composition described herein for use in the method of treatment of the invention. In one embodiment, said other anticancer therapy is surgery. In another embodiment, said anticancer therapy has cytotoxic or anti-proliferative effects, which includes radiation therapy, chemotherapy, immunotherapy, hormone therapy, or targeted therapy.

It is additionally provided herein the compound for use in a method of treatment of the invention wherein the method of treatment of the invention is performed in combination with another drug. Moreover, the invention provides a method of inhibiting, or reducing the frequency of, cancer stem cells in a subject in need thereof, comprising administering to a subject in need thereof a therapeutically effective amount of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof to inhibit, or reduce the frequency of, cancer stem cells by at least about 50%, in combination with another anticancer drug.
The term "another drug" as used herein may refer to one, two, three or more drugs. It includes any therapeutic agent and preferably anticancer drugs, drugs with palliative or supportive effects; and combinations thereof. The term "drugs with palliative or supportive effects" includes but is not limited to the following group of compounds: antianemic, antiemetic, anticachexia (e.g., megestrol acetate), inhibitors of bone resorption (e.g., clodronate, ibandronic acid, pamidronate, zoledronic acid, denosumab), corticosteroids, antimucositis (e.g., palifermin), hematopoietic growth factors, opioids (e.g. morphine, fentanyl), gabapentin, antibiotics (e.g., minocycline, doxycycline, tetracycline), cannabis and cannabinoids (e.g., dronabinol, nabnilone), vitamins and nutritional supplements.

The present invention also provides a pharmaceutical composition comprising a compound as defined herein, another drug, and a pharmaceutically acceptable excipient for use in a method of treating cancer of the invention.

It further provides a kit for use in a method of treating cancer of the invention which comprises a dosage form of a compound as defined herein, and/or a dosage form of another drug, and instructions for the use of both drugs in combination. The containers in which the compound or pharmaceutical composition is supplied can be any conventional container that is capable of holding the supplied dosage forms.

In preferred embodiments, said other drug is another anticancer drug. Generally a tumor comprises cancer cells that are not CSCs, these are herein referred as "bulk" cancer cells. Preferably, said another anticancer drug is capable of killing, inhibiting, or reducing the frequency of, said bulk cancer cells, typically resulting in the shrinkage of the tumor. Said another anticancer drug may be any cytotoxic or antiproliferative drug, and includes but it is not limited to, the group consisting of platinum coordination complexes (e.g., cisplatin, oxaliplatin, carboplatin, BBR3464, satraplatin, tetraplatin,ormiplatin, and iproplatin); antimetabolites (e.g., 5-fluorouracil, gemcitabine, cytarabine, capicabine, decitabine, floxuridine, 6-mercaptopurine, methotrexate, fludarabine, aminopterin, pemetrexed, raltitrexed, cladribine, clofarabine, fludarabine, mercaptopurine, pentostatin, and thioguanine); mitotic inhibitors (e.g., paclitaxel, docetaxel, vinblastine, vincristine, vindesine, and vinorelbine); anthracycline antibiotics (e.g., bleomycin, daunorubicin, doxorubicin, epirubicin, idarubicin, mitomycin, mitoxantrone, pixantrone, and valrubicin); topoisomerase I and/or II inhibitors (e.g., topotecan, SN-38, irinotecan, camptothecine, rubitecan, etoposide, and teniposide); antitumor monoclonal antibodies (e.g., bevacizumab, cetuximab, panitumumab, trastuzumab, rituximab, tositumomab, alemtuzumab, and gemtuzumab); tyrosine kinase inhibitors (e.g., erlotinib, sorafenib, axitinib, bosutinib, cediranib, dasatinib, gefitinib, imatinib, canetinib, lapatinib, lestaurtinib, nilotinib, smanekanib, sunitinib, and vandetanib); metabolic modulators (e.g., mTOR inhibitors) and epigenetic inhibitors (e.g., DNMT inhibitors).

In another embodiment, said another drug has activity against CSCs. In a further embodiment, the use of a compound as described herein in combination with another anticancer drug in a method of treating cancer of the invention provides synergistic effects in the inhibition of CSCs, e.g., in inhibiting the ability of CSCs of forming spheres. In a particular embodiment, said other anticancer drug is selected from the group consisting of metabolic modulators, epigenetic inhibitors, anthracycline antibiotics, a multitargeted antifolate, a biguanide, a PARP inhibitor and an anti-HER2 agent.
In a preferred embodiment, said other drug is a metabolic modulator. Metabolic modulators as used herein refer to drugs that interfere with cancer-associated alterations in metabolic pathways and include mTOR inhibitors. Several mTOR inhibitors have been used preclinically and clinically in the treatment of cancer and are well known in the art. These include but are not limited to rapamycin or analogs thereof (also collectively known as rapalogs), and ATP-competitive mTOR kinase inhibitors. Rapalogs are well known in the art and include sirolimus (also known as rapamycin), temsirolimus, everolimus, and deforolimus (also referred as ridaforolimus). ATP-competitive mTOR kinase inhibitors comprise mTORC1/mTORC2 dual inhibitors (e.g., OSI027, INKI 28, AZD8055, AZD2014, PP242, PP30, WAY600, WYE687, WYE354, WYE132, Ku0063794); PI3K/mTOR dual inhibitors (e.g., NVPBEZ235, SF1126, GSK2126458, XL765, BGT226, GDC0980, PI103, PI540, PI620, NVPBB130, and PKI402); and other mTOR inhibitors. Further details on the above-mentioned compounds and other mTOR inhibitors are provided in Zhang et al., 2011 (Drug Discov Today, 2011, 16(7-8), 325-31) which is hereby incorporated by reference, especially Tables 1-3. In a more preferred embodiment, said other drug is an mTOR inhibitor. In a particularly preferred embodiment, said other drug is rapamycin.

In another preferred embodiment, said other anticancer drug is an epigenetic inhibitor or modulator. Epigenetic changes are responsible for cellular plasticity that enables cellular reprogramming and response to the environment. Epigenetic events, i.e., DNA methylation and histone deacetylation have been shown to silence the expression of suppressor genes, thus being a promising target for oncologic treatment.

Some epigenetic inhibitors have already been approved and other are currently under clinical development for use in the treatment of cancer. These include but are not limited to DNA methyltransferases (DNMT) inhibitors; and histone deacetylases inhibitors (vorinostat, romidepsin, panobinostat, belinostat, entinostat, mocetinostat, resminostat, and givinostat). In a more preferred embodiment, said other drug is a DNMT inhibitor. DNMT inhibitors may be nucleoside inhibitors (e.g., 5-azacytidine, 5-aza-2'-deoxycytidine (decitabine), 5-fluoro-2'-deoxycytidine (FdCyd), 1-beta-D-arabinofuranosyl-5-azacytosine and dihydro-5-azacytidine) and non-nucleoside inhibitors (e.g., curcumin, procaine, procanamid, hydralazine, epigallocatechin gallate, psammaplin A, 3-nitro-2-(3-nitrophenyl)flavone and delta 2-isoxazoline). In a particular preferred embodiment, said other drug is a nucleoside inhibitor, preferably 5-azacytidine (5-AZA).

In a further preferred embodiment, said other anticancer drug is a chemotherapeutic agent, preferably an anthracycline antibiotic. Anthracycline antibiotics are characterized by containing an anthracylene ring in its chemical structure and include but are not limited to bleomycin, daunorubicin, doxorubicin, epirubicin, idarubicin, mitomycin, mitoxantrone, pixantrone, and valrubicin. Preferably, said anthracycline antibiotic is doxorubicin.

In another preferred embodiment, said other anticancer drug is a multi-targeted antifolate agent, preferably acting by inhibition of folate-dependent one-carbon enzymes, more preferably pemetrexed. Interestingly, pemetrexed is the only of those compounds which has resulted in a synergistic inhibition of
CSCs in the absence of significant cytotoxicity when used in combination with DOA (Example 13) for which activity against CSCs has not previously been disclosed.

In an additional preferred embodiment, said other anticancer drug is a biguanide, preferably metformin.

In an also preferred embodiment, said other anticancer drug is a PARP inhibitor. PARP inhibitors are a group of pharmacological inhibitors of the enzyme poly ADP ribose polymerase (PARP). These include but are not limited to olaparib, niraparib, iniparib, talazoparib, veliparib, rucaparib. Preferably said PARP inhibitor is olaparib.

In further preferred embodiment, said other anticancer drug is an anti-HER2 agent, Trastuzumab is a humanized monoclonal antibody designed to target the extracellular domain of the HER2 receptor, and is the foundation of care of women with early and advanced HER2-positive breast cancer. New anti-HER2 agents are currently being developed. These include small molecules that inhibit the HER2 tyrosine kinase activity (lapatinib, neratinib), monoclonal antibodies directed at other epitopes of the HER2 extracellular domain (pertuzumab), antibody-drug conjugates (T-DM1), and heat shock protein 90 inhibitors (tanespimycin). Preferably, said anti-HER2 agent is trastuzumab emtansine (T-DM1).

In a preferred embodiment, said other anticancer drug is selected from the group consisting of rapamycin, 5-azacytidine, pemetrexed, metformin, olaparib and trastuzumab emtansine (T-DM1). These drugs have shown to provide synergistic effects in reducing the size of spheres in the Cell2Sphere™ kit assay (Example 13).

Therapeutic agents and methods of administration, dosages, etc., are well known to those of skill in the art (see for example, the "Physicians Desk Reference", Klaassen’s "The Pharmacological Basis of Therapeutics", "Remington's Pharmaceutical Sciences", and "The Merck Index, Eleventh Edition", incorporated herein by reference), and may be combined with the invention in light of the disclosures herein. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject, and such individual determinations are within the skill of those of ordinary skill in the art.

In preferred embodiments, the compound, the pharmaceutical composition or the kit of parts for use in a method of treatment of the invention are used for the treatment of a cancer which is resistant to the other anticancer drug used in the combination therapy and administration of said compound or pharmaceutical composition as described herein, reverses the resistance.

COMBINATION THERAPIES FOR THE TREATMENT OF CANCER

In another aspect, the invention provides a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, for use in a method of treating a subject suffering from cancer in combination therapy with another anticancer drug.
It further provides, a method of treating cancer comprising administering to a subject in need of such treatment a therapeutically effective amount of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, and a therapeutically effective amount of another anticancer drug, administered prior, concomitantly or after its administration. The two drugs may form part of the same composition, or be provided as a separate composition for administration at the same time or at a different time.

In another aspect, the invention also relates to a method of increasing or potentiating the therapeutic efficacy of an anticancer drug in the treatment of cancer, which comprises administering to a patient in need thereof a therapeutically effective amount of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof.

The invention also relates to the use of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, for the manufacture of a medicament for use in combination therapy with another anticancer drug in the treatment of cancer.

The invention also provides a pharmaceutical composition comprising a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, another anticancer drug, and a pharmaceutically acceptable excipient for use in combination therapy for the treatment of cancer.

The invention further provides a kit for use in the treatment of cancer which comprises a dosage form of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof; and/or a dosage form of another anticancer drug; and instructions for the use of both drugs in a combination treatment.

In a preferred aspect, the present invention is concerned with synergistic combinations of a compound, pharmaceutical composition or kit as described herein, and another anticancer drug.

In preferred embodiments of any of the above aspects, said another anticancer drug is a compound selected from the group consisting of an mTOR inhibitor, preferably rapamycin, a DNA methyltransferase inhibitor, preferably 5-azacytidine, a multi-targeted antifolate agent, preferably permetrexed, a biguanide, preferably metformin, a PARP inhibitor, preferably olaparib and an anti-HER2 agent, preferably T-DM1.

The invention further provides a method of treating cancer, comprising administering to a subject in need thereof a therapeutically effective amount of a compound which is a multiple epigenetic modulator,
preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, in combination with a compound selected from the group consisting of a mTOR metabolic inhibitor, preferably rapamycin, a DNA methyltransferase epigenetic inhibitor, preferably 5-azacytidine, a multi-targeted antifolate agent, preferably perimetrexed, a biguanide, preferably metformin, a PARP inhibitor, preferably olaparib and an anti-HER2 agent, preferably T-DM1.

It also relates to a method of treating cancer, comprising administering to a subject in need thereof a pharmaceutical composition comprising a synergistically effective amount of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I) and a compound selected from the group consisting of a mTOR metabolic inhibitor, preferably rapamycin, a DNA methyltransferase epigenetic inhibitor, preferably 5-azacytidine, a multi-targeted antifolate agent, preferably perimetrexed, a biguanide, preferably metformin, a PARP inhibitor, preferably olaparib and an anti-HER2 agent, preferably T-DM1.

In a related aspect it refers to a composition, comprising a synergistically effective amount of a compound which is a multiple epigenetic modulator, preferably a dual mTOR inhibitor and multiple epigenetic modulator, more preferably a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, and a compound selected from the group consisting of a mTOR metabolic inhibitor, preferably rapamycin, a DNA methyltransferase epigenetic inhibitor, preferably 5-azacytidine, a multi-targeted antifolate agent, preferably perimetrexed, a biguanide, preferably metformin, a PARP inhibitor, preferably olaparib and an anti-HER2 agent, preferably T-DM1.

Further details of said compound which is a multiple epigenetic modulator, said compound of formula (I), pharmaceutical composition and kit for use in a method of treating a subject suffering from cancer in combination therapy with another anticancer drug, methods of combination treatment, as well as preferred combinations of features, including said other anticancer drugs, are as described above for the other aspects of the invention.

A COMPOUND OF FORMULA (I), A PHARMACEUTICALLY ACCEPTABLE SALT OR PRODRUG THEREOF, FOR USE IN THE PREVENTION OR TREATMENT OF A DISEASE MEDIATED BY A DEREGLULATION IN A EPIGENETIC ENZYME, SUCH AS INCREASED METHYLTRANSFERASE ACTIVITY

In a further aspect, the present invention also provides a compound, pharmaceutical composition or kit as described above for the other aspects of the invention, for use in a method of preventing or treating a disease or disorder mediated by a deregulation in a epigenetic enzyme, such as increased methyltransferases activity (e.g., DNA methyltransferases activity) wherein said disease is not a solid tumor or an age-related disease.

In addition, the present invention provides a method for preventing or treating a disease or disorder mediated by a deregulation in a epigenetic enzyme, such as increased methyltransferases activity (e.g.,
DNA methyltransferases activity) wherein said disease is not a solid tumor or an age-related disease, said method comprising administering to a subject in need of such treatment a therapeutically effective amount of a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof.

It further provides the use of a compound of formula (I), or a pharmaceutically acceptable salt or prodrug thereof, in the manufacture of a medicament for the treatment or prevention of a disease or disorder mediated by a deregulation in an epigenetic enzyme, such as increased methyltransferases activity (e.g., DNA methyltransferases activity) wherein said disease is not a solid tumor or an age-related disease. Methods to determine methyltransferase activity (are well known in the art and for DNMT activity have been described above.

Several diseases mediated by increased methyltransferase activity, in particular increased DNMT activity, wherein said disease is not a solid tumor or an age-related disease have been described. These include but are not limited to:

- Fibrotic disease (Sun X et al., Cell Signal. 2013 Sep;25(9):1870-6);
- Hematological malignancies, such as myelodysplastic syndromes (MDSs) and acute myeloid leukemia (AML) (Gore SD, Nat Clin Pract Oncol. 2005 Dec;2 Suppl 1:S30-5; Griffiths EA and Gore SD, . Semin Hematol. 2008 Jan;45(1):23-30);
- Neuropsychiatric disorders, such as schizophrenia (SZ), Rett syndrome, and ICF syndrome (Grayson DR, et al., Pharmacol Ther. 2006 Jul;111(1):272-86; Feng J, Fan G., Int Rev Neurobiol. 2009;89:67-84);
- Cardiovascular diseases (Duthie SJ., Proc Nutr Soc. 2011 Feb;70(1):47-56);
- Thalassemia and sickle cell disease (Saunthararajah Y, DeSimone J, Semin Hematol. 2004 Oct;41 (4 Suppl 6):11-6); and

Diseases other than a solid-tumor or an age-related disease which have also been related with other methyltransferases are CNS-related disorders. These include for instance Parkinson's disease, Schizophrenia, Huntington's disease, and cognitive disorders,

Said disease mediated by increased methyltransferases activity can be one or more of the diseases mentioned herein. Preferably, said disease mediated by increased methyltransferases activity (e.g., DNMT activity) is not cancer or an age-related disease.

Additional details and preferred embodiments on the compound of formula (I), the pharmaceutical composition or kit comprising thereof for use in a method for preventing or treating a disease or disorder mediated by increased DNA methyltransferases activity wherein said disease is not a solid tumor or an age-related disease are as provided above for other aspects of the invention.

ITEMS OF THE INVENTION
1. A compound of formula (I)

\[
R^1 \quad R^2
\quad O
\quad O
\quad A
\]

(I)

wherein A is selected from the group consisting of:

\[
\begin{align*}
R^3 & \quad R^4 \\
\quad R^3 & \quad R^4 \\
\quad R^3 & \quad R^4 \\
\quad R^3 & \quad R^4 \\
\quad R^4 & \quad R^3 \\
\quad R^4 & \quad R^3 \\
\quad R^4 & \quad R^3 \\
\quad R^4 & \quad R^3 \\
\end{align*}
\]

wherein

15. \( R^1 \) and \( R^2 \) are independently selected from hydrogen, C1-C8 alkyl, C2-C8 alkenyl, C2-C8 alkynyl and -OR;

R3 is selected from hydrogen, C1-C8 alkyl, C2-C8 alkenyl or C2-C8 alkynyl, -OR, -COR, -COOR, and -OCOR;

20. \( R^4 \) is selected from hydrogen, C1-C8 alkyl, C2-C8 alkenyl, C2-C8 alkynyl and -COOR;

R5 is selected from C1-C8 alkyl, C2-C8 alkenyl or C2-C8 alkynyl, -OR, -COR, -COOR, and -OCOR;

25. \( R^6 \) is selected from hydrogen, C1-C8 alkyl, C2-C8 alkenyl, C2-C8 alkynyl, -COR, and -COOR.
each of $R^7$, $R^8$, $R^9$, and $R^{10}$ is independently selected from hydrogen, saturated or unsaturated C5-C7 cycloalkyl, saturated or unsaturated C5-C7 heterocycloalkyl, C5-C7 aryl, C5-C7 heteroaryl, C1-C8 alkyl, C2-C8 alkenyl C2-C4 alkynyl, and a protecting group of -OH;

wherein the C5-C7 cycloalkyl, C5-C7 heterocycloalkyl, C5-C7 aryl, C5-C7 heteroaryl, C1-C8 alkyl, C2-C8 alkenyl or C2-C8 alkynyl group is unsubstituted or substituted with one or more of a -OR$^{12}$, -SR$^{12}$, -NHCOR$^{12}$, -CONHR$^{12}$, -COR$^{12}$, -COOR$^{12}$, -NHR$^{12}$R$^{13}$, -SR$^{12}$, -SO$_2$R$^{12}$, -SO$_2$NR$^{12}$R$^{13}$, -CF$_3$, -OCF$_3$ or -CN, wherein $R^{12}$ and $R^{13}$ are independently of each other hydrogen, C1-C4 alkyl, C2-C4 alkenyl, C2-C4 alkynyl, and a protecting group of -SH, -OH or amino;

or a pharmaceutically acceptable salt or prodrug thereof; for use in a method of treating a subject suffering from cancer by inhibiting, or reducing the frequency of, cancer stem cells (CSCs).

2. The compound for use in a method of treatment according to item 1, wherein the compound of formula (I) is selected from one or more of the following enantiomeric forms:

\[
\text{(I)}
\]

\[
A = R^3 \quad \text{or}
\]

wherein

\[
A = R^3 \quad \text{or}
\]

3. The compound for use in a method of treatment according to any of items 1 or 2, wherein $R^1$ and $R^2$ are independently selected from hydrogen, C1-C4 alkyl, and -OR$^7$; preferably wherein $R^7$ is hydrogen or C1-C4 alkyl.

4. The compound for use in a method of treatment according to any of items 1 to 3, wherein $R^3$ is selected from hydrogen, C1-C4 alkyl, and -OR$^8$; preferably wherein $R^8$ is hydrogen or C1-C4 alkyl.

5. The compound for use in a method of treatment according to any of items 1 to 4, wherein $R^4$ is selected from hydrogen, C1-C4 alkyl and -COOR$^9$, preferably wherein $R^9$ is hydrogen or C1-C4 alkyl.

6. The compound for use in a method of treatment according to any of items 1 to 5, wherein $R^5$ is OR$^{10}$ and $R^{10}$ is selected from hydrogen and a saturated or unsaturated C5-C6 heterocycloalkyl; preferably
wherein said C5-C6 heterocycloalkyl is a O-cycloalkyl, more preferably a hydroxy substituted O-cycloalkyl.

7. The compound for use in a method of treatment according to any of items 1 to 6, wherein

5 R¹ and R² are independently selected from hydrogen and -OH, preferably R¹ and R² are -OH;
R³ is selected from -CH₃, -CH2OH, and -OH, preferably is -CH₃;
R⁴ is selected from hydrogen and -COOCH₃; and
R⁶ and R⁷ are independently selected from hydrogen and D-glucose, preferably is hydrogen.

8. The compound for use in a method of treatment according to any of items 1 to 7, wherein said

10 compound of formula (I) is selected from one or more of the group consisting of oleuropein,
oleuropein aglycone (OA), decarboxymethyl oleuropein aglycone (DOA), hydroxy oleuropein
aglycone, ligstroside, ligstroside aglycone, decarboxymethyl ligstroside aglycone, and hydroxy
ligstroside aglycone.

9. The compound for use in a method of treatment according to any of items 1 to 8, wherein said

15 compound is a mTOR inhibitor and/or a DNA methyltransferase (DNMT) inhibitor, preferably a dual
mTOR - DNMT inhibitor.

10. The compound for use in a method of treatment according to any of items 1 to 9, wherein said

20 compound is selected from the group consisting of DOA and OA, or a combination thereof; preferably
wherein said compound is DOA.

11. The compound for use in a method of treatment according to any of items 1 to 10, wherein said
inhibition or reduction of cancer stem cells frequency is characterized by the suppression or reduction of
one or more of the following:

25 i. the expression of stem cell marker(s) by cancer cells;
ii. the ability of cancer cells to form spheres when grown in suspension; and
iii. the ability of cancer cells to reproduce a tumor in vivo when injected into an
immunocompromised animal.

12. The compound for use in a method of treatment according to any of items 1 to 11, wherein said
compound of formula (I), or pharmaceutically acceptable drug or prodrug thereof, is capable of
achieving selective inhibition of cancer stem cells, wherein selective inhibition is herein understood as
inhibition of the ability to form spheres when grown in suspension culture, in the absence of inhibition of
cell growth in a monolayer adherent culture.

13. The compound for use in a method of treatment according to any of items 1 to 12, wherein said cancer
is a solid tumor, preferably carcinoma, more preferably breast or ovarian carcinoma.

14. The compound for use in a method of treatment according to any of items 1 to 13, wherein said cancer is resistant to an anticancer drug or therapy.
15. A pharmaceutical composition comprising a compound as defined in any of items 1 to 10, and a pharmaceutically acceptable excipient, for use in a method of treatment according to any of items 1 to 14.

16. The compound for use in a method of treatment according to any of items 1 to 14, or the pharmaceutical composition according to item 15, wherein the method of treatment is performed in combination with another anticancer therapy; preferably wherein said anticancer therapy is selected from the group consisting of surgery, radiation therapy, chemotherapy, immunotherapy, hormone therapy, and targeted therapy.

17. The compound for use in a method of treatment according to any of items 1 to 14, or the pharmaceutical composition according to item 15, wherein the method of treatment is performed in combination with another drug.

18. The compound for use in a method of treatment or the pharmaceutical composition according to item 17, wherein said other drug is an anticancer drug.

19. A compound as defined in any of items 1 to 10, for use in a method of treating cancer wherein the method of treatment is performed in combination with another anticancer drug.

20. A pharmaceutical composition comprising a compound as defined in any of items 1 to 10, another anticancer drug, and a pharmaceutically acceptable excipient, for use in a method of treating cancer.

21. A kit for use in a method of treating cancer which comprises a dosage form of a compound as defined in any of items 1 to 10, and/or a dosage form of another anticancer drug, and instructions for the use of both drugs in combination in said method of treating cancer.

22. The compound for use in a method of treatment according to item 19, the pharmaceutical composition according to item 20 and/or the kit of parts according to item 21, wherein said other anticancer drug is selected from the group consisting of metabolic modulators, preferably a mTOR inhibitor; epigenetic modulators, preferably a DNMT inhibitor; and anthracycline antibiotics.

23. The compound for use in a method of treatment according to any of items 19 or 22, the pharmaceutical composition according to any of items 20 or 22, and/or the kit of parts according to any of items 21 or 22, wherein said other anticancer drug is selected from the group consisting of rapamycin, 5-azacytidine, and doxorubicin.

24. The compound for use in a method of treatment according to any of items 19 or 22-23, the pharmaceutical composition according to any of items 20 or 22-23, and/or the kit of parts according to any of items 21 or 22-23 wherein said cancer is resistant to said other anticancer drug and administration of said compound or pharmaceutical composition reverses the resistance.
25. A method of inhibiting, or reducing the frequency of, cancer stem cells in a subject in need thereof, comprising administering to said subject a therapeutically effective amount of a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, wherein said amount inhibits, or reduces the frequency of, cancer stem cells by at least about 50%.

26. A method of treating cancer, comprising administering to a subject in need thereof a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, in an amount effective to inhibit, or reduce the frequency of, cancer stem cells by at least about 50%.

27. A method of treating a cancer wherein said cancer is a solid tumor, comprising administering to a subject in need thereof a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, in an amount effective to inhibit, or reduce the frequency of, cancer stem cells by at least about 50%.

28. A method of inhibiting, or reducing the frequency of, cancer stem cells in a subject in need thereof, comprising administering to a subject in need thereof a therapeutically effective amount of a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof to inhibit, or reduce the frequency of, cancer stem cells by at least about 50%, in combination with another anticancer drug.

29. A method of treating cancer, comprising administering to a subject in need thereof a therapeutically effective amount of a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, in combination with a compound selected from the group consisting of a mTOR metabolic inhibitor, preferably rapamycin, a DNA methyltransferase epigenetic inhibitor, preferably 5-azacytidine, and a chemotherapeutic agent, preferably doxorubicin.

30. A method of treating cancer, comprising administering to a subject in need thereof a pharmaceutical composition comprising a synergistically effective amount of a compound of formula (I) and a compound selected from the group consisting of a mTOR metabolic inhibitor, preferably rapamycin, a DNA methyltransferase epigenetic inhibitor, preferably 5-azacytidine, and a chemotherapeutic agent, preferably doxorubicin.

31. A composition, comprising a synergistically effective amount of a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, and a compound selected from the group consisting of a mTOR metabolic inhibitor, a DNA methyltransferase epigenetic inhibitor, and a chemotherapeutic agent.

32. A method of treating a cancer resistant to an anticancer drug or therapy, comprising administering to a subject suffering from a cancer resistant to an anticancer drug or therapy effective amount of a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, wherein said amount inhibits, or reduces the frequency of, cancer stem cells by at least about 50%.
33. A method of treating solid tumors resistant to anticancer drug or therapy, comprising administering to a subject suffering from said cancer resistant to an anticancer drug or therapy a therapeutically effective amount of a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, wherein said amount inhibits, or reduces the frequency of, cancer stem cells by at least about 50%.

34. A method of reducing the frequency of cancer stem cells in a subject having a solid tumor with at least a 1% of CSCs comprising administering to a subject in need thereof a therapeutically effective amount of a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, to inhibit, or reduce the frequency of, cancer stem cells by at least about 50%.

35. A method of inhibiting, or reducing the frequency of, cancer stem cells in a subject having an increased risk of developing cancer, comprising administering to said subject a preventative or therapeutically effective amount of a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, wherein said subject has one or more risk factors selected from the group consisting of:
   a. a preliminary diagnosis of cancer but no confirmatory test,
   b. a preliminary diagnosis of cancer but no confirmatory test about the stage of cancer, and
   c. a cancer in remission.

36. A dosage form, comprising an effective amount of a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, wherein said dosage form is suitable for administration said compound in an amount effective to inhibit, or reduce the frequency of, cancer stem cells by at least about 50%.

37. A pharmaceutical composition, comprising a compound of formula (I) or a pharmaceutically acceptable salt or prodrug thereof, in an amount effective to inhibit, or reduce the frequency of, cancer stem cells in a subject in need thereof by at least about 50%, and a pharmaceutically acceptable excipient.

It is contemplated that any embodiment discussed in this specification can be implemented with respect to any medical use, pharmaceutical composition, kit, method of treatment, method of manufacturing a medicament and combination therapies of the invention, and vice versa. It will be understood that particular embodiments described herein are shown by way of illustration and not as limitations of the invention. The principal features of this invention can be employed in various embodiments without departing from the scope of the invention. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the claims.
All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The use of the word "a" or "an" may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one." The use of the term "another" may also refer to one or more. The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive.

As used in this specification and claim(s), the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, unrecited elements or method steps. As used herein, the phrase "consisting essentially of" limits the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristic(s) of the claimed invention. As used herein, the phrase "consisting of excludes any element, step, or ingredient not specified in the claim except for, e.g., impurities ordinarily associated with the element or limitation.

The term "or combinations thereof" as used herein refers to all permutations and combinations of the listed items preceding the term. For example, "A, B, C, or combinations thereof" is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is important in a particular context, also BA, CA, CB, CBA, BCA, ACB, BAC, or CAB. Continuing with this example, expressly included are combinations that contain repeats of one or more item or term, such as BB, AAA, AB, BBC, AAABCCCC, CBAAA, CABABB, and so forth. The skilled artisan will understand that typically there is no limit on the number of items or terms in any combination, unless otherwise apparent from the context.

As used herein, words of approximation such as, without limitation, "about", "around", "approximately" refers to a condition that when so modified is understood to not necessarily be absolute or perfect but would be considered close enough to those of ordinary skill in the art to warrant designating the condition as being present. The extent to which the description may vary will depend on how great a change can be instituted and still have one of ordinary skilled in the art recognize the modified feature as still having the required characteristics and capabilities of the unmodified feature. In general, but subject to the preceding discussion, a numerical value herein that is modified by a word of approximation such as "about" may vary from the stated value by at least ±1, 2, 3, 4, 5, 6, 7, 10, 12 or 15%.

EXAMPLES

EXAMPLE 1. Material and Methods

1. Isolation and purification of DOA and OA
Isolation, purification and characterization of DOA (Figs. 1A-E) and OA (Figs. 2A-E) was performed as described in Lozano-Sanchez et al. (J Agric Food Chem. 2010, 58(18):9942-9955). Semi-synthetic DOA was obtained as described in Vougogiannopoulou K et al., J Nat Prod. 2014, 77(3): 441-445.

I. 1. Chemical and reagents. All chemicals were of analytical reagent grade. Methanol and n-hexane were purchased from Merck (Darmstadt, Germany). Acetic acid was purchased from Fluka, Sigma-Aldrich (Steinheim, Germany). Double-deionized water with conductivity less than 18.2 MΩ was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). The vacuum pump used for this study was a Millipore pump model WP6222050 (Millipore, Billerica, MA, USA).

I. 2. Sample preparation. A concentrated EVOO phenolic extract of the Arbequina olive variety of Olea europaea was used in this study. The phenolic fraction was isolated from EVOO using solid-phase extraction (SPE) with Diol-cartridges (bed weight: 10 g, 600 mL tube size) following a method that was previously described by Lozano-Sanchez et al. (J Agric Food Chem. 2010, 58(18):9942-9955). Briefly, EVOO (600 g) was dissolved in n-hexane and loaded onto the column, and the cartridge was washed with 150 mL n-hexane. Finally, the sample was recovered by passing 400 mL of methanol through the column and removing the solvent using vacuum evaporation. The residue was dissolved in 5 mL of methanol and filtered through a 0.25-µm filter.

I. 3. DOA and OA Isolation and Purification. Isolation of DOA and OA from the EVOO phenolic extract was achieved using a Gilson preparative HPLC system (Gilson, Middleton, USA) equipped with a binary pump (model 331/332), automated liquid handling solution (model GX-271), and a UV-Vis detector (model UV-Vis 156). The compounds in the EVOO-PE were fractionated at room temperature. A 250 mm x 10 mm i.d. 5-µm Phenomenex RP-C18 column was used to separate the phenolic compounds. The mobile phases consisted of 0.25% acetic acid (A) and methanol (B). The following multi-step linear gradient was applied: 0 min: 5% B, 1 min: 35% B, 35 min: 45% B, 45 min: 50% B, 57 min: 55% B, 70 min: 60% B, 74 min: 95% B, 82 min: 5% B, and 85 min: 5% B. The initial conditions were maintained for 15 min. The injection volume was 200 µL. The flow rate was set at 3 mL/min. The separated compounds were monitored by UV-Vis at 240 and 280 nm. The fraction collection step consisted of mass spectrometry (MS)-based purification to determine the elution time window for collecting the target compound. MS was performed using the micro time-of-flight (TOF) mass spectrometer detector (Bruker Daltonik, Bremen, Germany) equipped with a model G1607A ESI interface (Agilent Technologies) operating in the negative ion mode. The TOF detector was coupled to the HPLC system. At this stage, the use of a makeup pump and MRA splitter (model 307, Gilson, Middleton, USA) was required for coupling with the MS detector because the flow arriving at the TOF detector had to be 0.2 mL/min to obtain reproducible results and stable spray. Finally, the target compound was collected, and the solvent was evaporated under a vacuum. The residue was weighed and dissolved with an appropriate volume of 100 µg/mL methanol. The fractions were filtered through a 0.25-µm filter before HPLC analysis.
I. 4. Analytical characterization of the isolated compounds by HPLC-DAD-ESI-TOF-MS. Analysis of the collected fraction was performed using the Agilent 1200 Series Rapid Resolution LC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, autosampler, binary pump, and diode array detector (DAD). HPLC was coupled to a TOF detector, which was equipped with a model G1607A ESI interface (Agilent Technologies) operating in negative ion mode. A 150 mm x 4.6 mm i.d., 1.8 µm Zorbax Eclipse Plus RP-C18 column (Agilent Technologies, Palo Alto, CA, USA) was used for analysis. The mobile phases included 0.25% acetic acid as eluent A and methanol as eluent B. The total run time was 27 min, and a previously reported multistep linear gradient was used (Lozano-Sanchez et al., J Agric Food Chem. 2010; 58 (18):9942-9955). The flow rate was 0.80 mL/min; consequently, the use of a splitter was required for coupling with the MS detector because the flow arriving at the TOF detector had to be 0.2 mL/min to ensure reproducible results and a stable spray. External mass spectrometer calibration was performed with sodium acetate clusters (5 mmol/L sodium hydroxide in water/2-propanol 1/1 (v/v) with 0.2% of acetic) in a quadratic p high-precision calibration (HPC) regression mode. The optimum values of the source and transfer parameters were established according to Lozano-Sanchez et al. (J Agric Food Chem. 2010; 58 (18):9942-9955). The widely accepted accuracy threshold for confirmation of the elemental composition was established at 5 ppm.

I. 5. DOA semi-synthesis. First, a sample (2 g) of Olea europaea leaf dry extract (Euromed SA) was purified by flash chromatography on silica (CH₂Cl₂:MeOH from 99:1 to 9:1) to afford oleuropein (520 mg) as a solid. TLC (silica): Rₜ 0.16 (CH₂Cl₂:MeOH 9:1; UV and p-anisaldehyde developer). Second, a mixture of oleuropein (100 mg, 0.18 mmol), NaCl (21 mg, 0.36 mmol) and water (32 mL, 1.8 mmol) in DMSO (2 mL) was heated at 150 °C in a round bottom flask provided with a reflux condenser for 10 h. After cooling, the reflux condenser was removed and the flask was connected to an oil vacuum pump provided with a cold trap. The resulting magnetically stirred mixture was heated at -50 °C under vacuum for 4-5 h to remove most of the DMSO. The residue was then purified by flash chromatography on silica (CH₂Cl₂/MeOH 80:20) to obtain DOA (1.15 mg, 0.036 mmol, 20%) as an amorphous solid. TLC (silica): Rₜ 0.30 (CH₂Cl₂/MeOH 80:20; UV and p-anisaldehyde developer).

II. Cell lines

Table 4 below provides histological and molecular classification as well as the tumor source and the percentage of CSCs according to the ALDEFLUOR™ assay (ALDH activity determination) and ability to form spheres in the sphere formation assay for each of the tested cell lines (i.e., MCF-7, MCF-7/HER2, T47D, ZR-75-1, DCIS.com and SUM-159 referred in Fig.5D). All cell lines were routinely maintained in recommended culture media.

Table 4. Characterizing properties of the tested breast cancer cell lines.

<table>
<thead>
<tr>
<th>BC Molecular Subtype</th>
<th>MCF-7</th>
<th>MCF-7/HER2</th>
<th>T47D</th>
<th>ZR-75-1</th>
<th>SKBR3</th>
<th>DCIS.com</th>
<th>MDA-MB-231</th>
<th>SUM-159</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>Luminal B</td>
<td>Luminal A</td>
<td>Luminal B</td>
<td>HER2</td>
<td>Claudin-low</td>
<td>Claudin-low</td>
<td>Luminal-low</td>
<td>Luminal-low</td>
</tr>
</tbody>
</table>
5 III. Laboratory animals and maintenance conditions

The animal experiments described in this proposal were performed on the minimal number of animals required to give statistically significant results. Appropriate licenses were obtained for the experiments involving living animals in order to comply with national regulations (Animal Use Protocol #6302 authorized by the Animals Experimental Commission form the Catalan Government). According to the ESF (European Science Foundation), our protocols endorsed the principles of the "Three Rs", Replacement, Reduction and Refinement (The Principles of Humane Experimental Technique, WMS Russel and RL Burch. http://altweb.jhsph.edu/publications/humane_exp/het-toc.htm; 1. Replacement: When it was possible, we used another biological model without the use of animals; 2. Reduction: The number of animals used in each experiment was the minimum for obtaining meaningful results. For this purpose, an expert in statistics in this field assessed each group; 3. Refinement: The protocols were designed for minimizing the degree of suffering of the animals. For this reason, procedures that caused more than momentary or minimal pain or distress in animals were performed, where appropriate with sedation, analgesia, anaesthesia or any other suitable means to reduce pain or distress, in accordance with accepted veterinary practice. Moreover, all the procedures included a severity index, where some characteristics of the animals (lose of weight, loss of movement, etc.) observed and scored to detect any pain or suffering in the animal. Researchers and other personnel involved in the design and performance of animal-based experiments had the adequate education and training, with accredited courses on laboratory animal science, including information on animal alternatives, welfare and ethics. At the end of the experiment, the animals were humanely killed in order obtain the samples necessary for the analysis. Furthermore, the care and health of the animals were under the supervision of veterinarians or specialists in the field of laboratory animals.

All experiments with animal models included in this proposal were performed at the animal facility of the IDIBELL (Instituto de Investigacion Biomedica de Bellvitge - Institute for Biomedical Research of Bellvitge), which is located in L'Hospitalet del Llobregat (Barcelona, Catalonia, Spain). The IDIBELL Animal Facility Service (Servicio de Estabulario IDIBELL) complies with state and autonomic rules for the use of animals for experimentation. The IDIBELL also has an Animal Experimentation Ethics Committee
(AEEC), which will supervise and evaluate the research procedures related to the use of animals for experiments in our project. Due to the safety conditions and its quality policy, the IDIBELL Animal Facility Service has obtained “full accreditation” from AAALAC International (Association for Assessment and Accreditation of Laboratory Animal Care International). AAALAC is a reference organisation involved in the accreditation of centres in which research is performed on animals. The IDIBELL Animal Facility Service is the only one in Spain to have obtained this international accreditation.

III. Characterization of anti-CSC activity

All compounds were prepared at 10 mmol/L in 100% DMSO, and aliquots were stored at -20°C. All compounds were diluted to the desired concentrations immediately before each experiment. Final DMSO concentration was kept below 0.1% in control and compound-treated cells.

III. 1. Mammosphere assays (culture in suspension under non-differentiating conditions) and mammosphere-forming efficiency. Mammospheres were generated using individual cells from different breast cancer cell lines seeded at 10^3 cells/cm^2 in six-well ultralow attachment plates (Corning Inc.) in sphere medium (i.e., DMEM medium containing 1% L-glutamine, 1% penicillin/streptomycin, 30% F12 (Sigma), 2% B27 (Invitrogen, Carlsbad, CA), 20 ng/ml EGF (Sigma, St. Louis, MO) and 20 ng/ml FGFb (Invitrogen, Carlsbad, CA). The medium was made semi-solid by the addition of 0.5% methylcellulose (R&D Systems, Minneapolis, MN), which prevents cell aggregation. Mammosphere-forming efficiency (MSFE) was calculated as the number of sphere-like structures (large diameter >50 m) formed in 7 days divided by the original number of cells seeded and expressed as the mean percentage (± SD). MSFEs were calculated in the absence (DOA vehicle v/v) or presence of graded concentrations of DOA or OA, as specified. Mammosphere cultures were re-fed with DOA or OA every 3 days (i.e., twice throughout each sphere formation assay).

III. 2. Metabolic status assessment (MTT-based cell viability assays) - culture in adherence under differentiating conditions-. Cell viability was determined using a standard colorimetric MTT (3-4,5-dimethylthiazol-2-yl-2, 5-diphenyl-tetrazolium bromide) reduction assay. Exponentially growing cells were harvested by trypsinization and seeding at a concentration of 2.5 x 10^5 cells/200 μL/well in 96-well plates, and they were allowed to attach overnight. The medium was then removed, and fresh medium containing various concentrations of DOA/OA was added to the cultures as specified. Control cells without DOA/OA were cultured in parallel using the same conditions with comparable media changes. Following treatment (5-7 days), the medium was removed and replaced with fresh drug-free medium (100 μL/well), and MTT (5 mg/mL in PBS) was added to each well at a 1/10 volume. After incubation for 2-3 h at 37°C, the supernatants were carefully aspirated, and 100 μL of DMSO was added to each well. The plates were agitated to dissolve the crystal product. The optical density (OD) was measured at 570 nm with a multi-well plate reader (Model Anthos Labtec 2010 1.7 reader). The cell viability effects resulting from exposure to DOA/OA were analyzed as percentages of the control cell absorbance, which was obtained from control wells treated with appropriate concentrations of the DOA vehicle that were simultaneously processed. For each treatment, cell viability was evaluated as a percentage using the following equation: (OD_{treated} sample/OD_{untreated} sample) x 100.
III. 3. ALDEFLUOR™ activity assay. The ALDEFLUOR® assay (Stem Cell Technologies) quantifies ALDH activity by measuring the conversion of the ALDH substrate BODIPY aminoacetaldehyde into the fluorescent product BODIPY aminoacetate. Briefly, SUM-159 cells were incubated in the absence or presence of DOA/OA or vehicle for 3 days with daily DOA/OA re-feeding were suspended in ALDEFLUOR assay buffer containing the fluorescent ALDH substrate BODIPY-aminoacetaldehyde (BAAA) and incubated for 45 min at 37°C. The assay buffer also contained a transport inhibitor to prevent the efflux of BAAA from the cells. BAAA passively diffuses into live cells and is then converted by intracellular ALDH into a negatively charged product (BODIPY-aminoacetate) that is retained inside cells, labeling cells with a bright fluorescent signal (Fig. 6, top; Fig. 8, top). After a washing step, brightly fluorescent ALDExpressing cells (ALDH<sup>bright</sup>) were detected in the green fluorescence channel (FL1; 520-540 nm) with a FACSCalibur instrument (BD Biosciences). A sample of cells was further stained with a specific ALDH inhibitor, diethylaminobenzaldehyde (DEAB, Sigma), to serve as a negative control for each experiment. Because only cells with intact cellular membranes can retain the ALDH1 reaction product, only viable ALDH<sup>bright</sup> cells were identified. Cells incubated with BAAA and DEAB were used to establish the background signals and define the ALDH<sup>bright</sup> region. The incubation of cells with substrate in the absence of DEAB induced a shift in the BAAA fluorescence and defined the ALDH<sup>bright</sup> population.

III. 4. Tumor xenograft studies. To produce xenografts, approximately 2 x 10^6 SUM-159 cells were subcutaneously injected into the dorsal flanks of female athymic nude mice (4-5 weeks old, 23-25 g; Harlan Laboratories). The cells were incubated in the absence (DOA vehicle v/v) or presence of DOA or vehicle for 3 days with daily DOA refeeding before injection. In both pre-treatment regimens, the body weight and diet consumption were determined weekly after dosing, tumor size was measured daily with electronic calipers, and tumor volumes were calculated using the following formula: volume (mm<sup>3</sup>) = length x width<sup>2</sup> x 0.5. The experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Institut d'Investigacio Biomedica de Bellvitge (IDIBELL; Animal Use Protocol #6302 authorized by the Animal Experimental Commission of the Catalan Government, Barcelona, Spain). The mice were euthanized by cervical dislocation.

III. 5. Tumor orthotopic studies. SUM-159 cells were cultured in non-adherent and non-differentiating conditions for 48 h. SUM-159 cells grown in mammosphere medium were collected by centrifugation, and cells that were visibly anoikis-resistant with intact plasma membranes, i.e., excluding trypan blue stain, were then treated in the absence or presence of graded concentrations of DOA for 2 h before being orthotopically implanted (5 x 10<sup>3</sup> cells) into the second right mammary fat pad of female SCID/Beige mice (Harlan Laboratories). The mice were palpated twice per week, and the tumor volumes were calculated as mentioned above.

III. 6. Synergism studies. To infer the DOA's mechanism of action (MOA) we utilized the Phenotype Microarray (PM) system, marketed and sold by Biolog (www.biolog.com), by measuring the sensitivity of CSC-enriched SUM-159 cells to a wide variety of 92 antibiotics and other growth inhibitors in microplates (PM-M11 to PM-M14). Although the PM chemical sensitivity technology has been commonly employed to analyze components of pathways that might be involved in the occurrence of resistance phenotypes, the
major role for this system is for use in drug development by interference of the MOA of a given compound. Because earlier studies revealed the importance of performing experiments using a sub-minimum inhibitory concentration (MIC), the MIC for DOA was determined using the same methodology as the Biolog experiments, as the composition of the media and the colorimetric redox-sensitive dye employed to indicate growth are different to standardized conditions. We choose a DOA concentration of 20 µM/L, which was 4 to 5 times lower than the IC_{50} value ("half maximal inhibitory concentration", i.e., the concentration needed to inhibit cell growth by half) against SUM-159 cells and consistently reduced cell growth by solely 5% in multiple independent experiments using the colorimetric redox-sensitive dye employed by the Biolog technology. On the one hand, a negative control plate set ("plus zero" controls) served as a way to assess the inherent response of CSCs-enriched SUM-159 cells to a wide variety of 92 antibiotics (4 graded concentrations/each) and other growth inhibitors irrespective of DOA. On the other hand, a positive plate set ("plus DOA") served as a way to assess the nature of the interaction between DOA and 92 different anti-cancer drugs. We grouped chemosensitivity responses into "synergistic", "additive" or "antagonistic" categories based on an arbitrarily defined ratio of observed effect/theoretical effect, the so-called fractional effect (FE) method (Li et al., Antimicrob Agents Chemother. 1993;37:523-531). Briefly, the theoretical effect of the combination was calculated by adding the effects of each antibiotic used alone at the concentration tested in the combination to that obtained when DOA was tested alone (i.e., "plus zero" control plates + effect of single agent DOA). This theoretical effect was compared with the actual effect obtained during the combinatorial experiment ("plus DOA" plates, i.e., antibiotics in simultaneous combination with DOA) carried out strictly in parallel. The different interactions were then defined as follows: "additivity" was an observed effect equal to the theoretical effect, and the ratio between them was equal to 1.0; "synergy" was an observed effect higher than the theoretical effect, and the ratio between them was less than 1.0; "antagonism" was an observed effect lower than the theoretical effect, and the ratio between them was more than 1.0. The interaction between DOA and a given antibiotic was scored as truly "synergistic" when at least two FMEs were < 1. The interaction between DOA and a given antibiotic was considered as truly "antagonistic" when at least two FMEs were > 1.0.

IV. Mechanistic studies

IV. 1. mTOR activity

IV. 1. 1. mTOR activation status: Phosphorylation of P70S6K1 at T389. SUM-159 cells grown to 80-90% confluence were exposed to 20 µM/L DOA for 0, 30, 180, and 360 minutes; alternatively, SUM-159 cells grown to 80-90% confluence were exposed to graded concentrations of DOA (5, 10 and 20 µM/L) for 1 h. Cells were then collected and subjected to standard Western blot analysis with antibodies against phospho-p70S6K1 (Thr389) and total p70S6K1 (Cell Signaling Technology). β-actin (Sigma-Aldrich) was used as loading control.

IV. 1. 2. In silico molecular modelling studies of the DOA's docking into the ATP-binding pocket of mTOR. The chemical structure of DOA was sketched in Chemdraw ultra (7.01), and saved in MDL mol file format. The binding site was generated from the cocrystallized inhibitor within PI3K-gamma protein
Docking was performed using VINA AUTODOCK. Definition of the cavity volume and cavity radius to employed 15, 25 and 35 cubic angstroms for the volume and 10 angstroms for the radius as default settings. After these docking studies to elucidate stable DOA-binding modes, values of the DOA-mTOR binding energy (AE_{bin, ding}) were calculated using molecular mechanics procedures. AE_{bin, ding} was defined as the difference between the potential energy of the DOA-mTOR (E_{mTOR, bin, plex}) and the sum of potential energies of the DOA (E_{ligand}) and mTOR (E_{mTOR, yme}): \[ \Delta E_{\text{binding}} = E_{mTOR, bin, plex} - (E_{\text{ligand}} + E_{mTOR, yme}) \]. A favorable (more negative) binding energy was taken as evidence that DOA possesses high affinity for the ATP-binding pocket of mTOR.

**IV. II. DNMT activity**

**IV. II. 1. DNMT activity/inhibition assay.** DNMT activity was evaluated in nuclear extracts using the DNMT activity/inhibition assay (Active Motif, Carlsbad, CA, USA), according to manufacturer’s instructions. Briefly, 10 µg of nuclear extracts, obtained with the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA), were incubated in presence of 20 µl/L DOA for 2 hours. Optical density (OD) was measured on a microplate reader at 450 nm, and DNMT activity (OD/h/mg) was calculated according to the following formula: DNMT activity= 1000*(average sample OD-average blank OD)/[protein amount *g]* incubation time (h)]. All samples were assayed in duplicate. As positive control, nuclear extracts were incubated in presence of 5 µl/L 5-aza.

**IV. II. 2. In silico molecular modelling studies of the DOA’s docking into the DNMT catalytic domain.** To investigate the possible inhibition mechanism of DOA to DNMT1, we used the crystal structure of mouse DNMT1 (531-1602) in the free state (PDB code:3PT9) (Song et al., Science 2011, 331 (6020):1036-1040) as the modelling template. Docking was performed using VINA AUTODOCK. Definition of the cavity volume and cavity radius to employed 15, 25 and 35 cubic angstroms for the volume and 10 angstroms for the radius as default settings. After these docking studies to elucidate stable DOA-binding modes, values of the DOA-DNMT1 binding energy (AE_{bin, ding}) were calculated using molecular mechanics procedures. AE_{bin, ding} was defined as the difference between the potential energy of the DOA-mTOR (E_{mTOR, bin, plex}) and the sum of potential energies of the DOA (E_{ligand}) and DNMT1 (E_{DNMT1, yme}): \[ AE_{\text{binding}} = E_{mTOR, bin, plex} - (E_{\text{ligand}} + E_{DNMT1, yme}) \]. A favorable (more negative) binding energy was taken as evidence that DOA possesses high affinity for the “active site” (DNA-binding) pocket of DNMT1.

**IV. 3. Transcriptional effects of DOA**

**IV. 3. 1. RNA isolation and reverse transcription.** Total RNA was extracted from cells using Qiagen miRNeasy mini kit (QIAGEN, Qiagen Iberia, S.L., Spain) according to manufacturer’s instructions. 2 µg of total RNA was reverse-transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (LifeTechnologies, Carlsbad, CA, USA) according to manufacturer’s instructions. RNA concentration and quality were determined a ND-1000 spectrophotometer (NanoDrop™ ND-1000, NanoDrop Technologies, USA).
IV. 3.2. TaqMan OpenArray. Gene expression was assessed by quantitative Real-Time PCR using the TaqMan OpenArray Human Stem Cell panel (Lifetechnologies), which contains 609 assays specific to stem cell-related genes and 22 endogenous controls for normalization. Thermal cycling and fluorescence detection were performed on an OpenArray® Real-Time PCR System (NT Cycler) (Lifetechnologies) according to user’s guide.

**EXAMPLE 2. Purified DOA reverses EMT-induced de novo acquired cancer stem cell phenotype**

HMLER\textsuperscript{shEcad} is an immortalized human mammary epithelial cell line with an acquired CSC-like phenotype further to epithelial-mesenchymal transition (EMT) induction by experimentally and stably reducing the expression of E-cadherin; and presents mammosphere initiating capacity (Gupta et al., Cell 2009, 138, 645-659). As an initial test for the effects of the purified form of DOA on the self-renewal capacity of CSC populations, the impact of DOA on the enhanced ability of HMLER\textsuperscript{shEcad} cells to form colonies in suspension culture was assayed. It was observed that HMLER\textsuperscript{shEcad} tumorsphere formation under suspension non-differentiating conditions was completely prevented with 20 µmol/L DOA (Fig. 3A-C). Thus, DOA reversed the EMT-induced de novo acquired CSC-like phenotype of HMLER\textsuperscript{shEcad} cells. Furthermore, cell viability as measured by an MTT assay on a monolayer of HMLER\textsuperscript{shEcad} cells under differentiating conditions was not significantly inhibited by exposure to DOA relative to untreated (treated with vehicle) control HMLER\textsuperscript{shEcad} cells. The lack of reduction of cell viability in the MTT assay (Fig. 3C) is indicative that DOA-mediated CSC inhibition, evidenced by the lack of sphere formation (Fig. 3A-B), cannot be attributed to a general (non-specific) inhibition of cell proliferation and survival, thus evidencing that CSCs are selectively targeted.

**EXAMPLE 3. Treatment with DOA efficiently suppresses CSCs-driven mammosphere-initiating capacity in a wide panel of human-derived breast cancer cell lines**

The MCF-7 cell line, a human breast cancer cell line with a high level of mammosphere forming cells, was used to explore the ability of DOA to inhibit mammosphere formation (Fig. 4A-B). A subset (2.2 ± 0.2%) of untreated MCF-7 breast cancer cells likewise formed bona fide mammospheres upon initial plating, thus confirming previous reports (e.g. Ciocca et al., Cell Cycle 2010, 9(14):2878-87, Iglesias et al. 2013, PLoS ONE 8(10): e77281) that MCF-7 cell cultures intrinsically contain a CSC-like population. Notably, in the presence of a gradient of micromolar concentrations of DOA, MCF-7 cells lost their ability to develop mammospheres in a dose-dependent manner with the highest dose of DOA (20 µmol/L) eliciting consistently greater inhibitory effects than the lowest dose (1 µmol/L; data not shown). Indeed, 20 µmol/L DOA dramatically suppressed the MSFE by > 90% (Fig. 4B). The amount of tight MCF-7 spheroids in the presence of DOA was drastically reduced, and the suspension cultures were characterized by a high prevalence of less rounded, irregular cell aggregates with lower size with respect to untreated cultures.

It was then examined whether DOA exposure was sufficient to prevent the well-recognized ability of the HER2 oncogene to expand the CSC population in breast carcinoma cell populations. MCF-7 cells engineered to overexpress HER2 (MCF-7/HER2 cells) exhibited a notably enhanced efficiency for forming mammospheres in suspension culture (3.6 ± 0.4%) compared with parental MCF-7 cells (Fig. 5A-
B). Notably, the strong spheroid formation capability of MCF-7/HER2 cells was completely prevented by treatment with DOA, and the DOA-mediated full suppression of MCF-7/HER2 mammosphere formation was not due to non-specific toxicity cells (Fig. 5C). Equivalent results were observed when employing SKBR3 cells, a human breast cancer cell line naturally overexpressing HER2 (data not shown).

We finally examined the effects of DOA treatment on four additional breast cancer cell lines namely, DCIS.com (a basal-like model for ductal carcinoma in situ), T47D and ZR-75-1 (two luminal breast cancer models), and SUM-159 (a highly aggressive model for claudin-low, triple-negative breast cancer (TNBC)). DOA drastically decreased the mammosphere formation ability of all cell lines relative to their respective untreated controls (Fig. 5D). Equivalent results were observed when employing MDA-MB-231 cells, another model of highly aggressive, claudin-low, TNBC (data now shown). The observed decrease in tumor sphere formation were not due to non-specific DOA toxicity because identical DOA concentrations had no impact on cell viability under adherent culture conditions (data not shown), illustrating that the mechanism of action targeted by DOA is not essential for the majority of breast cancer cells.

EXAMPLE 4. DOA treatment eliminates chemoresistant breast cancer cells expressing the CSC marker ALDEFLUOR

The drastic decrease in tumor initiation rates observed following the pre-treatment of SUM-159 cancer cell populations with DOA prior to implantation in nude mice provided preliminary insight into the mechanism of action of DOA, particularly its ability to specifically remove CSCs from the bulk population. Because previous studies revealed that only the ALDH-positive sub-population of SUM-159 cells was able to form tumors capable of serial passage in NOD/SCID mice (Korkaya et al., Oncogene. 2008, 27(47):6120-6130), the inventors hypothesized that the DOA-reduced tumorigenic potential of biologically aggressive SUM-159 cancer cell populations might be due to a DOA-induced specific suppression of chemoresistant ALDH-positive CSC-like cells.

The ALDEFLUOR™ assay quantifies ALDH activity by measuring the conversion of the ALDH substrate BODIPY aminoacetaldehyde to the fluorescent product BODIPY aminoacetate. Using flow cytometry and the ALDEFLUOR™ reagent, it was confirmed the presence of a notable ALDEFLUOR™-positive sub-population of cells within the SUM-159 cell line. Addition of the inhibitor DEAB (as a control negative assay) reduced the ALDH-dependent fluorescence, thus confirming the correct identification of ALDH-positive cells. To identify ALDEFLUOR™-positive SUM-159 cells, control (+DEAB) untreated SUM-159 cells were analyzed by flow cytometry to establish parameters for detecting only the brightest ALDH-positive cells. Using a cut-off of 0.1%, the ALDH-positive SUM-159 cell content was analyzed in a test (no DEAB) aliquot. In DOA-untreated SUM-159 cell populations, approximately 25% of cells express high ALDH activity (Fig. 6). Interestingly, in DOA-treated SUM-159 cell populations, only 3% of cells remain ALDH-positive (Fig. 6); therefore, the ALDEFLUOR™-positive cell content in DOA-treated SUM-159 cell populations drastically decreases by approximately 97% compared with untreated SUM-159 control cells.

EXAMPLE 5. Oleuropein aglycone (OA) decreases mammosphere formation in breast cancer cells
Established breast cancer cell lines have been shown to harbor a subpopulation of cells with stem cell-like properties, as defined experimentally by their functional ability to form spheroid multicellular "microtumors" under non-adherent and non-differentiating conditions (i.e., mammospheres) in vitro at markedly low frequencies (1-3%). Because the ability of certain breast cancer cells to survive and proliferate as floating mammospheres is commonly regarded as an in vitro model of the self-renewal and tumor-initiating capacities of CSCs, we first tested the ability of MCF-7 breast cancer cells to form mammospheres when grown in a suspension culture in the presence of a range of concentrations of OA (1, 5, 10, and 20 µmol/L) (Fig. 7A). The mammosphere-forming efficiency (MSFE) was calculated as the number of sphere-like structures (diameter >50 µm) divided by the original number of cells seeded and was expressed as the mean percentage (±SD). A subset (3.1 ± 0.2%) of untreated MCF-7 breast cancer cells formed typical mammospheres upon the initial plating, and this result confirmed previous reports that MCF-7 cell cultures intrinsically contain a stem cell-like population. Interestingly, the spheroid formation capability of the MCF-7 cells was significantly reduced following treatment with increasing concentrations of OA in a dose-dependent manner (-55% reduction at 20 µmol/L oleuropein; Fig. 7A). Importantly, this reduction in mammosphere formation was not due to non-specific toxicity, as MTT-based experiments of monolayer cultures of MCF-7 cells indicated that cell viability remained as high as 90% in the presence of identical micromolar concentrations of OA (data not shown).

We then examined the effects of oleuropein treatment on additional cancer cell lines such as ZR-75-1, another model of luminal breast cancer. Mammosphere formation of ZR-75-1 cells was drastically decreased by OA relative to their respective untreated controls (-65% reduction at 20 µmol/L oleuropein; Fig. 7B). Once again, none of these decreases in tumor sphere formation were due to non-specific OA toxicity against the entire breast cancer cell population. Indeed, identical concentrations of OA did not produce any drastic effects on cell viability under adherent culture conditions (data not shown), illustrating that the mechanism of action targeted by OA is not essential for the majority of non-CSC, differentiated breast cancer cells.

**EXAMPLE 6.** OA treatment eliminates the percentage of breast cancer cells expressing the CSC marker ALDEFLUOR.

The drastic decrease in mammosphere formation rates observed following the treatment of breast cancer cell populations with OA provided preliminary insight into OA's mechanism of action, particularly with regard to its ability to specifically remove CSCs from the overall population. Previous studies have revealed that only ALDH-positive (but not ALDH-negative) cells are capable of mammosphere formation and of generating mammary structures in the humanized fat pads of NOD/SCID mice (Korkaya et al., Oncogene. 2008, 27(47):6120-6130). We therefore envisioned that the OA-reduced MSFE of breast cancer cells might be due to an OA-specific suppression of ALDH-positive CSC-like cells.

Using flow cytometry and the ALDEFLUOR™ reagent, we first confirmed the presence of a notable ALDEFLUOR™-positive sub-population of cells in the SUM-159 cell line, a highly aggressive model of claudin-low, triple-negative breast cancer. Adding the inhibitor DEAB reduces ALDH-dependent
fluorescence, thereby confirming the correct identification of ALDH-positive cells. To identify the ALDEFLUOR™-positive SUM-159 cells, a control aliquot (+DEAB) of untreated SUM-159 cells was analyzed using flow cytometry, and the parameters were adjusted to detect only the brightest of the ALDH-positive cells. Using this cut-off (0.1%), the number of ALDH-positive SUM-159 cells was analyzed in the test (no DEAB) aliquot. In oleuropein-naive SUM-159 cell populations, approximately 24% of the cells expressed high ALDH activity. Interestingly, in the OA-treated SUM-159 cell populations, only 4% of the cells remained positive for ALDH (Fig. 8); therefore, the ALDEFLUOR™ positive cell population in OA-treated cultures of SUM-159 cells was drastically decreased by approximately 90% compared with the untreated SUM-159 control cells. To confirm that the OA-induced repression of ALDH-positive cells could not be attributed to non-specific cytotoxic effects, we conducted MTT-based cell viability assays under adherent culture conditions with unsorted populations of SUM-159 cells. It is worth noting that the SUM-159 cells only exhibited a cytostatic response to OA concentrations that significantly suppressed ALDH-positive CSC-like cells (data not shown).

**EXAMPLE 7. Xenotransplantation assay showing that DOA significantly reduces the tumorigenicity of cancer cells in vivo**

Because CSCs are suggested to play a key role in cancer initiation, we assessed the impact of DOA on the functional presence of CSCs by assaying their in vivo tumor-seeding ability after treatment with DOA in vitro. For these experiments, SUM-159 breast cancer cells were pre-treated with either vehicle or 50 µM OA/L DOA for 3 days with daily re-feeding of the compound (Fig. 9A). Subsequently, SUM-159 cells that were fully viable (>95%) as determined by trypan-blue exclusion were subcutaneously injected (2 x 10⁶) into the left rear flanks of female nude mice.

Examination of the palpable tumor lesions (>50 mm³) revealed that all mice injected with vehicle-pretreated SUM-159 cells formed tumors 6 weeks after injection (Fig. 9C). Compared with animals in the vehicle-pretreated group (mean xenografted SUM-159 tumor volume: 1176 mm³), animals that received DOA for 3 days immediately before the subcutaneous implantation of SUM-159 cells dramatically decreased tumor growth by more than 15 times, reaching a final mean tumor volume of 76 mm³ 64 days after injection (Fig. 9B).

None of the mice injected with the DOA-pretreated SUM-159 cells developed well-established tumors at 6 weeks, strongly suggesting that DOA might delay tumor formation by directly suppressing the tumorigenicity of SUM-159 cells; the median tumor-free survival significantly increased from 35 days for vehicle-pretreated SUM-159 cells to 64 days for DOA-pretreated SUM-159 cells (Fig. 9C). These differences remained highly significant when monitoring the time-dependent appearance of smaller tumor lesions (>10 mm³); thus, all mice injected with vehicle-pretreated SUM-159 cells formed ≥10 mm³ lesions by week 5 after injection, whereas all mice implanted with DOA-pretreated SUM-159 cells failed to develop visible tumor lesions at the same time point. In this setting, the median tumor-free survival increased 2.3-fold i.e., from 28 days for vehicle-pretreated cells to 64 days for DOA-pretreated SUM-159 cells (Fig. 9D).
EXAMPLE 8. DOA treatment suppresses the orthotopic implantation of CSC-enriched breast cancer cell populations

It was further assessed the effect of DOA pretreatment in the orthotopic implantation of low-numbers of SUM-159 cells cultured under non-adherent and non-differentiating conditions for 48 hours, a condition where most cancer cells undergo anoikis (a form of programmed cell death), and select for CSC-like cells with anchorage-independent growth ability (Fig. 10A). Thus, viable single cell suspensions (1 x 10^3 cells) obtained from SUM-159 cell mammosphere cultures were treated in the absence or presence of a DOA concentration gradient for 2 hours before injection into the mammary fat pads of SCID/Beige mice. Examination of tumor formation (lesions >50 mm^3) revealed that all of the mice injected with CSC-like SUM-159 cells formed tumors 9 weeks after injection.

Treatment of CSC-like SUM-159 single cell suspensions with the lowest dose of DOA (5 μg/mL) for 2 hours was sufficient to induce that more than half of the mice failed to develop visible tumors at the same time point; indeed, the median tumor-free survival increased 2.5-fold i.e., from 47 days for vehicle-treated CSC-like SUM-159 cells to 120 days for CSC-like SUM-159 cells pre-treated with 5 μg/mL DOA for 2 h. Strikingly, mice that were orthotopically injected with CSC-like SUM-159 cells pre-treated with either an intermediate (10 μg/mL) or the highest concentration of DOA (20 μg/mL) remained free from tumors for more than 2 months after the control arm was ended (Fig. 10B-C).

The major increase in the overall survival for mice bearing orthotopically inoculated CSC-like SUM-159 cells strongly suggests that treatment with DOA for 2 hours successfully was shown to be sufficient to fully impair cancer cells with stem-like characteristics from forming tumors in mouse mammary fat pads even though the compound is not present 4 months after injection.

EXAMPLE 10. Synergism of DOA in combination with other antitumoral drugs

A ready-to-use, highly pure DOA was rapidly and efficiently semi-synthesized by applying a Krapcho decarboxalcoholysis to perform the deglucosylation, ring opening, and removal of the carbomethoxy group of oleuropein-glycoside (Fig. 11A; see also section 15, Example 1). First, the strong anti-CSC activity of semi-synthetic DOA was confirmed in mammosphere assays using SUM-159 cells. 20 μg/mL of semi-synthetic DOA likewise reduced MSFE of SUM-159 by >95% (data not shown). Second, phenotype microarrays for mammalian cells (Biolog Inc., Hayward CA, US) were employed to simultaneously evaluate the ability of semi-synthetic DOA to interact with 92 different anti-cancer drugs (Fig. 11B). Using the TNBC SUM-159 cell model, which naturally has high levels of aldehyde dehydrogenase (ALDH)-positive chemoresistant CSC-like subpopulations, it was confirmed that DOA exerts strong synergistic effects when used in combination with the mTOR metabolic inhibitor rapamycin, with the DNA methyltransferase (DNMT) epigenetic inhibitor 5-azacytidine (5-AZA), and with the chemotherapeutic agent doxorubicin. A strong antagonistic interaction was observed when DOA was combined with the alkaloid sanguinarine. Altogether, these findings strongly suggested a dual metabolically-epigenetic activity of DOA, which likely involves a direct inhibitory binding to the ATP active site of mTOR.
 EXAMPLE 11. Anti-mTOR activity of DOA

Prompted by the observation that DOA synergistically interacted with the mTOR inhibitor rapamycin, it was hypothesized that DOA might exhibit significant inhibitory effects against mTOR activity. Early studies aimed to search for the specific rapamycin-sensitive phosphorylation site(s) on p70S6K1 revealed that, although the phosphorylation of the T389, T229, S404 and S411 residues were inhibited by the well-known mTOR inhibitor rapamycin, the loss of T389 phosphorylation was the most closely associated with the loss of p70S6K1 activation (Pearson et al., EMBO J. 1995, 14(21):5279-5287; Dennis et al., Mol Cell Biol. 1996, 16(11): 6242-6251). Indeed, the mutation of T389 to a non-phosphorylatable alanine residue (A389) has been found to completely abolish p70S6K1 activity, providing evidence that the phosphorylation of T389 by mTORC1 is a major event in the activation of p70S6K1 (Pearson et al., EMBO J. 1995, 14(21):5279-5287). Combined, these data demonstrate that the phosphorylation the T389 residue is critical for p70S6K1 function, and that the T389 residue is a major rapamycin-sensitive phosphorylation site on p70S6K1, and thus a major target of mTOR signaling. Because of the well-characterized mTOR’s role in the phosphorylation of p70S6K1, combined with the relative ease of assessing T389 phosphorylation via western blotting analysis, p70S6K1 T389 phosphorylation remains one of the most commonly used read outs of changes in mTORC1 signaling in human, animal and cell culture studies that investigate the activation of mTOR signaling. When testing the phosphorylation status of the mTOR substrate p70S6K1 in the absence or presence of DOA, the results indicated that the phosphorylation of p70S6K1 on T389 was time- and dose-dependently suppressed by DOA treatment (Fig. 12A). Particularly, at the same range of concentrations at which DOA fully suppressed mammosphere-initiating capacity of ALDH-positive CSCs in SUM-159 cells, short-term treatment with DOA led to a strong inhibition of mTOR kinase activity to an extend comparable to that achieved with rapamycin, a specific inhibitor of mTOR.

To test the hypothesis that mTOR was a direct target for DOA, we commenced by computer-aided docking of DOA into the ATP-binding pocket of a close mTOR homolog, i.e., the phosphatidylinositol 3-kinase (PI3K) catalytic subunit (PDP code: 1E8W). Because high structural similarity has been observed between the ATP-binding sites of mTOR and the catalytic domain of PI3K, particularly for the conserved residues that interact with ATP and surround the ATP-binding site (Sturgill and Hall, ACS Chem Biol. 2009, 4(12):999-1 015), we first validated our docking settings by comparing the docked pose of DOA with that of well-characterized mTOR inhibitors targeting the ATP-binding site such as LY294002, PI-103, or NVP-BEZ235. Clearly from the figure, our docking settings confirmed that DOA apparently shares the pharmacophore space utilized by potent ATP-competitive dual inhibitors of PI3K and mTOR to achieve their specificity (Fig. 12B, top). The binding pose of DOA-mTOR obtained from the docking results suggested that the binding of DOA to mTOR extended beyond the ATP site to form a good interaction with the mTOR active site (deltaG = -6 kcal/mol; usually a strong interaction between ligand and protein means around -10 kcal/mol or less in delta G), which might be the basis of the direct binding of DOA to
Mtor (Fig. 12B, bottom). In conclusion, our molecular docking experiments and in vitro cell culture assays show that DOA binds and significantly inhibits mTOR enzyme.

**EXAMPLE 12. Anti-DNMT activity of DOA**

Total DNMT activity was assessed using an ELISA-based assay. A significant reduction (approx. 50%) in enzyme activity from purified nuclear extracts was seen in the presence of DOA compared to that observed in untreated (control) cell nuclei (Fig. 13A). The DOA’s ability to reduce DNMT activity was significantly higher than that observed in the presence of 5-AZA, a well known DNMT inhibitor/hypomethylating agent that was found to reduce DNMT activity by less than 20%. To test the hypothesis that DNMTs was a direct target for DOA, we carried out computer-aided docking of DOA into the DNA-binding site of DNMT1 (PDB code: 3PT9). Computational modeling studies confirmed a high-affinity (i.e., deltaG = -8 kcal/mol; usually a strong interaction between ligand and protein means around -10 kcal/mol or less in delta G), direct inhibitory interaction of DOA with the catalytic site of the DNMT1 (Fig. 13B, top). These findings suggest that DOA can exert its inhibitory effect on DNMT1 function by blocking entry of the nucleotide cytosine into its active site and this, prevents methylation. The modeling data on the precise molecular mode of DOA’s inhibitory interaction with DNMT1 agrees perfectly with our experimental findings using a DNMT activity/inhibition assay to screen for DNMTs regulators. Beyond the direct, strong inhibitory effect of DOA on DNMT-mediated DNA methylation, it might be possible that DOA can inhibit further DNA methylation in vitro by increasing the formation of S-adenosylmethionine (a potent competitor of DNMTs) during the catechol-O-methyltransferase (COMT)-mediated methylation of DOA (Fig. 13B, bottom).

**EXAMPLE 13. DOA reduces the self-renewal capacity of CSCs**

Cancer stem cells (CSC) typically have the capacity to grow as spherical clusters of self-replicating cells and, based on this characteristic, tumoursphere-forming platforms are commonly used a sorting method to study self-renewal and tumorigenicity of CSCs. Because the number of mammospheres reflects the quantity of CSC-like cells capable of self-renewal in vitro, while the size of mammosphere measures the self-renewal capacity of each mammosphere-generating CSC-like cell, we finally took advantage of the recently developed Cell2Sphere™ kit (http://stemtektherapeutics.com/en/cell2sphere#cell2sphere _kit) to re-evaluate the impact of DOA on the size of the mammospheres formed by the BRCA1mut/PTENmul MDA-MB-436 cells, a model of highly aggressive claudin-low triple-negative (ER-/PR-/HER2-) breast cancer, and by the ER+/HER2+ p53mut/PI3KCAmut BT-474 cells, a model of the Luminal B breast cancer subtype.

The Cell2Sphere™ kit provides a simple and ready-to-use method for rapidly testing compound activity in CSC-derived 3D spheroids as a surrogate assay for tumor formation. The Cell2Sphere™ kit includes three different elements:

1. A standard low-attachment 96-well microplate that can be directly used manually or integrated into existing drug screening platforms.
2. CSCs enriched single cells in each well; the laboratory can directly add any compound to the wells to be analyzed in its capacity to alter spheroid formation as an indirect measurement of the quantity and/or self-renewal capacity of CSCs.

3. A proprietary culture medium.

By combining these three elements, the Cell2Sphere™ kit informs about the efficacy of a given compound against spheroid formation efficiency and growth. Using this approach, Cell2Sphere™ kit were performed as per manufacturer’s instructions in MDA-MB-436 and BT-474 cells. We confirmed that DOA should target signaling pathways required for the survival or self-renewal of tumor sphere-forming CSCs. DOA treatment significantly reduced the mean tumorsphere size in a dose-dependent manner compared with untreated vehicle controls, indicating the DOA’s ability to target CSCs self-renewal (Figure 14).

EXAMPLE 14. DOA potentiates the ability of anti-cancer drugs to decrease the self-renewal capacity of CSCs.

We then evaluated the DOA’s capacity to potentiate the ability of a variety of anti-cancer agents with well-characterized mechanisms of action to decrease CSCs self-renewal (Figure 15, 16; Table 5). DOA was used at a concentration of 1 µmol/L (a sub-optimal concentration to evaluate the DOA’s sensitizing efficacy, i.e., to enhance the anti-CSC therapeutic effect of other anti-cancer drugs). DOA co-supplementation was able to notably enhance the ability of the mTOR inhibitory rapamycin [100 nmol/L], the DNMT1 inhibitor 5-azacytidine [5 µmol/L], and the multi-targeted anti-folate pemetrexed (Alimta®) [500 nmol/L] to decrease the self-renewal capacity of CSCs in MDA-MB-436 (Figure 15) and BT-474 (Figure 16) models. Whereas a weak interaction was observed between DOA and the biguanide metformin [1 mmol/L], a more pronounced enhancement occurred when combining DOA with the poly(ADP-ribose) polymerase (PARP) inhibitor olaparib (Lynparza®) [5 µmol/L] in BRCA7-mutated MDA-MB-436 cells and with trastuzumab emtansine (T-DM1; Kadcyla®) [1 µg/mL] in HER2-ger® amplified BT-474 cells. Although DOA co-supplementation apparently enhanced the ability of the chemotherapeutic agents paclitaxel [50 nmol/L] and doxorubicin [100 nmol/L] to decrease the self-renewal capacity of CSCs, these findings were unreliable due to the extreme toxicity of the high doses employed (> 80% cell toxicity in parallel MTT-based cell viability experiments). We failed to observe any significant interaction between DOA and mdivi-1, [10 umol/L] an inhibitor of mitochondrial fission machinery.
Table 5.- Interaction between DOA and well-known anti-cancer drugs against CSC-derived 3D spheroids

<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug class</th>
<th>MoA*</th>
<th>Anti-CSC activity</th>
<th>MDA-MB-436</th>
<th>BT-474</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Paclitaxel</strong></td>
<td>Taxanes</td>
<td>Microtubule-targeted antimitotic drug</td>
<td>NO</td>
<td>Yes (NR)</td>
<td>Yes (NR)</td>
</tr>
<tr>
<td><strong>Doxorubicin</strong></td>
<td>Anthracyclines</td>
<td>Topoisomerase II inhibition, DNA intercalation, and free radical production</td>
<td>NO</td>
<td>Yes (NR)</td>
<td>Yes (NR)</td>
</tr>
<tr>
<td><strong>Rapamycin</strong></td>
<td>mTOR inhibitors</td>
<td>The FKBP12-rapamycin complex binds and inhibits the kinase mTOR</td>
<td>YES&lt;sup&gt;12&lt;/sup&gt;</td>
<td>Yes (strong)</td>
<td>Yes (moderate)</td>
</tr>
<tr>
<td><strong>5-Azacytidine</strong></td>
<td>DNMT inhibitors</td>
<td>DNMT1 inhibition</td>
<td>YES&lt;sup&gt;3,4&lt;/sup&gt;</td>
<td>Yes (strong)</td>
<td>Yes (strong)</td>
</tr>
<tr>
<td><strong>Metformin</strong></td>
<td>Biguanides</td>
<td>Energy stress</td>
<td>YES&lt;sup&gt;5-7&lt;/sup&gt;</td>
<td>Yes (moderate)</td>
<td>Yes (weak)</td>
</tr>
<tr>
<td><strong>Mdivi-1</strong></td>
<td>Drp1 inhibitors</td>
<td>Inhibition of mitochondrial fission</td>
<td>YES&lt;sup&gt;6&lt;/sup&gt;</td>
<td><strong>No</strong></td>
<td><strong>No</strong></td>
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<tr>
<td><strong>Pemetrexed</strong></td>
<td>Multi-targeted anti-folates</td>
<td>Inhibition of folate-dependent one-carbon enzymes</td>
<td>NO</td>
<td>Yes (moderate)</td>
<td>Yes (strong)</td>
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<tr>
<td><strong>Olaparib</strong></td>
<td>PARP inhibitors</td>
<td>Catalytic inhibition and trapping of PARP</td>
<td>YES&lt;sup&gt;9,10&lt;/sup&gt;</td>
<td>Yes (strong)</td>
<td>NE</td>
</tr>
<tr>
<td><strong>T-DM1</strong></td>
<td>HER2 antibody-drug conjugate</td>
<td></td>
<td>YES&lt;sup&gt;11,12&lt;/sup&gt;</td>
<td>NE</td>
<td>Yes (weak)</td>
</tr>
</tbody>
</table>

* MoA: Mechanism of Action; NR: Not reliable; NE: Not evaluated.

The anti-CSC activity has previously been reported for those compounds where it has been indicated “YES” under the relevant column. The corresponding scientific references are indicated below:


Virtual profiling was performed using the Ixchel® software (https://www.mindthebyte.com/saas-platform/ixchel/; Felix E. Santamaria-Navarro E, Sanchez-Martinez M and Nonell-Canals A, Ixchel®, 2015, available at www.mindthebyte.com), a structure-based software which gives a computational prediction of the biological activity of compounds allowing to understand mechanisms of action as well as side effects and toxicity.

DOA’s 3D structure was used as a docking seed and tried against a protein database composed by 2,500 different proteins and 9,000 cavities, approximately. Cavities were obtained from filtering RSCPDB (Research Collaborator for Structural Bioinformatics: Rutgers and UCSD/SDSC-Protein Data Bank; http://www.rcsb.org/pdb/home/home.do; H.M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T.N. Bhat, H. Weissig, I.N. Shindyalov, P.E. Bourne (2000) The Protein Data Bank), using all relevant crystallographic cavities (binding sites) per protein. Cavities found in different crystallographic structures were used just once.

Ixchel® is based on autodock Vina, an open-source program for doing molecular docking (http://vina.scripps.edu; O. Trott, A. J. Olson, AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading, Journal of Computational Chemistry 31 (2010) 455-461). It is well-accepted that, for dockings done with Autodock Vina, binding energies lower than -5.5/-6.0 Kcal/mol are to be trusted, and the interactions between the ligand and the cavity are highly probable to occur. For our study, a threshold of -6.0 Kcal/mol was chosen for the docking to be considered successful and trusted.

The list of epigenetic targets for which binding energies with DOA were lower than -6.0 Kcal/mol are shown in Table 6 below.

Additionally, for one of the targets identified: DNMT1, a further docking study was performed using Kin® (a blind docking software tool that combines f pocket for cavity searching and AutoDock Vina for the docking simulations; Felix E. Santamaria-Navarro E, Sanchez-Martinez M and Nonell-Canals A, Kin®, 2015, available at www.mindthebyte.com) where different cavities of the DNMT1 molecule were assessed, and results were confirmed by short (1ns) Molecular Dynamics simulations using NAMD (Phillips, J. C.; Braun, R.; Wang, W.; Gumbart, J.; Tajkhorshid, E.; Villa, E.; Chipot, C.; Skeel, R. D.; Kale, L.; Schulten, K. J. Comput. Chem. 2005, 26 (16), 1781-1 802). MD integrates the Newton’s laws of motion, constructing trajectories that allow to describe the temporal evolution of the positions and velocities of the particles of a given system, thereafter, through Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) calculations the binding free energies are estimated (using module MMPBSA.py (Miller II, B. R.; McGee Jr., T.; Swails, J. M.; Homeyer, N.; Gohlke, H.; Roitberg, A. E. J. Chem. Theory Comput. 2012, No. 8, 3314-3321 ) from Amber Tools (Case, D. A.; et al. AMBER, 2015) ; M. Karplus & A. McCammon, Molecular Dynamics simulations of Biomolecules, Nature Structural & Molecular Biology, 9, 646-652 ,2002). The data obtained were used both as positive control, and as energy value to contrast against all other binding energies obtained for dockings between DOA and its potential target.
Table 6.- Epigenetic targets selected for presenting binding energies with DOA lower than -6.0 Kcal/mol in the virtual profiling performed using the Ixchel® software

<table>
<thead>
<tr>
<th>Uniprot ID</th>
<th>Protein Name</th>
<th>PDB ID</th>
<th>Binding Energy (Kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q96EB6</td>
<td>NAD-dependent protein deacetylase sirtuin-1 (hSIRT1) (EC 3.5.1.1) (Regulatory protein SIR2 homolog 1) (SIR2-like protein 1) (hSIRT2 [Cleaved into: Sirt17 75 kDa fragment (75Sirt17)])</td>
<td>4KXQ</td>
<td>-9.2</td>
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<tr>
<td>P40261</td>
<td>Nicotinamide N-methyltransferase (EC 2.1.1.1)</td>
<td>3ROD</td>
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<td>P11086</td>
<td>Phenylethanolamine N-methyltransferase (PNMTase) (EC 2.1.1.28) (Noradrenaline N-methyltransferase)</td>
<td>20BF</td>
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<td>P80276</td>
<td>Aldose reductase (AR) (EC 1.1.1.21) (Aldehyde reductase)</td>
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<td>-8.7</td>
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<td>P51580</td>
<td>Thiopurine S-methyltransferase (EC 2.1.1.67) (Thiopurine methyltransferase)</td>
<td>2BZG</td>
<td>-8.5</td>
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<tr>
<td>P06700</td>
<td>NAD-dependent histone deacetylase SIR2 (EC 3.5.1.1) (Regulatory protein SIR2) (Silent information regulator 2)</td>
<td>4IAO</td>
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<tr>
<td>Q8NB78</td>
<td>Lysine-specific histone demethylase 1B (EC 1.1.1.1) (Flavin-containing amine oxidase domain-containing protein 1) (Lysine-specific histone demethylase 2)</td>
<td>4FWE</td>
<td>-8.3</td>
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<tr>
<td>P09874</td>
<td>Poly [ADP-ribose] polymerase 1 (PARP-1) (EC 2.4.2.30) (ADP-ribosyltransferase diphtheria toxin-like 1) (ARTD1) (NAD(+)-ADP-ribosyltransferase 1) (ADPRT 1) (Poly[ADP-ribose] synthase 1)</td>
<td>4L6S</td>
<td>-8.2</td>
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<td>P0AES6</td>
<td>DNA gyrase subunit B (EC 3.99.1.3)</td>
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<td>-8.1</td>
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<td>Histone deacetylase-like amidohydrolase (HDAC-like amidohydrolase) (HDAL) (EC 3.5.1.1)</td>
<td>2VCG</td>
<td>-8.1</td>
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<td>Q9N4R6</td>
<td>N-lysine methyltransferase SMYD2 (EC 2.1.1.1) (HSKM-B) (Histone methyltransferase SMYD2) (EC 2.1.1.43) (Lysine-N-methyltransferase 3C) (SET and MYND domain-containing protein 2)</td>
<td>3RIB</td>
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<td>P43490</td>
<td>Nicotinamide phosphoribosyltransferase (NAmPTase) (Namp1) (EC 2.4.2.12) (Pre-B-cell colony-enhancing factor 1) (Pre-B cell-enhancing factor) (Visfatin)</td>
<td>4KFN</td>
<td>-8</td>
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<td>P53686</td>
<td>NAD-dependent protein deacetylase HST2 (EC 3.5.1.1) (Homologous to SIR2 protein 2) (Regulatory protein SIR2 homolog 2)</td>
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<td>-8</td>
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<tr>
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<td>Histone-arginine methyltransferase CARM1 (EC 2.1.1.125) (Coactivator-associated arginine methyltransferase 1) (Protein arginine methyltransferase 4)</td>
<td>2Y1W</td>
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<td>Q86N77</td>
<td>NAD-dependent protein deacetylase sirtuin-6 (EC 3.5.1.1) (Regulatory protein SIR2 homolog 6) (SIR2-like protein 6)</td>
<td>3PKI</td>
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<td>Betalaine--homocysteine S-methyltransferase 1 (EC 2.1.1.5)</td>
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<td>UDP-3-O-[3-hydroxyimistryl] N-acetylglucosamine deacetylase (EC 3.5.1.1) (Protein EnvA) (UDP-3-O-acyl-GlcNAc deacetylase)</td>
<td>4FW3</td>
<td>-7.9</td>
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<td>O60341</td>
<td>Lysine-specific histone demethylase 1A (EC 1.1.1.1) (BRAF35-HDAC complex protein BHC110) (Flavin-containing amine oxidase domain-containing protein 2)</td>
<td>2IW5</td>
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<td>Histone acetyltransferase p300 (p300 HAT) (EC 2.3.1.48) (E1A-associated protein p300)</td>
<td>4PZT</td>
<td>-7.8</td>
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<td>P50135</td>
<td>Histamine N-methyltransferase (HMT) (EC 2.1.1.8)</td>
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<tr>
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<td>DNA (cytosine-5)-methyltransferase 3A (Dnmt3a) (EC 2.1.1.37) (DNA methyltransferase HsallA) (DNA MTase HsallA) (M.HsallA)</td>
<td>2QRV</td>
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<td>Q96LA8</td>
<td>Protein arginine N-methyltransferase 6 (EC 2.1.1.125) (Heterogeneous nuclear ribonucleoprotein methyltransferase-like protein 6) (Histone-arginine N-methyltransferase PRMT6) (EC 2.1.1.125)</td>
<td>4QPP</td>
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<td>Q8TEK3</td>
<td>Histone-lysine N-methyltransferase, H3 lysine-79 specific (EC 2.1.1.43) (DOT1-like protein) (Histone H3-K79 methyltransferase) (H3-K79-MTase) (Lysine N-methyltransferase 4)</td>
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<td>Q9NXA8</td>
<td>NAD-dependent protein deacetylase sirtuin-5, mitochondrial (EC 3.5.1.1) (Regulatory protein SIR2 homolog 5) (SIR2-like protein 5)</td>
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<td>-7.6</td>
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<tr>
<td>Code</td>
<td>Description</td>
<td>Value</td>
<td>Weight</td>
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<tr>
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<td>Histone-arginine methyltransferase CARM1 (EC 2.1.1.125) (Coactivator-associated arginine methyltransferase 1)</td>
<td>2V74</td>
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<td>Q9UGN5</td>
<td>Poly ADP-ribose polymerase 2 (PARP-2) (hPARP-2) (EC 2.4.2.30) (ADP-riboylltransferase diphtheria toxin-like 2) (ARTD2) (NAD+ ADP-riboyltransferase 2) (ADPRT-2) (Poly[ADP-ribose] synthase 2) (pADPR-2)</td>
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<td>Q29495</td>
<td>Serotonin N-acetyltransferase (Serotonin acetylase) (EC 2.3.1.87) (Aralylamine N-acetyltransferase) (AA-NAT)</td>
<td>1IB1</td>
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<td>UDP-3-O-[3-hydroxymyristoyl] N-acetylgalactosamine deacetylase (EC 3.5.1.1) (UDP-3-O-acyl-GlcNac deacetylase)</td>
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<td>Histone deacetylase 7 (HD7) (EC 3.5.1.98) (Histone deacetylase 7A) (HD7a)</td>
<td>3C10</td>
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<td>Q9NQR1</td>
<td>N-lysine methyltransferase SETD8 (EC 2.1.1.-) (H4-K20-HMTase SETD8) (Histone-lysine N-methyltransferase SETD8) (EC 2.1.1.43) (Lysine N-methyltransferase 5A) (PR/SET domain-containing protein 07) (PR/SET7) (PR/SET07) (SET domain-containing protein 8)</td>
<td>4IJ8</td>
<td>-7.4</td>
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<td>P56524</td>
<td>Histone deacetylase 4 (HD4) (EC 3.5.1.98)</td>
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<td>Lysine-specific demethylase 4C (EC 1.14.11.-) (Gene amplified in squamous cell carcinoma 1 protein) (GASC-1 protein) (JmJ-C domain-containing histone demethylase protein 3C) (Jumonji domain-containing protein 2C)</td>
<td>2XML</td>
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<td>P21673</td>
<td>Diamine acetyltransferase 1 (EC 2.3.1.57) (Polyamine N-acetyltransferase 1) (Putrescine acetyltransferase) (Spermidine/spermine N(1)-acetyltransferase 1) (SSAT) (SSAT-1)</td>
<td>2FXF</td>
<td>-7.3</td>
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<tr>
<td>O15550</td>
<td>Lysine-specific demethylase 6A (EC 1.14.11.-) (Histone demethylase UTX) (Ubiquitously-transcribed TPR protein on the X chromosome) (Ubiquitously-transcribed X chromosome tetrapartecopeptide repeat protein)</td>
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<td>Histone deacetylase 8 (HD8) (EC 3.5.1.98)</td>
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<td>Q92831</td>
<td>Histone acetyltransferase KAT2B (EC 2.3.1.48) (Histone acetyltransferase PCAF) (Histone acetylase PCAF) (Lysine acetyltransferase 2B) (P300/CBP-associated factor) (P/CAF)</td>
<td>1CM0</td>
<td>-7.2</td>
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<td>P28329</td>
<td>Choline O-acetyltransferase (CHOACTase) (Chat) (Choline acetylase) (EC 2.3.1.6)</td>
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<td>3BW5</td>
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<td>O60885</td>
<td>Bromodomain-containing protein 4 (Protein HUNK1)</td>
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<td>Lanosterol 14-alpha-demethylase (EC 1.14.13.70)</td>
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<tr>
<td>Q8WTS6</td>
<td>Histone-lysine N-methyltransferase SETD7 (EC 2.1.1.43) (Histone H3-K4 methyltransferase SETD7) (H3-K4-HMTase SETD7) (Lysine N-methyltransferase 7) (SET7/9) (SET domain-containing protein 7)</td>
<td>4E+47</td>
<td>-7.1</td>
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<tr>
<td>Q7Z1V1</td>
<td>Sterol 14-alpha demethylase (Tc14DM) (EC 1.14.13.70) (Cytochrome P450 51) (Lanosterol 14-alpha demethylase)</td>
<td>4BMM</td>
<td>-7</td>
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<td>P0C559</td>
<td>DNA gyrase subunit B (EC 5.99.1.3)</td>
<td>4BAE</td>
<td>-7</td>
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<tr>
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<td>Lanosterol 14-alpha demethylase (LDM) (EC 1.14.13.70) (CYPL1) (Cytochrome P450 51A1) (Cytochrome P450 14DM) (Cytochrome P45014DM) (Cytochrome P450LI) (Sterol 14-alpha demethylase)</td>
<td>3LD6</td>
<td>-7</td>
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<tr>
<td>O15054</td>
<td>Lysine-specific demethylase 6B (EC 1.14.11.-) (JmJ-C domain-containing protein 3) (Jumonji domain-containing protein 3) (Lysine demethylase 6B)</td>
<td>4ASK</td>
<td>-7</td>
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<tr>
<td>O75164</td>
<td>Lysine-specific demethylase 4A (EC 1.14.11.-) (JmJ-C domain-containing histone demethylase protein 3A) (Jumonji domain-containing protein 2A)</td>
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<td>-7</td>
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<td>Catechol O-methyltransferase (EC 2.1.1.6)</td>
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<td>-7</td>
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<td>Arylamine N-acetyltransferase (EC 2.3.1.5)</td>
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<td>Histone-lysine N-methyltransferase EHMT2 (EC 2.1.1.-) (EC 2.1.1.43) (Euchromatic histone-lysine N-methyltransferase 2) (H3-K9-HMTase 3) (H3-K9-HMTase 3) (Lysine N-methyltransferase 1C) (Protein G9a)</td>
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<td>-6.9</td>
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<tr>
<td>P0671 0</td>
<td>DNA polymerase III subunit tau (EC 2.7.7.7) (DNA polymerase III subunit gamma)</td>
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<td>-6.8</td>
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<td>Q53H47</td>
<td>Histone-lysine N-methyltransferase SETMAR (SET domain and mariner transposase fusion protein) (Mtnase) [Includes: Histone-lysine N-methyltransferase (EC 2.1.1.43); Transposon Hsmal transposase (EC 3.1.-.-)]</td>
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<td>-6.7</td>
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<tr>
<td>Q6ZMT4</td>
<td>Lysine-specific demethylase 7A (EC 1.14.11.-) (JmjC domain-containing histone demethylation protein 1D) (Lysine-specific demethylase 7)</td>
<td>3KV6</td>
<td>-6.7</td>
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<td>P26358</td>
<td>DNA (cytosine-5)-methyltransferase 1 (Dnmt1) (EC 2.1.1.37) (CXXC-type zinc finger protein 9) (DNA methyltransferase Hsal) (DNA MTase Hsal) (M.Hsal) (MCMT)</td>
<td>3SWR</td>
<td>-6.6</td>
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<td>P24752</td>
<td>Acetyl-CoA acetyltransferase, mitochondrial (EC 2.3.1.9) (Acetoacetyl-CoA thiolase) (T2)</td>
<td>2IB9</td>
<td>-6.6</td>
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<tr>
<td>Q9H9B1</td>
<td>Histone-lysine N-methyltransferase EHMT1 (EC 2.1.1.-) (EC 2.1.1.43) (Euchromatic histone-lysine N-methyltransferase 1) (Eu-HMTase 1) (G9a-like protein 1) (GLP) (GLP1) (Histone H3-K9 methyltransferase 5) (H3-K9-HMTase 5) (Lysine N-methyltransferase 1D)</td>
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<td>Q9NTG7</td>
<td>NAD-dependent protein deacetylase sirtuin-3, mitochondrial (hSIRT3) (EC 3.5.1.-) (Regulatory protein SIR2 homolog 3) (SIR2-like protein 3)</td>
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<td>-6.6</td>
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<td>Q92769</td>
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<td>-6.5</td>
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<td>Histone deacetylase 3 (HD3) (EC 3.5.1.98) (RPD3-2) (SAM45)</td>
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<td>-6.4</td>
</tr>
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<td>Q8IXJ6</td>
<td>NAD-dependent protein deacetylase sirtuin-2 (EC 3.5.1.-) (Regulatory protein SIR2 homolog 2) (SIR2-like protein 2)</td>
<td>3ZGO</td>
<td>-6.3</td>
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<td>Histone lysine demethylase PHF8 (EC 1.14.11.27) (PHD finger protein 8)</td>
<td>2WWU</td>
<td>-6.1</td>
</tr>
</tbody>
</table>
CLAIMS

1. A compound of formula (I)

\[
R^1 \begin{array}{c}
\text{O} \\
\text{O} \\
\hline
\text{A} \\
\hline
\end{array} \\
R^2
\]

wherein A is selected from the group consisting of:

\[
\begin{array}{c}
\text{R}^4 \\
\text{R}^3 \\
\text{O} \\
\text{O} \\
\hline
\text{R}^6 \\
\text{R}^5 \\
\hline
\end{array}
\]

\[
\begin{array}{c}
\text{R}^4 \\
\text{R}^3 \\
\text{OR}^6 \\
\text{O} \\
\hline
\text{R}^5 \\
\text{R}^6 \\
\hline
\end{array}
\]

wherein

R\textsuperscript{1} and R\textsuperscript{2} are independently selected from hydrogen, C\textsubscript{1}-C\textsubscript{8} alkyl, C\textsubscript{2}-C\textsubscript{8} alkenyl, C\textsubscript{2}-C\textsubscript{8} alkynyl and -OR\textsuperscript{7};

R\textsuperscript{3} is selected from hydrogen, C\textsubscript{1}-C\textsubscript{8} alkyl, C\textsubscript{2}-C\textsubscript{8} alkenyl or C\textsubscript{2}-C\textsubscript{8} alkynyl, -OR\textsuperscript{8}, -COR\textsuperscript{8}, -COOR\textsuperscript{8}, and -OCOR\textsuperscript{8};

R\textsuperscript{4} is selected from hydrogen, C\textsubscript{1}-C\textsubscript{8} alkyl, C\textsubscript{2}-C\textsubscript{8} alkenyl, C\textsubscript{2}-C\textsubscript{8} alkynyl and -COOR\textsuperscript{9};

R\textsuperscript{5} is selected from C\textsubscript{1}-C\textsubscript{8} alkyl, C\textsubscript{2}-C\textsubscript{8} alkenyl or C\textsubscript{2}-C\textsubscript{8} alkynyl, -OR\textsuperscript{10}, -COR\textsuperscript{10}, -COOR\textsuperscript{10}, and -OCOR\textsuperscript{10};

R\textsuperscript{6} is selected from hydrogen, C\textsubscript{1}-C\textsubscript{8} alkyl, C\textsubscript{2}-C\textsubscript{8} alkenyl, C\textsubscript{2}-C\textsubscript{8} alkynyl, -COR\textsuperscript{11}, and -COOR\textsuperscript{11};
each of R<sup>7</sup>, R<sup>8</sup> R<sup>9</sup>, and R<sup>10</sup> is independently selected from hydrogen, saturated or unsaturated C5-C7 cycloalkyl, saturated or unsaturated C5-C7 heterocycloalkyl, C5-C7 aryl, C5-C7 heteroaryl, C1-C8 alkyl, C2-C8 alkenyl C2-C4 alkenyl, and a protecting group of -OH;

wherein the C5-C7 cycloalkyl, C5-C7 heterocycloalkyl, C5-C7 aryl, C5-C7 heteroaryl, C1-C8 alkyl, C2-C8 alkenyl or C2-C8 alkenyl group is unsubstituted or substituted with one or more of a -OR<sup>12</sup>, -SR<sup>12</sup>, -NHCOR<sup>12</sup>, -CONHR<sup>12</sup>, -COR<sup>12</sup>, -COOR<sup>12</sup>, -OCOR<sup>12</sup>, -NR<sup>12</sup>R<sup>13</sup>, -SO<sub>2</sub>R<sup>12</sup>, -SO<sub>2</sub>NR<sup>12</sup>R<sup>13</sup>, -CF<sub>3</sub>, -OCF<sub>3</sub> or -CN, wherein R<sup>12</sup> and R<sup>13</sup> are independently of each other hydrogen, C1-C4 alkyl, C2-C4 alkenyl, C2-C4 alkenyl, and a protecting group of-SH, -OH or amino;

or a pharmaceutically acceptable salt thereof; for use in a method of selectively inhibiting cancer stem cells (CSCs),

wherein selective inhibition of CSCs refers to the inhibition of the self-renewal and tumor initiating capacity of CSCs in the absence of a significant reduction in cell viability.

2. The compound for use in a method for selectively inhibiting CSCs according to claim 1, wherein the compound of formula (I) is selected from one or more of the following enantiomeric forms:

![Chemical structure](image)

wherein

![Chemical structures](image)

3. The compound for use in a method for selectively inhibiting CSCs according to any of claims 1 or 2, wherein R<sup>1</sup> and R<sup>2</sup> are independently selected from hydrogen, C1-C4 alkyl, and -OR<sup>7</sup>; preferably wherein R<sup>7</sup> is hydrogen or C1-C4 alkyl.

4. The compound for use in a method for selectively inhibiting CSCs according to any of claims 1 to 3 wherein R<sup>3</sup> is selected from hydrogen, C1-C4 alkyl, and -OR<sup>8</sup>; preferably wherein R<sup>8</sup> is hydrogen or C1-C4 alkyl.

5. The compound for use in a method for selectively inhibiting CSCs according to any of claims 1 to 4 wherein R<sup>4</sup> is selected from hydrogen, C1-C4 alkyl and -COOR<sup>9</sup>, preferably wherein R<sup>9</sup> is hydrogen or C1-C4 alkyl.
6. The compound for use in a method for selectively inhibiting CSCs according to any of claims 1 to 5, wherein R₅ is OR\(^{10}\) and R\(^{10}\) is selected from hydrogen and a saturated or unsaturated C₅-C₆ heterocycloalkyl; preferably wherein said C₅-C₆ heterocycloalkyl is a O-cycloalkyl, more preferably a hydroxy substituted O-cycloalkyl.

7. The compound for use in a method for selectively inhibiting CSCs according to any of claims 1 to 6, wherein R¹ and R² are independently selected from hydrogen and -OH, preferably R¹ and R² are -OH; R³ is selected from -CH₃, -CH₂OH, and -OH, preferably is -CH₃; R⁴ is selected from hydrogen and -COOCH₃; and R⁵ and R\(^{10}\) are independently selected from hydrogen and D-glucose, preferably is hydrogen.

8. The compound for use in a method for selectively inhibiting CSCs according to any of claims 1 to 7, wherein said compound of formula (I) is selected from one or more of the group consisting of oleuropein, oleuropein aglycone (OA), decarboxymethyl oleuropein aglycone (DOA), hydroxy oleuropein aglycone, ligstroside, ligstroside aglycone, decarboxymethyl ligstroside aglycone, and hydroxy ligstroside aglycone.

9. The compound for use in a method for selectively inhibiting CSCs according to any of claims 1 to 8, wherein said compound is selected from the group consisting of DOA and OA, or a combination thereof; preferably wherein said compound is DOA.

10. The compound for use in a method for selectively inhibiting CSCs according to any of claims 1 to 9, wherein said inhibition of the self-renewal and tumor initiating capacity of CSCs is characterized by the suppression or significant reduction of one or more of the following:
   i. the ability of CSCs to form spheres when grown in suspension under non-differentiating conditions; and
   ii. the ability of CSCs to reproduce a tumor in vivo when injected into an immunocompromised animal.

11. The compound for use in a method for selectively inhibiting CSCs according to claim 10, wherein the ability of CSCs to form spheres measured as sphere formation efficiency is reduced at least 50%, preferably at least 65%, more preferably at least 90%.

12. The compound for use in a method for selectively inhibiting CSCs according to any of claims 1 to 11, wherein the cell viability is reduced less than 15%, preferably less than 10%, more preferably less than 5%.

13. The compound for use in a method for selectively inhibiting CSCs according to any of claims 1 to 12, wherein cell viability is determined in a monolayer adherent cell culture, preferably by an assay based on tetrazolium salts reduction, more preferably by the MTT assay.

14. The compound for use in a method for selectively inhibiting CSCs according to any of claims 1 to 13, wherein prior to inhibition, said CSCs are characterized by a stem cell marker profile ALDH\(^{+}\), CD44\(^+\)/CD24\(^{low}\) or ALDH\(^{+}\) and CD44\(^+\)/CD24\(^{low}\).
15. The compound for use in a method for selectively inhibiting CSCs according to any of claims 1 to 14, wherein said cancer is a solid tumor, preferably carcinoma, more preferably breast or ovarian carcinoma.

16. The compound for use in a method for selectively inhibiting CSCs according to any of claims 1 to 14, wherein said cancer is a hematologic tumor, preferably leukemia or lymphoma.

17. The compound for use in a method for selectively inhibiting CSCs according to any of claims 1 to 16, wherein said cancer is resistant to an anticancer drug or therapy.

18. The compound for use in a method for selectively inhibiting CSCs according to any of claims 1 to 17, wherein said compound is used in combination with another anticancer drug or therapy.

19. The compound for use in a method for selectively inhibiting CSCs according to claim 18, wherein said other anticancer drug is selected from the group consisting of metabolic modulators, preferably a mTOR inhibitor; epigenetic modulators, preferably a DNMT inhibitor; a multitargeted antifolate, a biguanide, a PARP inhibitor and an anti-HER-2 agent.

20. The compound for use in a method for selectively inhibiting CSCs according to claim 18, wherein said other anticancer drug is selected from the group consisting of rapamycin, 5-azacytidine, pemetrexed, metformin, olaparib and trastuzumab emtansine (T-DM1).

21. A pharmaceutical composition comprising a compound as defined in any of claims 1 to 9, and a pharmaceutically acceptable excipient, for use in a method for selectively inhibiting CSCs according to any of claims 1 to 20.

22. The pharmaceutical composition for use according to claim 21, wherein said pharmaceutical composition further comprises another anticancer drug.

23. The pharmaceutical composition for use according to claim 22, wherein said other anticancer drug is selected from the group consisting of metabolic modulators, preferably a mTOR inhibitor; epigenetic modulators, preferably a DNMT inhibitor; a multitargeted antifolate, a biguanide, a PARP inhibitor and an anti-HER-2 agent.

24. The pharmaceutical composition for use according to claim 22, wherein said other anticancer drug is selected from the group consisting of rapamycin, 5-azacytidine, pemetrexed, metformin, olaparib and trastuzumab emtansine (T-DM1).
FIG. 4

A

Untreated

20 μmol/L DOA

MCF-7

MCF-7

B

C

MSFE (%)

Cell Viability (% of untreated control)

DOA [μmol/L] 0 20

DOA [μmol/L] 0 20

n.s.
A

Inject cells pre-treated with DOA

Treat with DOA

2 x 10^6 cells s.c.

n = 10 (untreated)

n = 10 (pre-treated with MS-001)

Measure tumor volume

Day 0 → Day 3 → Day 75

Tumor volume (mm³)

Untreated

DOA

Time (Days)

0 0 10 20 30 40 50 60 70

0 200 400 600 800 1000 1200 1400 1600

** **
FIG. 10

A

50,000 cells CSC-like cells orthotopically implanted into the mammary fat pads of SCID Beige mice

SUM-159 CSC-like cells were not treated with graded concentrations of DOA [12.5, 25, 50 μmol/L] for 2 h before injection

Enrichment for CSC-like cellular states:
Culture of SUM-159 cells in non-adherent non-differentiating conditions (48 h)

DOA
[Diclofenac sodium form]

[Henestrol form]
FIG. 11 (cont.)

C

Antagonism

Fractional Effect (FE)

Additivity

Synergy

D1 D2 D3 D4

mTOR

Rapamycin

Antagonism

Fractional Effect (FE)

Additivity

Synergy

D1 D2 D3 D4

DNMT1

5-Azacytidine

Antagonism

Fractional Effect (FE)

Additivity

Synergy

D1 D2 D3 D4

Energetic stress
Oxidative stress
Genomic stress

Doxorubicin
mTOR catalytic domain

mTOR ATP-binding site

DOA

mTOR INHIBITORS targeting the ATP-binding site
(LY294002, PI-103, NVP-BEZ235)
FIG. 13

DNMT Activity / Inhibition Assay activity assay to screen for DNA methyltransferases

% inhibition of DNMT activity

DOA

5-Aza

Untreated

**
FIG. 13 (cont.)

SAM-dependent, DNMT-catalyzed DNA methylation
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K31/222 A61K31/225 A61K31/351 A61K31/7048 A61K45/06
ADD.

According to International Patent Classification (IPC) as to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal , CHEM ABS Data, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<tr>
<th>Category*</th>
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Date of the actual completion of the international search: 5 January 2017

Date of mailing of the international search report: 16/01/2017

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Authorized officer:
Hoff, Philippe

Further documents are listed in the continuation of Box C.

See patent family annex.
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### Categories Considered Relevant

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