SELECTIVE REMOVAL OF OCT-4 POSITIVE CELLS USING LUTEOLIN

Field of Invention

This invention relates to a composition for inhibiting the growth of cancer stem cells and pluripotent stem cells. In particular, the composition comprises luteolin. More particularly, luteolin is able to selectively remove Oct-4 positive cancer stem cells from a tumour, as well as pluripotent stem cells from a mixed pool of differentiating cells.

Background

The Stem Cell Hypothesis of Cancer Origin

Stem cells are small subsets of cells found in all multicellular organisms that can either mitotically divide to self-renew and produce more of stem cells or differentiate into mature cells of a particular tissue. Interestingly, unlike somatic cells, stem cells can self-renew for a long time. (Bose and Shenoy (2014) Stem Cell versus Cancer and Cancer Stem Cell: Intricate Balance Decides Their Respective Usefulness or Harmfulness in the Biological System. *J Stem Cell Res Ther 2014,* 4:2)

Similarly, cancer cells also have the potential to self-renew for a long time. Cancer cells are cells having unlimited proliferation potential but divide at an unregulated, fast pace leading to failure of regulation of tissue growth. In order for a normal cell to transform into a cancer cell, the genes which regulate cell growth and differentiation must be altered (Croce CM (2008) Oncogenes and cancer. *N Engl J Med 358*: 502-511). The development of cancer is a complex multi-step process that requires the accumulation of mutations resulting in a cell acquiring the essential hallmarks of cancer: evasion of apoptosis, self-sufficiency in growth signals, and insensitivity to anti-growth signals, invasive and metastatic abilities, limitless replicative potential, and sustained angiogenesis (Hanahan D, Weinberg RA. (2000) The hallmarks of cancer. *Cell. 100:* 57-70).

Given that normal adult stem cells already exhibit limitless replicative potential, it has been hypothesized that transformed stems cells may be the cells of origin for many cancers (Blanpain C, Fuchs E (2009) Epidermal homeostasis: a balancing act of stem cells in the skin. *Nat Rev Mol Cell Biol 10:* 207-217 and Kangsamaksin T, Park HJ, Trempus CS, Morris

RJ (2007) A perspective on murine keratinocyte stem cells as targets of chemically induced skin cancer. *Mol Carinog 46*: 579-584) and hence, can also be termed as cancer stem cells (CSCs).

CSCs, also known as cancer initiating cells, are defined as the fraction of cells within a tumour that are long lived and possess the potential to proliferate indefinitely. Importantly, CSCs that survive treatment could account for tumour recurrence as a result of reactivation of proliferation in surviving CSCs (Ricci-Vitiani L, Fabrizi E, Palio E, De Maria R (2009) Colon cancer stem cells. *J Mol Med (Berl) 87*: 1097-1104). Traditional chemotherapy regimens target proliferating cells, potentially missing slower dividing CSCs that must be eradicated to provide long-term disease-free survival (Visvader JE, Lindeman GJ (2008) Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer 8*: 755-768).

The idea that cancer is primarily driven by a smaller population of stem cells has important implications. For instance, many new anti-cancer therapies are evaluated based on their ability to shrink tumours, but if the therapies are not killing the CSCs, the tumour will soon grow back. Another important implication is that it is the CSCs that give rise to metastases (when cancer travels from one part of the body to another) and can also act as a reservoir of cancer cells that may cause a relapse after surgery, radiation or chemotherapy has eliminated all observable signs of a cancer.

Since cancer stem cells are the small aggressive population within the entire population of cancer cells, it is important to identify and, hence, target such population of CSC for successful cancer therapies, thereby avoiding a cancer relapse. CSCs of various origins have been reported to express different marker proteins such as Oct-4. The presence of Oct-4 can be taken as indicative of CSCs. Non-CSC tumour cells are Oct-4 low or negative (Hui Sun Leong et al. (2014) Targeting Cancer Stem Cell Plasticity Through Modulation of Epidermal Growth Factor and Insulin-Like Growth Factor Receptor Signalling in Head and Neck Squamous Cell Cancer. *Stem Cells Trans Med* published online July 14, 2014).

Oct-4 (octamer-binding transcription factor 4) is a master transcription factor which controls pluripotency and is critically involved in the self-renewal of undifferentiated embryonic stem cells. As such, it is frequently used as a marker for undifferentiated cells, including pluripotent stem cells (PSCs), which are a type of stem cells (Richards M et al. (2002) Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. *Nat Biotechnol. 20*: 933-6; Richards M et al. (2003) Comparative

evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem cells. *Stem Cells 21*: 546-56; Richards M et al. (2004) The transcriptome profile of human embryonic stem cells as defined by SAGE. *Stem Cells 22*: 51-64).

Pluripotent stem cells (PSCs) can either divide to give rise to more of PSCs, or can specialize and become any cell type. Because of this versatility, these cells have the highest potential for use to regenerate or repair diseased tissue and organs in people. Furthermore, stem cell therapy is still at the research stage, in relation to, robust and validated protocols for *in vitro* to differentiation of PSCs into various cells types of all three germ layers and their respective validation in animal disease models (Bose and Shenoy (2014) Stem Cell versus Cancer and Cancer Stem Cell: Intricate Balance Decides Their Respective Usefulness or Harmfulness in the Biological System. *J Stem Cell Res Ther 2014,* 4:2).

PSCs and CSCs have similar, unique and biased signatures of pro- and anti-apoptotic gene expression which is well described in the literature (Iacovides D et al. *Front Cell Infect MIcrobiol.* 2013 Dec 25; 3:66). In particular, the anti-apoptotic gene, BIRC5 has been found to be selectively enriched in PSCs and CSCs. Inhibitors of programmed cell death like BIRC5 that directly contribute to the aberrant prolonging of cell viability may lead to cancer by facilitating the insurgence of mutations and by promoting resistance to therapy. BIRC5 is also present in stem cells and during fetal development but is undetectable in terminally differentiated adult tissues. BIRC5 inhibition leads to selective cell death of PSCs but does not affect lineage-specific differentiation or functionality of differentiated cells. Therefore, BIRC5 antagonists have been proposed as a strategy to avoid teratoma formation for clinical application of PSCs. BIRC5 is also an important factor for CSC proliferation, renewal and dedifferentiation, and its expressions correlates with resistance to radiotherapy and chemotherapy in most human cancers (Lin Y et al. *Curr Cancer Drug Targets.* 2008 Nov; 8(7): 634-46 and Seelinger G et al. *Molecules.* 2008 Oct 22; 13(10): 2628-51). Interestingly, BIRC5 becomes prominently expressed in transformed cell lines and is highly up-regulated in all the most common human cancers of lung, colon, pancreas, prostate and breast *in vivo.*

Colorectal cancer has provided an important model to test the stem cell hypothesis of cancer origin, which suggests that cancer arises as a result of genetic aberrations in stem cells leading to deregulation of the proliferation / differentiation balance. Colorectal cancer is sustained by a rare subset of CSCs with high BIRC5 expression (Seelinger G et al. *Molecules.* 2008 Oct 22; 13(10): 2628-51). These CSCs enable the tumour to survive conventional anticancer treatments because they harbour efficient mechanisms allowing escape from apoptosis and ultimately triggering tumour recurrence. To improve therapy

outcomes, it is critical that conventional anticancer therapies be replaced with specific approaches targeting CSCs.

For the reasons above, it is advantageous to develop a strategy using drugs that inhibit BIRC5 function and expression for selectively targeting PSCs and CSCs.

Flavonoids and BIRC5

Flavonoids are polyphenols that play an important role in defending plant cells against microorganisms, insects and UV irradiation. Evidence from cell culture, animal and human population studies has also suggested that flavonoids are beneficial to general human and animal health. Flavonoids are abundant in vegetables, fruits and medicinal herbs and are part of a pool of common nutrients in vegetables that comprise of antioxidants, estrogenic regulators and antimicrobial agents. It has been noticed that flavonoids may be cancer preventive (*Sak K. Pharmacogn Rev.* 2014 Jul; 8(16): 122-46). Quercetin, which belongs to the family of flavonoids, is a known BIRC5 inhibitor in PSCs (Lee MO et al. *ProcNatlAcadSci USA* 2013 Aug 27; 110(35): E3281-90).

Since flavonoids are found commonly in human diet and quercetin, a type of flavonoid, is found to inhibit BIRC5 in PSCs, it is useful to compare the effects of quercetin against a panel of other types of flavonoids such as luteolin, to determine their ability to act as an inhibitor on the growth of CSCs and PSCs.

Summary of Invention

The present invention generally relates to a composition for treating cancer by inhibiting the growth of cancer stem cells (CSCs) and pluripotent stem cell (PSCs), the composition comprising luteolin. Particularly, the CSCs and PSCs over express Oct-4.

Preferably, the concentration of luteolin present in the composition is at least 500nM.

In a different aspect, the invention relates to a composition for treating cancer via BIRC5 inhibition in the PSCs and CSCs, the composition comprising luteolin.

In a different aspect, the invention relates to a method for the selective removal of cancer stem cells and Oct-4 positive cell population in a tumour by administering to a subject in need thereof a therapeutically effective amount of a composition comprising luteolin.

The invention also provides a method for the selective removal of undifferentiated Oct-4 positive pluripotent stem cells from a mixed pool of differentiating cells *in vitro* and *in vivo* by administering a composition comprising luteolin.

In yet another aspect, the invention provides a method for treating or preventing cancer by administering to a subject in need thereof a therapeutically effective amount of a composition comprising luteolin to prevent tumour formation by Oct-4 positive cells.

Brief Description of Figures

In order that the present invention may be fully understood and readily put into practical effect, there shall now be described by way of non-limitative examples only preferred embodiments of the present invention, the description being with reference to the accompanying illustrative figures.

In the Figures:

Figure 1 illustrates the comparative cytotoxicity data which shows luteolin to be the most potent flavonoid PSC inhibitor;

Figure 2 illustrates the comparative cytotoxicity data which shows luteolin to be the most potent flavonoid CSC inhibitor;

Figure 3 illustrates the comparative cytotoxicity data which shows luteolin to be highly specific and does not target non-PSCs and non-CSCs;

Figures 4A, 4B and 4C show the phase contrast micrographs of H1-feeder co-culture treated with different inhibitors;

Figure 5 is a bar graph showing the qPCR Oct-4 expression data for four Oct-4 expressing cell lines which are used to test the effect of luteolin;

Figure 6 is a summary table indicating luteolin to have greater potency in cell lines with greater Oct-4 expression; and

Figure 7 is a line graph showing the dose response for luteolin activity against Oct-4 expressing cell lines.

Detailed Description of the Preferred Embodiments

The present invention generally relates to a composition for treating cancer by inhibiting the growth of cancer stem cells (CSCs) and pluripotent stem cell (PSCs), the composition comprising luteolin. Particularly, the CSCs and PSCs over express Oct-4. In a preferred embodiment, it is shown that a composition comprising luteolin is the most potent flavonoid PSC and CSC inhibitor.

A non-limiting example is described below.

EXAMPLE

MATERIALS AND METHODS

1. Cell Culture Protocols

All cell lines were cultured in Dulbecco's modified Eagle's medium / Ham's F-12 medium containing GlutaMax supplemented with 10% fetal serum. Cell lines between passages 15 and 20 were used in this study. Cancer stem cell lines were obtained from Celprogen (www.celprogen.com). All other cell lines were obtained from American Type Culture Collection (ATCC).

2. Cell Viability Analysis

Cells were seeded in 96 well multi-well dish format at a density of 10,000 cells per well. Cells were washed with PBS before Resazurin™ (Sigma) solution (40μg/ml final concentration) was added to each well. Following an incubation period of 2 hours at 37°C before fluorescence readouts were obtained a Tecan™ plate reader (Emission wavelength: 590nm, Excitation wavelength: 535nm, Gain = 25). All experiments and controls were performed in quadruplicate. All flavonoid compounds were diluted in DMSO. Final DMSO concentration was fixed at 0.5% v/v.

3. Total RNA Isolation and First-Strand cDNA Synthesis

Total RNA was extracted using TRIZOL (Life Technologies) and reverse transcribed using the SuperScript IITM first-strand synthesis system (Life Technologies). Total RNA was quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) and contaminating genomic DNA removed with DNA-freeTM reagent (Life Technologies) before reverse transcription.

4. Real-time quantitative reverse transcriptase polymerase chain reaction gene expression analysis

Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis was conducted using the ABI PRISM® 7500 Fast Sequence Detection System using Predesigned Assays on Demand TaqMan™ probes and primer pairs obtained from Life Technologies. After an initial incubation step for 2 minutes at 50°C and denaturation for 10 minutes at 95°C, qRT-PCR was carried out using 40 cycles of PCR (95°C for 15 seconds, 60°C for 60 seconds). Equal amounts of input first strand cDNA were used for all qRT-PCR reactions, reactions were performed in quadruplicate, and ACTB RNA levels served as internal controls.

RESULTS AND DISCUSSION

1. Luteolin was found to be the most potent flavonoid PSC and CSC inhibitor

The Applicants conducted a comparative analysis of the effects of a panel of flavonoids with structural similarity to quercetin. The flavonoids used are luteolin, baicaelin, chrysin, galangin, and kampeferol. Non-flavonoids but known PSC inhibitors such as JC011 and PluriSIn were also tested.

With reference to the figures, Figure 1 describes comparative cytotoxicity data for a flavonoid inhibitor panel (10μM) against H1 PSC cell line (Wicell). The result shows that luteolin is the most potent flavonoid against the H1 PSC cell line.

Figure 2 describes comparative cytotoxicity data for a flavonoid inhibitor panel (10μM) against colon CSC cell line (Celprogen#36112-39). The result shows that luteolin is the most potent flavonoid against the colon CSC cell line.

Figure 3 describes comparative cytotoxicity data for a flavonoid inhibitor panel (10μM) against BJ foreskin fibroblasts. The result shows that luteolin is non-toxic to normal, untransformed BJ foreskin fibroblasts.

Figures 4A to 4C are phase contrast micrograph pictures of feeder layer supported H1 PSC colonies which have been treated with the flavonoid panel for 24 hours. Flavonoids which are toxic to PSC colonies produce a "hollowing-out" effect of the feeder layer after 24 hours. It can be seen in Figure 4C that the "hollowing-out" effect is seen most clearly when the flavonoid used is luteolin.

The comparative cytotoxicity data shows that, amongst all the agents tested, luteolin was the most potent inhibitor against PSCs and CSCs. A single exposure of PSCs and CSCs with luteolin is sufficient to eliminate PSCs and CSCs *in vitro*.

The data also shows that luteolin preferentially targets the Oct-4 positive/high cell population. Figure 5 compares the qPCR OCT4 gene expression data for the H1, NCCIT, 2102Ep & Celprogen #36112-39 cell lines. The figure shows that the H1 PSC line has the highest Oct-4 expression. The Celprogen #36112-39 colon cancer line has the lowest Oct-4 expression. In Figure 6, the summary table for flavonoid activity against four Oct-4 expressing cell lines used in our study shows that luteolin has greatest potency for the H1 cell line having the highest Oct-4 expression, while potency was reduced for the Celprogen #36112-39 colon cancer line with lowest Oct-4 expression. This is further supported by Figure 7, which represents dose response data for Luteolin activity against H1, NCCIT & Celprogen #36112-39. Luteolin is most cytotoxic to the H1 PSC line which has the highest Oct-4 expression. Conversely, Luteolin is least cytotoxic towards Celprogen #36112-39 the colon cancer stem cell line which has the lowest Oct-4 expression among the three cell lines assayed.

Since Oct-4 can be taken as indicative of CSCs, the result shows that luteolin is able to selectively remove CSCs from a tumour.

In theory, PSC-derived mixed populations can be exposed *in vitro* to luteolin for a short time, washed off, and can be further differentiated. The resulting cells may represent a safe transplantable cell source with no or minimal risk for tumour / teratoma formation. Furthermore, our data also suggests that luteolin may be used as safe and potent anti-colon CSC lead compound which could warrant further investigation. Luteolin may also potentially mediate its anti-CSC and anti-PSC activity via BIRC5 inhibition.

Our comparative data of luteolin with other published or disclosed PS inhibitors such as quercetin, JC011 and PluriSIn also indicate that luteolin is far more superior in terms of safety, potency and specificity. Luteolin has great potential to be developed into useful research tool or even into therapeutics for regenerative medicines and oncology.

CLAIMS

1. A composition for treating cancer by inhibiting the growth of cancer stem cells (CSCs) and pluripotent stem cells (PSCs), the composition comprising luteolin.

2. The composition of claim 1, wherein the CSCs and PSCs over express Oct-4.

3. The composition of claim 1, wherein luteolin inhibits CSC and PSC growth via BIRC5 inhibition.

4. A method for the selective removal of cancer stem cells and Oct-4 positive cell population in a tumour by administering to a subject in need thereof a therapeutically effective amount of a composition comprising luteolin.

5. A method for the selective removal of undifferentiated Oct-4 positive pluripotent stem cells from a mixed pool of differentiating cells *in vitro* and *in vivo* by administering a composition comprising luteolin.

6. A method for treating or preventing cancer by administering to a subject in need thereof a therapeutically effective amount of a composition comprising luteolin to prevent tumour formation by Oct-4 positive cells.

7. A method of treating cancer, the method comprising administering to a subject a composition according to claim 1.

8. The method of claim 7, wherein the cancer is lung, colon, pancreas, prostate or breast cancer associated with high BIRC5 expression.