

SENOLYTIC COMPOSITIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. provisional application No. 63/604,464 filed November 30, 2023, the entire contents of which are hereby incorporated by reference.

FIELD

[0002] The present disclosure relates to therapy-induced senescence in cancer cells and therapeutic strategies for overcoming senescence-escape and improving senolytic activity.

BACKGROUND

[0003] Cellular senescence is the irreversible arrest of cell proliferation in response to a wide range of cellular stress. Telomere shortening during replication, sustained genomic damage, oxidative stress, oncogene activation or tumour suppressor activation and other forms of stress that activate the DNA damage response pathway can induce cellular senescence. Although most of these cells have permanently lost their proliferative capacity, senescent cells have an intense paracrine activity with distinct biological effects on the surrounding cells depending on the physiological context. Although senescent cells no longer replicate, they remain metabolically active and have an immunogenic phenotype: a pro-inflammatory secretome, the up-regulation of immune ligands, a pro-survival response, promiscuous gene expression (pGE), and stain positive for senescence-associated β -galactosidase activity.

[0004] Despite the significant progression in the *in vitro* identification and investigation of senescent cancer cells, clinically applicable diagnostic tests to detect senescent cells in cytological or histological samples with high levels of confidence are still lacking. Some hallmarks of senescence observed in cell cultures, such as morphological changes or cell cycle associated proteins are difficult to detect in patient samples, due to the structure of tumours and the non-specific expression of senescence-associated proteins. Moreover, the heterogeneity of senescent phenotypes together with the intrinsic complexity of tumours further restricts the assessment of senescence *in vivo*. Although SA- β -galactosidase staining is the most widely used marker of senescence in tissue samples, it can only be detected in fresh or cryopreserved samples due to its incompatibility with paraffin fixation, which is limiting its applicability. Therefore, identifying new markers and developing the *in vivo* detection of senescent cancer cells are crucial to improve the diagnostic and therapeutic strategies used in clinical settings.

[0005] Senescent cells have been implicated in several adverse biological processes: inflammation, DNA damage, protein aggregation, fibrosis, lipotoxicity, mitochondrial dysfunction, immune cell dysfunction, and the production of reactive oxygen species (ROS), among others. However, senescence has long been considered as a defense mechanism against cancer, namely through halting cell division before uncontrolled growth occurs.

[0006] Cellular senescence was considered to function as a tumour suppressor, halting the proliferation of potentially malignant cells. However, the Senescence-Associated Secretory Phenotype (SASP) factors secreted by the senescent cells, along with the ability of cancer cells to escape from the senescence program can promote the proliferation of non-senescent tumour cells and induce the acquisition of stem cell properties. Recent studies have shown that the detrimental effects of senescence in cancer outweigh the beneficial effects, although, the impact of senescent cells in various types of tumours and the features of different senescent cell types are still not completely understood.

[0007] The induction of senescence has been detected in tumour tissues of patients with different types of cancer after receiving chemotherapy, indicating that it is a clinically relevant response to cancer therapy. Despite its potential initial benefit, chemotherapy or radiotherapy induced senescence can have a detrimental impact on cancer treatment due to the secretion of SASP or the ability of cancer cells to eventually escape from senescence and regain their proliferative capacity. It has been demonstrated that the increased expression of senescence markers in breast cancer patients is associated with tumour recurrence and poor overall survival of patients.

[0008] Senescent cells, particularly those that are developed in response to DNA-damaging therapy, are able to secrete chemo-protective SASP factors protecting neighbouring tumour cells from the same chemotherapeutic agents. Moreover, DNA damage-induced senescent tumour cells can escape from senescence, and potentially lead to a poor outcome of cancer treatment. The SASP secretome consists of different chemokines, cytokines, growth factors and proteases, with both tumour-promoting and antitumorigenic effects depending on the secreted molecules and the physiological conditions.

[0009] In most cases the prominent feature of the SASP is the ability to cause local chronic inflammation, promoting the recruitment and activity of immune cells to eliminate both senescent and cancer cells by CD4(+) T-cell-mediated adaptive immune response. The two major

inflammatory cytokines, the IL-6 and IL-8 are expressed in response to different senescence inducers, and they can directly regulate the immune response along with the other inflammatory factors. SASP factors derived from senescent tumour cells and the associated stroma can promote an immune response against senescent cancer cells *in vivo*. Moreover, inducing senescence in cancer by ionizing radiation resulted in the expression of immunostimulatory cytokines, that activated the antitumor response in mice. The beneficial effect of the SASP can be exploited to increase the cytotoxic antitumor activity of immune cells. The cancer-associated inflammation caused mainly by the secretion of IL-6 and IL-8 by senescent cancer cells, can contribute to the spread of cancer cells to distant sites via lymphovascular systems and ultimately lead to metastasis.

[0010] Cancer cells can challenge the common definition of senescence. Now, it is known that the halted proliferation of cancer cells is not always terminal, and some senescent cells can restore their proliferation capacity, implying a dynamic nature of senescence in cancer. Therefore, senescence should not always be considered as an end point in the life cycle of cancer cells, but rather as a resilient physiological state. Tumour cells are known to revert to active proliferation after a non-proliferative senescent state through a process that has been termed senescence-escape. Chemotherapy induced senescent cells can spontaneously escape from senescence and re-enter the cell cycle with an enhanced proliferation capacity and tumour-initiating potential compared to cancer cells that had never been senescent. After the termination of cancer treatment, the surviving senescent tumour cells could eventually restart their proliferation, that can possibly lead to cancer recurrence and poor outcome of cancer treatment.

[0011] What is needed, then, is a more thorough understanding of senescence and senescent escape in cancer cells. What is further needed are approaches to overcome senescence escape to improve the efficacy of senolytics. What is further needed are therapeutic strategies that overcome senescence escape in senescent cancer cells among a broad range of cancer stem cells (CSCs), with minimal or negligible impact on normal, healthy cells.

SUMMARY

[0012] In view of the foregoing background, it is an object of this disclosure to describe combinations of therapeutic agents or compounds, that may be used to reduce or minimize senescence-escape capability of senescent cancer cells and inhibit CSC propagation. It is an object of this disclosure to describe therapeutic agents for use in eradicating CSCs and senescent cells. It is an object of this disclosure to describe therapeutic agents for use in preventing and reducing the

likelihood of tumor recurrence and metastasis. It is further an object of this disclosure to describe compositions, such as pharmaceutical compositions, and methods for treating and preventing cancer, including tumor recurrence and/or metastasis. It is also an object of this disclosure to describe compositions, such as pharmaceutical compositions, and methods for senolytic therapeutic agents

[0013] The presence of senescent tumour cells and the ability of cancer cells to escape from senescence should be considered as a detrimental side-effect of cancer therapy that could potentially contribute to therapy resistance and tumour recurrence. Senolytic drugs target non-dividing senescent cells through interfering with the anti-apoptotic pathways of senescent cells, allowing the cells to undergo apoptosis. Despite the general concept that therapy-induced senescence (TIS) has a tumour-suppressive role, the presence of senescent tumour cells and the ability of cancer cells to escape from senescence could potentially lead to therapy resistance and tumour recurrence.

[0014] Although tamoxifen is a clinically relevant drug for the treatment of hormone receptor-positive breast cancer cells, in *in vitro* experiments tamoxifen treatment created a more heterogeneous population consisting of both senescent and non-senescent compared to the effect of DNA-damaging drugs. MDA-MB-231 cell line is a cellular model for triple-negative breast cancer, that is generally treated with chemotherapy, although it is not commonly used for studying TIS. One of the reasons why MDA-MB-231 cells are rarely used in senescence studies might be that they carry a mutation in the gene encoding p53, which has an important role in senescence-induction.

[0015] In view of the foregoing background, it is also an object of this disclosure to describe therapeutic compositions that may be used to reduce or minimize the accumulation of cellular senescence and inhibit senescence-escape and propagation in CSCs.

[0016] Azithromycin and certain conjugates of azithromycin have demonstrated senolytic activity. The senolytic effect of azithromycin was confirmed in senescent MCF-7 and MDA-MB-231 cells, and its inhibitory effect on autophagy was confirmed in MCF-7 cells. azithromycin treatment selectively decreased the viability of BrdU-, GEM- and PALBO-induced senescent cells in both MCF-7 and MDA-MB-231 senescence models. Senescent MCF-7 cells were able to escape after azithromycin (AZI) treatment, however, the senescence-escaping ability of AZI-treated cells was decreased by DPP4 inhibition (sitagliptin treatment).

[0017] Azithromycin is a senolytic drug, selectively targeting senescent fibroblast cells, however its effect has not been previously demonstrated in senescent cancer cells. To test its senolytic effect in senescent cancer cells as well, senescent MCF-7 cells were treated with azithromycin for 72 hours using the concentration of 50 μ M, 75 μ M and 100 μ M, based on the concentrations that had been used to target senescent fibroblasts. Fig. 1 illustrates the results of cell viability in MCF-7 cells following the azithromycin treatment. As shown, azithromycin concentration-dependently decreased the viability of senescent MCF-7 cells. At lower concentrations (50 μ M) it had a significant effect only on GEM-induced senescent cells, but not on BrdU- and PALBO-induced senescent cells. Although azithromycin decreased the viability of non-senescent MCF-7 and MDA-MB-231 cells as well, it showed no toxicity in normal epithelial and fibroblast cells.

[0018] After azithromycin treatment, a population of senescent MCF-7 cells remained alive, indicating resistance to azithromycin treatment, and in addition, they were able to escape senescence and continue to proliferate. Because the antibiotic activity of azithromycin is based on its ability to interfere with bacterial protein synthesis by the inhibition of the 50S subunit of bacterial ribosome, it can also inhibit the large subunit of mitochondrial ribosome, thus disrupting mitochondrial activity in cancer cells. Targeting metabolism in senescent cells increases their susceptibility to autophagy inhibition, which for example has been demonstrated by using a mitochondrial inhibitor (oleanolic acid) combined with an autophagy inhibitor (chloroquine), resulting in increased apoptosis in senescent cancer cells compared to using chloroquine alone.

[0019] The cell surface protein DPP4 (Dipeptidyl peptidase-4; CD26) is a marker of cellular senescence in cancer. Cell surface expression of DPP4/CD26 was highly increased in senescent MCF-7 and MDA-MB-231 cells, however, its expression was not essential for senescence-induction. The silencing or inhibition of DPP4 resulted in decreased senescence-escaping ability of MCF-7 cells and increased/unchanged senescence-escaping ability of senescent MDA-MB-231 cells. Senescence-escape increased the migration capacity of MCF-7 cells, but not MDA-MB-231 cells.

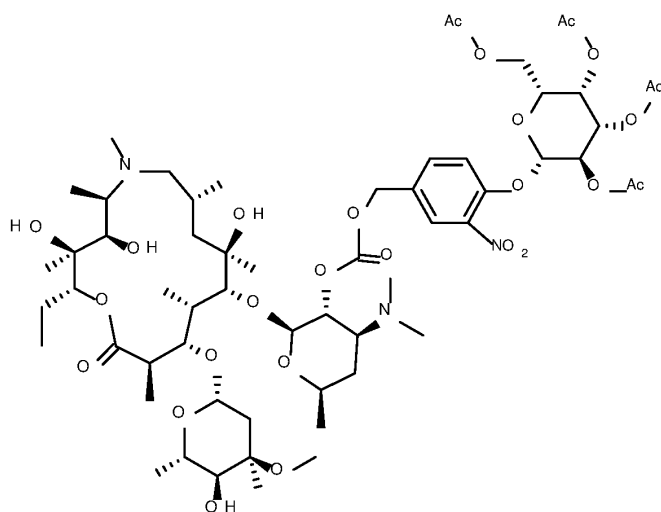
[0020] According to the previously described results, a small number of MCF-7 cells were able to survive after azithromycin treatment and resumed proliferation by escaping senescence. Fig. 3 shows the cell viability of control, BrdU-, GEM- and PALBO-induced senescent MCF-7 cells measured by SRB assay after 3 days and 5 days of treatment with azithromycin. Experiments

were repeated three times with six technical replicates, values were normalised respectively to vehicle-treated (DMSO) control BrdU-, GEM- and PALBO-induced senescent cells. The senescence-escaping cells were targeted using sitagliptin, an inhibitor of DPP4, to potentiate the senolytic effect of azithromycin in MCF-7 cells. Results demonstrated a synergistic effect of the combination treatment with azithromycin and sitagliptin, resulting in a significant reduction of senescence-escaped cells.

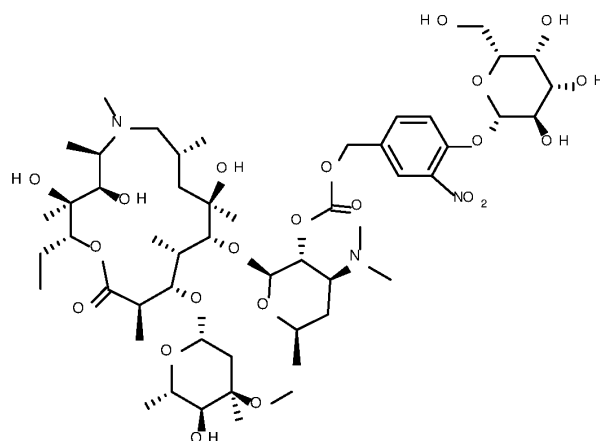
[0021] Fig. 4 shows the results of the combination of azithromycin (AZI) and sitagliptin (SITA) on senescence escape in MCF-7 cells. The senescence-escaping ability of the cells were assessed by the combination of Ki-67 expression and cell concentration represented as the number of Ki-67 positive cells. Values were normalised to the vehicle-treated cells. Bar graphs represent the mean of three independent experiments $\pm$ SEM. Statistical significance (in relation to control): ns $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

[0022] Altogether these results indicate that DPP4 inhibition could improve the effect of senolytics, by decreasing the senescence-escaping ability of the surviving cells, however, the distinct role of DPP4 in different types of cancer cells and its role in the inactivation of several chemokines could limit the application of DPP4 inhibitors in cancer therapy.

[0023] The present approach uses the synergy between inhibiting senescence escaping behavior and senolytics to inhibit the propagation of senescent cells and cancer stem cells. In some embodiments, the present approach takes the form of a composition comprising a dipeptidyl peptidase-4 (DPP-4) inhibitor and a senolytic selected from one of azithromycin, Compound [I], and Compound [II]:



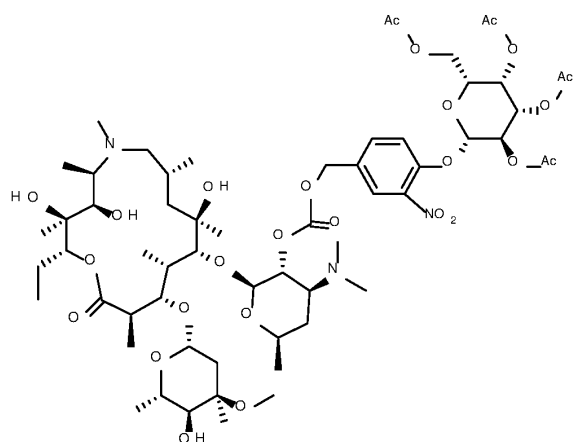
[I]



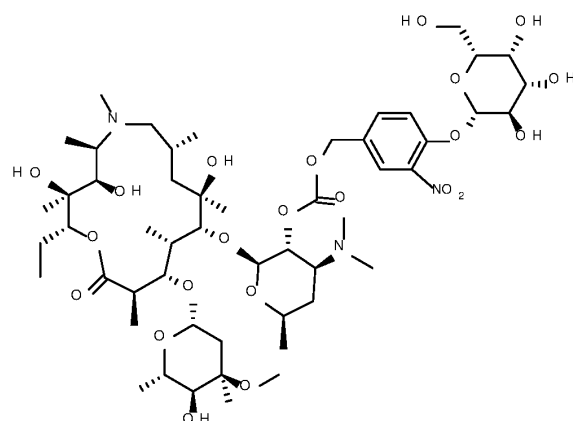
[II]

, or a pharmaceutically acceptable salt thereof. The DPP-4 inhibitor may be selected from sitagliptin, saxagliptin, linagliptin, alogliptin, and vildagliptin.

[0024] In some embodiments, the present approach takes the form of a pharmaceutical composition comprising a pharmaceutically effective amount of a dipeptidyl peptidase-4 (DPP-4) inhibitor and one of azithromycin, Compound [I], and Compound [II]:



[I]



[II]

, or a pharmaceutically acceptable salt thereof, and at least one pharmaceutically acceptable carrier. In some embodiments, the DPP-4 inhibitor is present at $100 \text{ mg} \pm 10 \text{ mg}$, and the one of azithromycin, Compound [I], and Compound [II] is present at $500 \text{ mg} \pm 50 \text{ mg}$. Some embodiments take the form of a method for treating or preventing tumor recurrence and/or metastasis. Some embodiments may take the form of methods for treating cancer. Some embodiments may take the form of methods for eradicating senescent cells. Some embodiments may take the form of methods for minimizing the accumulation of senescent cells in a subject. These methods involve administering to a patient at risk of tumor recurrence and/or metastasis a pharmaceutically effective amount of a composition having a DPP-4 inhibitor and one of azithromycin, Compound [I], and Compound [II]. Some embodiments may be for inhibiting the propagation of cancer stem cells in a patient. It should be appreciated that the administering of the

composition in these methods may be performed at least one of prior to a cancer treatment, with a cancer treatment, and following a cancer treatment.

DRAWINGS

[0025] Fig. 1 illustrates the results of cell viability in MCF-7 cells following the azithromycin treatment.

[0026] Fig. 2 illustrates toxicity of azithromycin was tested on MCF-10A non-malignant breast epithelial cells, showing that azithromycin treatment is not toxic to normal epithelial cells, and a reduction of cell viability was only detected at higher ($\geq 750 \mu\text{M}$) concentrations.

[0027] Fig. 3 shows the cell viability of control, BrdU-, GEM- and PALBO-induced senescent MCF-7 cells measured by SRB assay after 3 days and 5 days of treatment with azithromycin.

[0028] Fig. 4 shows the results of the combination of azithromycin (AZI) and sitagliptin (SITA) on senescence escape in MCF-7 cells.

[0029] Fig. 5 shows the impact of azithromycin treatment on senescence-escaped cells.

[0030] Fig. 6 shows the expression of Ki-67 represented as the percentage of positively stained cells compared to the total population.

[0031] Figs. 7A-7C illustrate the effect of DPP4 silencing on the senescence-escape of MCF-7 cells, with Fig. 7A comparing cell numbers for BrdU- and GEM-escaped siRNA cells, Fig. 7B shows the number of Ki-67 positive cells for BrdU- and GEM-escaped siRNA cells, and Fig. 7C shows colony formation assay results for BrdU- and GEM-escaped siRNA cells.

[0032] Figs. 8A and 8B show cell viability data of MCF-10A, MCF-7 cells and BrdU-, GEM- and PALBO-induced senescent MCF-7 cells, respectively.

[0033] Figs. 9A-9C illustrate the senescence-escaping ability of the cells by the combination of Ki-67 expression and cell concentration represented as the number of Ki-67 positive cells for BrdU-, GEM-, and PALBO- induced cells, respectively.

[0034] Figs. 10A and 10B show the effect of azithromycin and chloroquine in MDA-MB-231 cells, respectively.

[0035] Fig. 11A shows the expression of Ki-67 as the percentage of positively stained cells compared to the total population. Fig. 11B shows cell concentrations measured by flow cytometry using the samples from the measurement of Ki-67 expression represented as count/ μl (cell number/ μl). Fig. 11C shows the senescence-escaping ability of the cells were assessed by the

combination of Ki-67 expression and cell concentration represented as the number of Ki-67 positive cells. Fig. 11D shows Ki-67-positive cells treated at different concentrations of BrdU, and Fig. 11E shows Ki-67-positive cells treated at different concentrations of GEM.

[0036] Fig. 12 compares SRB assay results for different concentrations of azithromycin (100 μ M and 50 μ M) and Compound [I].

[0037] Fig. 13 shows xCELLigence data comparing the effect of Compound [I] of the present approach on control MRC-5 cells and MRC-5 cells treated with BrdU.

[0038] Figs. 14A and 14B are images of MRC-5 fibroblasts without and with BrdU pre-treatment, respectively.

[0039] Fig. 15A shows mammosphere formation assay results for MCF-7 cells treated with Compound [I], and Fig. 15B shows mammosphere formation assay results for MCF-7 cells treated with azithromycin.

[0040] Fig. 16 shows SRB assay results for Compound [III] on both control MRC5 cells and MRC5 cells treated with BrdU.

DETAILED DESCRIPTION

[0041] The following description illustrates embodiments of the present approach in sufficient detail to enable practice of the present approach. Although the present approach is described with reference to these specific embodiments, it should be appreciated that the present approach can be embodied in different forms, and this description should not be construed as limiting any appended claims to the specific embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the present approach to those skilled in the art.

[0042] This description uses various terms that should be understood by those of an ordinary level of skill in the art. The following clarifications are made for the avoidance of doubt.

[0043] The term “cancer” refers to physiological conditions in mammals that are typically characterized by uncontrolled cell growth. This definition includes benign and malignant cancers. Examples of cancers include cancer types, lymphomas, blastomas (including medullablastomas and retinoblastomas), sarcomas (including liposarcomas and synovial sarcomas), neuroendocrine tumors (carcinoid tumors, gastrin production Includes, but is not limited to, tumors and islet cell carcinomas), sarcomas, Schwannomas (including acoustic neuroma), medullary carcinomas, adenocarcinomas, melanomas, and leukemia or lymphocyte tumors. Specific examples of cancers

include bladder cancer, squamous cell carcinoma, small cell lung cancer, non-small cell lung cancer, lung adenocarcinoma, and lung cancer including squamous epithelial cancer of the lung, peritoneal cancer, hepatocellular carcinoma, gastric cancer including gastrointestinal cancer or stomach cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, liver cancer, breast cancer (including metastatic breast cancer), colon cancer, rectal cancer, colon rectal cancer, endometrial cancer or uterine cancer, salivary adenocarcinoma, kidney cancer (kidney cancer) or kidney cancer (renal cancer), prostatic cancer, genital cancer, thyroid cancer, liver cancer, anal cancer, penis cancer, testicular cancer, esophageal cancer, bile duct tumor, and head and neck cancer and multiple myeloma.

[0044] As used herein, the term “senolytic” refers to a therapeutic compound or drug that selectively target and kill senescent cells, which are cells that have stopped dividing. Generally, senolytics are small molecules that induce apoptosis in senescent cells, but not in non-senescent cells. Reducing senescent cell populations in mouse models resulted in numerous health benefits, including improvements in cardiac function, vascular function, and insulin sensitivity, reductions in vessel calcification, increases in vessel sensitivity to neurotransmitters, and relief from various age and obesity-related symptoms. Senolytic treatment appears to improve neurological and psychological dysfunction, as seen through reducing brain inflammation and anxiety in obese mice, and alleviating Alzheimer's disease-associated changes.

[0045] As used herein, the term “tumor” refers to the growth and proliferation of neoplastic cells, whether malignant or benign, including pre-cancerous and cancerous cells and tissues.

[0046] The term “metastasis” refers to the spread of cancer from its primary site to other parts of the body. Cancer cells can escape from the primary tumor, penetrate lymph vessels and blood vessels, circulate through the bloodstream, and grow or “metastasize” in distant lesions in normal tissue elsewhere in the body. Metastases can be local or distant. Metastasis is a sequential process that requires tumor cells to escape from the primary tumor, travel through the bloodstream, and stop at distant sites. At this new site, cells can establish a blood supply and grow to form a life-threatening mass. Both irritating and inhibitory molecular pathways within tumor cells control this behavior, and the interaction between tumor cells and host cells at distant sites is also important.

[0047] The terms “treat,” “treated,” “treating,” and “treatment” include the diminishment or alleviation of at least one symptom associated or caused by the state, disorder or disease being treated, in particular, cancer. In certain embodiments, the treatment comprises diminishing and/or

alleviating at least one symptom associated with or caused by the cancer being treated, by the compound of the invention. In some embodiments, the treatment comprises causing the death of a category of cells, such as senescent cells, SASP cells, or CSCs likely to be involved in metastasis or recurrence, of a particular cancer in a host, and may be accomplished through preventing senescent cells and/or cancer cells from further propagation, and/or inhibiting CSC function through, for example, depriving such cells of mechanisms for generating energy. For example, treatment can be diminishment of one or several symptoms of a cancer, or complete eradication of a cancer. As another example, the present approach may be used to inhibit mitochondrial metabolism in the cancer, eradicate (e.g., killing at a rate higher than a rate of propagation) CSCs in the cancer, eradicate TICs in the cancer, eradicate circulating tumor cells in the cancer, inhibit propagation of the cancer, target and inhibit CSCs, target and inhibit TICs, target and inhibit circulating tumor cells, prevent or reduce the likelihood of, metastasis, prevent recurrence, sensitize the cancer to a chemotherapeutic, sensitize the cancer to radiotherapy, sensitize the cancer to phototherapy. As another example, the treatment can reduce the accumulated senescent cells, and/or reduce the rate of senescent cell accumulation.

[0048] In the context of tumor recurrence and/or metastasis, the term “prevent” and “reduce the likelihood of” refer to reducing, in a subject, the presence of CSCs, TICs, and circulating tumor cells, likely to be involved in recurrence or metastasis, to a level at which tumor recurrence and/or metastasis from the primary site is unlikely, relative to a control (i.e., no treatment to prevent or reduce the likelihood of tumor recurrence and/or metastasis). In practice, a treatment to prevent and/or reduce the likelihood of tumor recurrence and/or metastasis as described herein targets and inhibits or eradicates CSCs, TICs, inhibit circulating tumor cells.

[0049] The terms “cancer stem cell” and “CSC” refer to the subpopulation of cancer cells within tumors that have capabilities of self-renewal, differentiation, and tumorigenicity when transplanted into an animal host. Compared to “bulk” cancer cells, CSCs have increased mitochondrial mass, enhanced mitochondrial biogenesis, and higher activation of mitochondrial protein translation. As used herein, a “circulating tumor cell” is a cancer cell that has shed into the vasculature or lymphatics from a primary tumor and is carried around the body in the blood circulation. The CellSearch Circulating Tumor Cell Test may be used to detect circulating tumor cells.

[0050] The phrase “pharmaceutically effective amount,” as used herein, indicates an amount necessary to administer to a host, or to a cell, tissue, or organ of a host, to achieve a therapeutic result, such as regulating, modulating, or inhibiting protein kinase activity, e.g., inhibition of the activity of a protein kinase, or treatment of cancer. A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required for a given subject, using methods well-known and available in the art. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. The determination of a pharmaceutically effective amount is deemed to be within the purview of the person having an ordinary level of skill in the art, having reviewed this disclosure.

[0051] The phrase “pharmaceutically acceptable carrier” as used herein, means a pharmaceutically acceptable material, composition, or vehicle, such as a liquid or solid filler, diluent, excipient, solvent, or encapsulating material. Each carrier must be “acceptable” in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

[0052] The present approach is not limited to the particular formulation of a given embodiment. Example formulations are as follows. Sitagliptin may come in the form of a film-coated tablet having sitagliptin phosphate monohydrate, microcrystalline cellulose, anhydrous calcium hydrogen phosphate, croscarmellose sodium, magnesium stearate, and sodium stearyl fumarate. Azithromycin and the azithromycin conjugates described herein may be provided in

formulations such as tablets (250 mg, 500 mg), packets (1 gram dissolved in ¼ cup or 60 ml of water), and suspension for reconstitution (100 mg/5 ml, 200 mg/5 ml).

[0053] The phrase “pharmaceutically acceptable salt” to the relatively non-toxic, inorganic and organic base addition salts of the compounds of the present approach. A pharmaceutically acceptable salt may be formed by, for example, reacting a compound in its free acid form with a base, such as hydroxide or carbonate of a pharmaceutically-acceptable metal cation, with ammonia or with a pharmaceutically-acceptable amine. Representative alkali or alkaline earth salts include sodium, potassium, calcium, magnesium, and aluminum salts, for example. Examples of amines that may be used for base addition salts include ethylamine, diethylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine. It should be appreciated that other salts may be used, of course, and that the person of ordinary skill in the art may use methods known in the art for identifying suitable salt forms, without departing from the present approach.

[0054] The emergence of senescent tumour cells following neoadjuvant chemotherapy has been observed in patients with different types of cancer. Patients with therapy induced senescent tumours showed a significant decrease in survival and the symptoms of accelerated physiological aging. Increased numbers of senescent tumour cells correlated with adverse clinical outcome and resistance to first- and second-line treatments.

[0055] Chemotherapies can induce cellular senescence in the surrounding stroma and in normal tissues as well, which can be responsible for debilitating side effects and the development of local and systemic inflammation. Furthermore, in a murine model, chemotherapy induced senescent non-tumour cells promoted the spread of cancer cells and led to cancer relapse, in response to a variety of DNA-damaging agents administered at a biologically effective dose. The elimination of therapy-induced senescent cells reduced the adverse side-effects of chemotherapy, suggesting that development of drugs to remove senescent cells might be a promising strategy to improve the outcome of current chemotherapies and to alleviate the post-therapeutic morbidities connected to physiological aging.

[0056] Senolytics are compounds that have been developed to selectively eliminate senescent cells without compromising the viability of non-senescent cells (Kirkland and Tchkonja, 2020). Using senolytics as adjuvant cancer therapy can be beneficial to improve therapeutic outcome and reduce the risk of tumour recurrence. Two well-known antibiotics, azithromycin and

roxithromycin have been identified as novel senolytic drugs targeting senescent fibroblast cells *in vitro*, indicating a promising approach to selectively eliminate senescent cells.

[0057] Senescence-targeted therapies represent a novel strategy to increase the senolytic effects and decrease the potential side-effects of senolytic drugs by selectively targeting the senescent cells based on the expression of a senescence marker. For example, a newly identified senescence marker, the cell surface protein DPP4/CD26, is a promising senescence-specific target for anti-DPP antibody-directed immunotherapy and for enhancing the selectivity of the previously described senolytics. DPP4 selectively sensitized senescent fibroblasts to cytotoxicity by natural killer cells.

[0058] Dipeptidyl peptidase IV (DPP4/CD26) is a transmembrane glycoprotein with serine exopeptidase activity capable of cleaving polypeptides, by removing the N-terminal dipeptides from several peptides preferably after proline or alanine. The expression of DPP4/CD26 has been investigated in various types of cancer, however, it shows a high diversity of expression and function in different cancer cell type. Due to its complex biological functions, DPP4/CD26 has been described as a tumour promoter and tumour suppressor as well, depending on tumour localisation, cell type and tumour microenvironment. The role of DPP4/CD26 in tumour progression can be attributed either to its enzymatic activity, or its connection to extracellular matrix proteins and cell adhesion proteins, as well as its immunologic function. It has been suggested that the role of DPP4/CD26 in the metastasis of breast cancer is connected to its extracellular matrix binding ability, which can increase the adhesion of breast cancer cells, leading to the formation of metastasis.

[0059] DPP4/CD26 can function as a tumour suppressor or tumour promoter in different tumour types, however, in certain types of cancer both functions has been reported. For example, non-small lung cancer cells present lower expression of DPP4/CD26 compared to normal lung epithelial cells, however, a significantly higher expression of DPP4/CD26 is present in lung adenocarcinoma samples compared to normal tissues. Moreover, the DPP4-inhibitor vildagliptin has been demonstrated to reduce lung cancer growth possibly via the regulation of macrophage and NK cell activity, and DPP4-inhibition was associated with improved overall survival of lung cancer patients. Similarly to these observations, DPP4/CD26 is expressed in normal endometrial cells and down-regulated in endometrial adenocarcinoma cells, although, DPP4 activity has also been associated with increased proliferation and tumorigenesis of endometrial carcinoma cells.

Although DPP4/CD26 has been described as a prognostic marker in different types of cancer and a marker of cancer stem cells in colorectal cancer, DPP4/CD26 has yet to be used as a therapeutic target in cancer treatment.

[0060] The work underlying this disclosure is encompassed in U.S. provisional application No. 63/604,464 filed November 30, 2023, and incorporated by reference. That work involved optimising, analysing, and comparing different *in vitro* models for therapy-induced senescence using breast cancer cells. This included inducing senescence in MCF-7 and MDA-MB-231 human breast cancer cells using bromodeoxyuridine (BrdU), gemcitabine (GEM) and Palbociclib (PALBO), and analysing the expression of different senescence markers, such as cell morphology, SA- β -galactosidase expression, cell cycle/proliferation arrest and cytokine (SASP) secretion.

[0061] The results and data led to the development of a two-hit treatment strategy using azithromycin as a senolytic drug. BrdU-, GEM- and PALBO-induced senescent cells with azithromycin and the senolytic effect was measured using the SRB assay. The effect of azithromycin was investigated in autophagic degradation by immunostaining assay. Cell surface protein DPP4 (Dipeptidyl peptidase-4; CD26) was investigated as a marker of cellular senescence in cancer and identifying its potential function. This included measuring the expression of DPP4/CD26 on the surface of BrdU-, GEM- and PALBO-induced senescent cells, and silencing the expression of DPP4 in MCF-7 and MDA-MB-231 cells and investigating its effect on senescence-induction and the expression of senescence markers (cell morphology, proliferation arrest, SA- β -gal expression).

[0062] Relevant to this disclosure, different cellular models of therapy-induced senescence (TIS) in cancer cells were evaluated. MCF-7 breast cancer cells were treated with three different drugs, namely bromodeoxyuridine (BrdU), gemcitabine (GEM) and Palbociclib (PALBO), each a potential senescence-inducer. Initially, the effect of BrdU, GEM and PALBO was tested on the cell viability of MCF-7 cells to determine the drug concentration to use for senescence-induction. The aim was to arrest the proliferation of the cells without inducing significant amount of cell death. The cell viability was measured by SRB assay as described below. The IC₅₀ of BrdU was 1 mM, the IC₅₀ of GEM was more than the highest tested concentration (100 μ M) and the IC₅₀ of PALBO was 10 μ M, however, apoptosis and proliferation arrest are not distinguishable with cell viability test. Based on these results and the relevant literature, the optimal drug concentrations and incubation times were empirically determined and described in detail below. Briefly, to induce

senescence in MCF-7 cells, the cells were treated for 7 days with 5 μ M BrdU and 100 nM GEM, and 14 days with 500 nM PALBO. To confirm the induction of senescence, different markers of cellular senescence were examined.

[0063] First, the morphology of the cells was examined under the microscope and using the measurements of forward scatter (FSC) and side scatter (SSC) by flow cytometry. Compared to the control cells, the BrdU- and GEM- and PALBO-induced senescent cells had a flattened shape and larger size, however, the shape of the PALBO-induced senescent cells was more similar to the control. Increased value of FSC/SSC measured by flow cytometry is a label-free method, that has been used to identify senescent cells based on their increased cell size (FSC) and granularity (SSC). Compared to control cells FSC/SSC values were both significantly increased in all of the senescent models; however, the SSC values increased more than the FSC. To investigate whether these three drugs induce DNA-damage as a senescence-inducing mechanism, the expression of γ H2AX (phosphorylated form of H2A histone family member X), a highly specific marker to detect double-stranded DNA damage, was analysed by flow cytometry (Mah et al., 2010). After senescence induction with BrdU ~15% of the cells expressed γ H2AX, while the ~80% of GEM-induced senescent cells expressed γ H2AX, this increased expression was detectable even after 2 days of BrdU/GEM treatment. PALBO-induced senescent cells did not have a significant increase of γ H2AX-expression compared to the control cells.

[0064] Based on the assessment of senescence-markers, such as the altered cell morphology (increased cell size, flattened shape, increased granularity), the increased SA- β -Gal expression, decreased Ki-67 expression and SASP secretion (IL-6, IL-8, CXCL12) the establishment of senescence models in MCF-7 cells was confirmed. The results of γ H2AX-expression indicates that in MCF-7 cells GEM induced senescence via DNA-damage induction, whereas BrdU and PALBO induced senescence through different mechanisms, independently from DNA-damage induction. Furthermore, results suggested that the SASP secretion of senescent MCF-7 cells were dependent on the mechanism of senescence-induction, and PALBO-induced senescent cells did not show SASP secretion.

[0065] To create senescence-escaped cells, after senescence-induction MCF-7 cells were incubated without the senescence-inducing drug, allowing cells to escape from senescence. For most of the experiments, cells were incubated in drug-free medium for 10 days to generate sufficient number of senescence-escaped cells for accurate detection. Compared to senescent cells,

senescence-escaped cells had an increased expression of Ki-67, indicating that they regained their proliferative capacity. During senescence-escape, MCF-7 cells form small colonies of Ki-67 expressing cells, that could be easily visualised by crystal-violet staining and quantified by counting the number of crystal-violet stained senescence-escaped colonies. The increase in cell proliferation during senescence-escape can also be detected and quantified by flow cytometry, by using the number of cells positively stained for Ki-67 compared to the total cell number, expressed as percentage of Ki-67 expressing cells. These methods for the detection and evaluation of senescence-escaped cells provides a basis for a quantitative approach to investigate senescence-escape. In subsequent experiments, the number of colonies consisting of senescence-escaped cells and the percentage of Ki-67 expressing cells are used to quantify the senescence-escaping ability of the cells.

[0066] Following the confirmation and analysis of senescence induction and escape, a two-hit treatment strategy was developed to utilize senescence-related vulnerability in cancer cells. The two-hit treatment strategy involved a senescence-inducing drug (first hit) and a senolytic drug (second hit) that eliminates the senescent cancer cells. Azithromycin is known to be a senolytic drug, selectively targeting senescent fibroblast cells. However its effect has not been tested in senescent cancer cells. To test its senolytic effect in senescent cancer cells as well, senescent MCF-7 cells were treated with azithromycin for 72 hours using the concentration of 50 μ M, 75 μ M and 100 μ M, based on the concentrations that had been used to target senescent fibroblasts. Based on the cell viability assays, azithromycin concentration-dependently decreased the viability of senescent MCF-7 cells, however, at lower concentration (50 μ M) it had a significant effect only on GEM-induced senescent cells, but not on BrdU- and PALBO-induced senescent cells. Fig. 1 illustrates the results of cell viability in MCF-7 cells following the azithromycin treatment. As shown, azithromycin concentration-dependently decreased the viability of senescent MCF-7 cells. At lower concentrations (50 μ M) it had a significant effect only on GEM-induced senescent cells, but not on BrdU- and PALBO-induced senescent cells. Although azithromycin decreased the viability of non-senescent MCF-7 and MDA-MB-231 cells as well, it showed no toxicity in normal epithelial and fibroblast cells.

[0067] Comparing the three senescent models, the PALBO-induced senescent cells were less affected by the azithromycin treatment, which had a significant effect only at 100 μ M concentration. Although using 100 μ M azithromycin decreased the viability of control MCF-7

cells to ~80%, the viability of BrdU- and PALBO-induced senescent cells were reduced to ~50%, and the viability of GEM-induced senescent cells was ~30%, indicating that the senescent cells were more sensitive to the treatment. Apoptosis and proliferation arrest are not distinguishable with cell viability test, however, based on the previous results demonstrating that senescent cells stopped proliferating, the decrease of cell viability of senescent cells indicates cell death. The toxicity of azithromycin was tested on MCF-10A non-malignant breast epithelial cells, showing that azithromycin treatment is not toxic to normal epithelial cells, and a reduction of cell viability was only detected at higher ($\geq 750 \mu\text{M}$) concentrations. Based on these results, the senolytic effect of azithromycin can be exploited in cancer treatment to reduce the number of senescent cancer cells generated by cancer therapy.

[0068] Besides its antibiotic effect, azithromycin has been described as having an impact on autophagic processes as well, which could be a potential mechanism explaining its senolytic effect. Measuring the expression of LC3B (LC3-II, microtubule-associated protein 1 light chain 3) and p62/SQSTM1 (sequestosome-1) is a widely used method to study autophagic flux in mammalian cells. LC3B/LC3-II is localised to autophagic membranes, and its expression correlates with the number of autophagosomes. p62/SQSTM1 is an adaptor protein of selective autophagy, and is itself degraded by autophagy, therefore, the reduced expression of p62 indicates induced autophagy, and the accumulation of p62 together with LC3B is an indicator of autophagy inhibition. According to results, azithromycin (AZI) treatment increased the expression of both LC3B and p62/SQSTM1, indicating that it inhibited autophagy in both control and senescent MCF-7 cells. The effect of azithromycin was compared with chloroquine (CQ), which has a well-known effect to inhibit autophagy by interrupting the fusion of autophagosomes with lysosomes and it has been described to act as a senolytic in several studies. As expected, CQ treatment of control and senescent MCF-7 cells increased the expression of both LC3B and p62, however, the expression of LC3B was higher and the expression of p62/SQSTM1 was lower compared to the effect of AZI. The analysis of immunostaining revealed that LC3B and p62/SQSTM1 was expressed heterogeneously in the AZI/CQ treated cell populations, especially in the senescent cells. The analysis also confirmed that the expression of LC3B increased after CQ treatment, while the expression of p62/SQSTM1 showed a higher increase after AZI treatment. Based on these results, AZI and CQ demonstrated a similar effect on autophagic processes in control and senescent MCF-7 cells. Based on the evaluation of LC3B and p62 expression, both AZI and CQ treatment

inhibited autophagy, however, the different levels of increase in LC3B and p62 expression suggests a different mechanism of action of AZI and CQ. The heterogeneous expression of both markers in response to AZI treatment indicates a heterogeneous response of senescent MCF-7 cells to AZI treatment, which could be responsible for the ~50% of surviving senescent cell populations measured by cell viability assay after 72 hours AZI treatment.

[0069] Although azithromycin treatment decreased the viability of senescent cells, ~50% of BrdU- and PALBO-induced senescent cells, and ~30% of GEM-induced senescent cells remained viable after the treatment with 100 μ M AZI as shown in Fig. 1. Moreover, 5 days treatment with 100 μ M AZI did not decrease the viability of senescent cells more than the 3-days treatment, indicating that a population of senescent MCF-7 cells could be resistant to AZI treatment as can be seen in Fig. 3. To test whether these remaining cells are able to escape from senescence, the Ki-67 expression of the cells along with the cell numbers (cell concentration) were measured 10 days after the removal of AZI treatment. According to the results, BrdU- and GEM-induced senescent cells were able to escape after AZI treatment, however, the Ki-67 expression was significantly decreased in the BrdU-induced senescent cells compared to the non-treated (without AZI) senescent cells, but not in GEM-induced senescent cells. Regarding the non-senescent MCF-7 cells, azithromycin treatment did not affect the Ki-67 expression of control cells, indicating that after the removal of AZI the cell proliferation rate of MCF-7 cells remained unchanged. The evaluation of cell concentration of BrdU- and GEM-induced senescent cells with or without AZI treatment showed that after 10 days drug-free incubation the number of AZI-treated senescence-escaped cells were significantly reduced compared to the number of senescence-escaped without AZI treatment. Results are shown in Fig. 5. The cell concentrations were measured by flow cytometry using the samples from the measurement of Ki-67 expression represented as count/ μ l (cell number/ μ l). The samples were collected with the same volume; therefore the cell concentration is proportional to the cell numbers of each sample. Values were respectively normalised to the untreated BrdU- and GEM-escaped cells. Bar graphs represent the mean of three independent experiments $\pm$ SEM. Statistical significance: ns $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

[0070] The decreased number of senescence-escaped cells would normally indicate decreased senescence-escaping ability, however, AZI treatment can influence the number of senescence-escaped cells by decreasing the number of senescent cells (as demonstrated before).

Due to this effect of AZI treatment, the decreased number of senescence-escaped cells is more likely to be a result of decreased cell viability rather than decreased senescence-escaping ability. PALBO-induced senescent cells can continue their growth shortly after the removal of PALBO, and although the AZI treatment decreased the viability of PALBO-induced senescent cells, it did not decrease the senescence-escaping ability of the remaining cell population. These results indicate that although AZI reduced the viability of senescent MCF-7 cells, the surviving cells can escape from senescence and regain their proliferative capacity.

[0071] The third objective of the project was to investigate the cell surface protein DPP4/CD26 as a marker of senescence in cancer and to identify its function in senescence. Although DPP4/CD26 was identified as a marker of senescence in fibroblast cells, its expression and potential function has not been investigated in senescent cancer cells (Kim et al., 2017a). Firstly, the expression of DPP4/CD26 was measured in control MCF-7 cells and in BrdU-, GEM- and PALBO-induced senescent cells by flow cytometry. Based on the results, senescent cells had a significantly increased expression of DPP4/CD26, demonstrated in a ~4-5-fold increase in PALBO- and GEM-induced senescent cells, and a ~10-fold increase in BrdU-induced senescent cells. Similar increase of DPP4/CD26 expression in senescent MCF-7 cells was detected by western blot as well. To further confirm the connection between the increased expression of DPP4/CD26 and senescence, the expression of DPP4/CD26 was monitored for 14 days, compared to the expression of SA- β -Gal. Both markers showed a time-dependent increase of expression; however, the levels of their increase were different in each senescent model, showing a maximal expression of DPP4/CD26 after 14 days of BrdU treatment. Based on these results, the expression of DPP4/CD26 is significantly increased in senescent cells, however, the levels of increase were dependent on the drugs used for senescence-induction. The expression of DPP4/CD26 was time-dependently increased during senescence-induction, indicating a strong connection between DPP4/CD26 expression and the development of senescence-phenotype.

[0072] To investigate the potential function of DPP4/CD26 in senescence, senescent MCF-7 cells with silenced expression of DPP4 (DPP4 siRNA) were generated by lentiviral vector-mediated gene silencing. The silencing was confirmed by both western blot and flow cytometry. However, the silencing of DPP4 expression had no effect on cell proliferation, and it did not affect the expression of SA- β -Gal and lysosomal mass in senescent cells. Due to the GFP-expression of the transduced cells, the fluorogenic SA- β -Gal staining (FDG) could not be detected, therefore the

assessment of lysosomal mass was used as an additional marker, that could be quantified easier than chromogenic SA- β -Gal staining (X-gal). These results indicate that although the expression of DPP4/CD26 is increased in senescent cells, its expression is not essential for senescence-induction in MCF-7 cells.

[0073] To investigate whether the expression of DPP4/CD26 has a role in senescence-escape, the senescence-escaping ability of DPP4-silenced (DPP4 siRNA) senescent cells was analysed by Ki-67 and crystal violet staining and compared with control senescent cells (ctrl siRNA). As described before, due to the transient growth-arrest/senescence induced by PALBO-treatment, PALBO-induced senescent cells might not be a suitable model to study senescence-escape, therefore senescence-escape was assessed only in BrdU- and GEM-induced senescent cells. Consistent with the previous observation that DPP4 is not required for senescence-induction in MCF-7 cells, DPP4 silencing did not change the Ki-67 expression in control and senescent (BrdU/GEM) cells. However, the proportion of Ki-67 expressing cells was significantly decreased in the senescence-escaped DPP4 siRNA cells compared to ctrl siRNA senescence-escaped cells. According to previous results, the silencing of DPP4 did not affect cell proliferation, therefore the decreased Ki-67 expression could indicate the decreased ability of senescent cells to regain their proliferative capacity. Although the percentage of Ki-67 expressing cells indicates the proportion of senescence-escaped (actively proliferating) cells, it does not give an information about the actual number of escaping-cells. To better demonstrate the difference in the senescence-escaping ability of DPP4 siRNA and ctrl siRNA senescence-escaped cells, the analysis of Ki-67 expression was combined with the measurement of cell numbers after senescence-escape. Based on this combined assessment, the number of senescence-escaped cells were significantly decreased to ~70% in BrdU-induced and to ~30% in GEM-induced senescent DPP4 siRNA cells compared to ctrl siRNA cells. These results were similar to the results of crystal violet staining, that showed that the silencing of DPP4 expression significantly reduced (~40%) the formation of senescence-escaped colonies in both BrdU- and GEM-induced senescent cells, while the colony formation ability of control cells were not affected by DPP4 silencing. Based on these results we concluded that DPP4 silencing decreased the senescence-escaping ability of BrdU- and GEM-induced senescent MCF-7 cells. However, further experiments are needed to identify the molecular pathways that could mediate the effect of DPP4 expression in senescence-escape.

[0074] Fig. 6 shows the expression of Ki-67 represented as the percentage of positively stained cells compared to the total population. Figs. 7A-7C illustrate the effect of DPP4 silencing on the senescence-escape of MCF-7 cells. Fig. 7A shows cell concentrations measured by flow cytometry using the samples from the measurement of Ki-67 expression represented as count/ μ l (cell number/ μ l). The samples were collected with the same volume; therefore, the cell concentration is proportional to the cell numbers of each sample. Values were respectively normalised to the BrdU- and GEM-escaped ctrl siRNA cells. Fig. 7B illustrates the senescence-escaping ability of the cells are assessed by the combination of Ki-67 expression and cell concentration represented as the number of Ki-67 positive cells. Values were respectively normalised to the BrdU- and GEM-escaped ctrl siRNA cells. Fig. 7C shows colony formation assay results of the BrdU- and GEM-escaped ctrl siRNA cells. The senescence-escaping ability of the cells were assessed by colony formation assay, represented as the number of colonies normalised respectively to the BrdU- and GEM-escaped ctrl siRNA cells. Experiments were repeated with three technical replicates. Bar graphs represent the mean of three independent experiments $\pm$ SEM. Statistical significance: ns $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

[0075] Based on the results of DPP4 silencing, the inhibition of enzymatic activity of DPP4 on decreasing the senescence escape of MCF-7 cells was analyzed. The effect of a widely used DPP4-inhibitor, sitagliptin, on cell viability was tested in MCF7 and MCF-10A cells. Figs. 8A and 8B show cell viability data of MCF-10A, MCF-7 cells and BrdU-, GEM- and PALBO-induced senescent MCF-7 cells, respectively, measured by SRB assay after 72 hours treatment of sitagliptin. Experiments were repeated three times with six technical replicates, values were normalised to vehicle-treated controls, error bars represent $\pm$ SEM. After senescence induction by BrdU- and GEM-treatment cells were incubated for 10 days with sitagliptin treatment.

[0076] The experiment demonstrated that sitagliptin treatment decreased the viability of MCF-7 cells at a concentration of 500 μ M or higher, and it showed no toxicity in normal epithelial MCF-10A cells, as seen in Fig. 8A. In addition, sitagliptin treatment did not decrease the viability of senescent cells up to a concentration of 1 mM, as seen in Fig. 8B. These results indicate that sitagliptin treatment does not affect the viability of control and senescent MCF-7 cells. Sitagliptin has been found to inhibit the enzymatic activity of DPP4 in MCF-7 cells at a concentration of $\sim$ 300 μ M or higher (Choi et al., 2015), therefore, in subsequent experiments it is used at a concentration

of 250 μ M and 500 μ M. Next, the effect of sitagliptin on senescence-escape was assessed by measuring the Ki-67 expression of senescence-escaped cells combined with the number of cells. Figs. 9A-9C illustrate the senescence-escaping ability of the cells by the combination of Ki-67 expression and cell concentration represented as the number of Ki-67 positive cells for BrdU-, GEM-, and PALBO, respectively. Values were normalised to the vehicle-treated cells. Graphs representing the Ki-67 expression and cell concentrations separately are included in supplementary [0077]

According to the results, sitagliptin treatment concentration-dependently decreased the number of Ki-67 expressing cells in BrdU- and GEM-induced senescent cells, however, in PALBO-induced senescent cells sitagliptin treatment only had an effect at the concentration of 500 μ M. The results were confirmed by crystal violet staining as well, that demonstrated decreased number of senescence-escaped colonies after sitagliptin treatment in BrdU- and GEM-induced senescent cells. Although 72 hours sitagliptin treatment showed no significant effect on the viability of non-senescent (control) MCF-7 cells at 250 μ M concentration, prolonged treatment (10 days) with 250 μ M sitagliptin decreased the colony formation of control MCF-7 cells. These results indicate that the effect of DPP4 in the senescence-escape of MCF-7 cells is based on the enzymatic activity of DPP4, and the senescence-escaping ability of MCF-7 cells could be decreased by DPP4-inhibition.

[0078] MCF-7 cells were able to escape from senescence after azithromycin treatment, therefore, to increase the effect of azithromycin, the combination of azithromycin (AZI) and sitagliptin (SITA) was tested on BrdU- and GEM-induced senescent cells. After senescence induction using BrdU or GEM, senescent cells were treated with 100 μ M AZI for 72 hours. Then, the cells were incubated with or without 250 μ M SITA treatment for 10 days, and the senescence-escaping ability of the cells was assessed by Ki-67 staining. Based on the results, using sitagliptin subsequent to the treatment with azithromycin improved the effect of azithromycin in BrdU- and GEM-induced senescence-models, by further decreasing the number of Ki-67 expressing cells to ~5-10%. However, the combination treatment was not effective to decrease senescence-escape in PALBO-induced senescent cells. These results indicate a synergistic effect of the combination treatment of azithromycin and sitagliptin in MCF-7 cells, resulting in a reduced number of senescence-escaped cells, compared to using azithromycin alone.

[0079] To further investigate the senolytic effect of azithromycin (AZI), AZI treatment was tested in senescent MDA-MB-231 cells and compared to the effect of chloroquine (CQ) treatment.

Figs. 10A and 10B show the effect of azithromycin and chloroquine in MDA-MB-231 cells, respectively. The cell viability of control, BrdU-, GEM- and PALBO-induced senescent MDA-MB-231 cells was measured by SRB assay after 72 hours treatment of azithromycin and chloroquine. Experiments were repeated three times with six technical replicates, values were normalised to vehicle-treated controls, error bars represent $\pm$ SEM. Statistical significance (in relation to control): ns $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

[0080] Based on the cell viability assays (SRB), AZI treatment concentration-dependently decreased the viability of both non-senescent (control) and senescent MDA-MB-231 cells, however, AZI was more selective towards senescent MDA-MB-231 cells at a concentration of 75 μ M and 100 μ M. The senolytic effect of AZI treatment on senescent MDA-MB-231 cells was dependent on the senescence-inducing drug. Similarly to the PALBO induced senescent MCF-7 cells, PALBO-induced senescent MDA-MB-231 cells were more resistant to AZI treatment. 100 μ M AZI treatment decreased the viability of control MDA-MB-231 cells to ~30%, and it almost eliminated the BrdU-, GEM- and PALBO-induced senescent cells by decreasing their viability to ~10%. Moreover, 5 days treatment with 100 μ M AZI further decreased the viability of senescent MDA-MB-231 cells to ~1-3%. Because senescent MDA-MB-231 cells were almost completely eliminated after 100 μ M AZI treatment, the senescence-escaping ability of AZI-treated MDA-MB-231 cells were not further investigated. The effect of chloroquine was similar to azithromycin, by decreasing the viability of both control and senescent MDA-MB-231 cells. These results indicate that azithromycin treatment was more effective in MDA-MB-231 cells compared to MCF-7 cells. Although AZI and CQ had similar effect in MDA-MB-231 cells, as described before, AZI was less toxic to fibroblast (MRC-5) cells compared to CQ, which demonstrates that AZI could be more suitable to use in cancer treatment.

[0081] Consistent with the results in MCF-7 cells, although the expression of DPP4/CD26 was increased in senescent MDA-MB-231 cells, its expression was not essential for senescence-induction. To investigate whether the expression of DPP4/CD26 has a role in senescence-escape, the senescence-escaping ability of DPP4-silenced (DPP4 siRNA) senescent cells was analysed by Ki-67 staining and compared with control senescent cells (ctrl siRNA). As described before, due to the transient growth-arrest induced by PALBO-treatment, PALBO-induced senescent cells might not be a suitable model to study senescence-escape, therefore senescence-escape was assessed only in BrdU- and GEM-induced senescent cells. Consistent with the previous

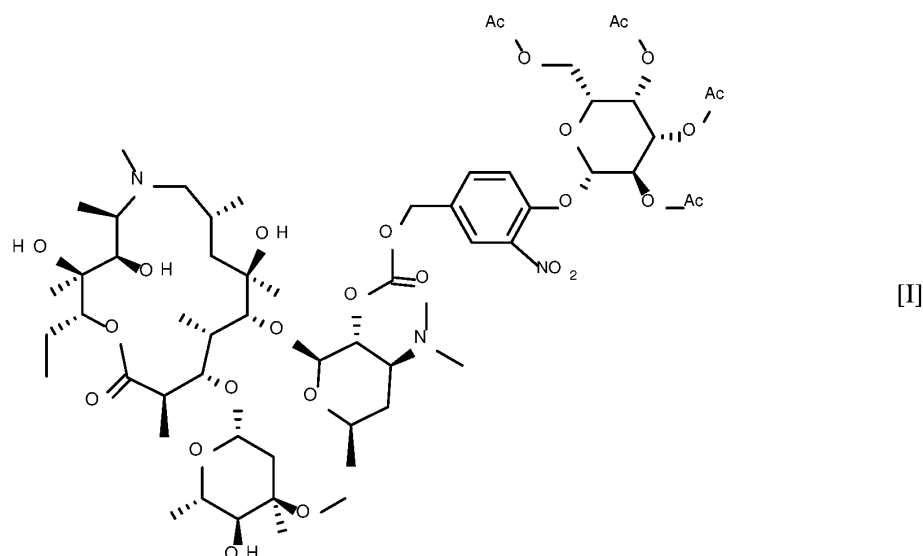
observation that DPP4 is not required for senescence-induction in MDA-MB-231 cells, DPP4 silencing did not change the Ki-67 expression in control and senescent (BrdU/GEM) cells. In contrast with the results in MCF-7 cells, the proportion of Ki-67 expressing cells was increased in the senescence-escaped DPP4 siRNA cells compared to ctrl siRNA senescence-escaped cells, even though this increase was only significant in the GEM-induced senescent cells.

[0082] Figs. 11A-11E show results relating to the effect of DPP4 silencing and DPP4 inhibition on the senescence-escape of MDA-MB-231 cells. Fig. 11A shows the expression of Ki-67 as the percentage of positively stained cells compared to the total population. The gates for Ki-67-positive cells were adjusted by using unstained cells. Fig. 11B shows cell concentrations measured by flow cytometry using the samples from the measurement of Ki-67 expression represented as count/ μ l (cell number/ μ l). The samples were collected with the same volume, therefore the cell concentration is proportional to the cell numbers of each sample. Values were respectively normalised to the BrdU- and GEM-escaped ctrl siRNA cells. Fig. 11C shows the senescence-escaping ability of the cells were assessed by the combination of Ki-67 expression and cell concentration represented as the number of Ki-67 positive cells. Values were respectively normalised to the BrdU- and GEM-escaped ctrl siRNA cells. Bar graphs represent the mean of three independent experiments $\pm$ SEM. Statistical significance: ns $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. Fig. 11D shows Ki-67-positive cells treated at different concentrations of BrdU, and Fig. 11E shows Ki-67-positive cells treated at different concentrations of GEM. After senescence induction by BrdU- and GEM-treatment cells were incubated for 10 days with sitagliptin treatment. The senescence-escaping ability of the cells were assessed by the combination of Ki-67 expression and cell concentration represented as the number of Ki-67 positive cells. Values were to the vehicle-treated cells, bar graphs represent the mean of two independent experiments $\pm$ SEM.

[0083] To better demonstrate the difference between the DPP4 siRNA and ctrl siRNA senescence-escaped cells, the analysis of Ki-67 expression was combined with the measurement of cell numbers, representing the number of senescence-escaped cells. The number of senescence-escaped cells was increased by ~50% in BrdU-induced and by ~2 folds in GEM-induced senescent DPP4 siRNA cells compared to ctrl siRNA cells, indicating that silencing of DPP4 promotes senescence escape in MDA-MB-231 cells. Unfortunately, these results could not be confirmed by crystal violet staining, since the senescence-escaped MDA-MB-231 cells did not form colonies (as

in the case of MCF-7 cells) that could be used to evaluate senescence-escape. Based on the results of DPP4 silencing, inhibition of DPP4 by sitagliptin treatment was expected to have a similar effect on the senescence escape of MDA-MB-231 cells. According to results of senescence-escape analysis by Ki-67 staining, 250 μ M sitagliptin treatment did not affect senescence-escape after BrdU-induced senescence, however, it seemed to increase senescence-escape in GEM-induced senescent MDA-MB-231 cells. Sitagliptin treatment at 500 μ M concentration resulted in decreased senescence-escape in both BrdU- and GEM-induced senescent cells, which might indicate a cytostatic or cytotoxic side-effect of long-term high concentration sitagliptin treatment. These results indicate that DPP4/CD26 has a cell-type dependent role in the regulation of senescence-escape, either preventing (MCF-7 cells) or promoting (MDA-MB-231 cells) it.

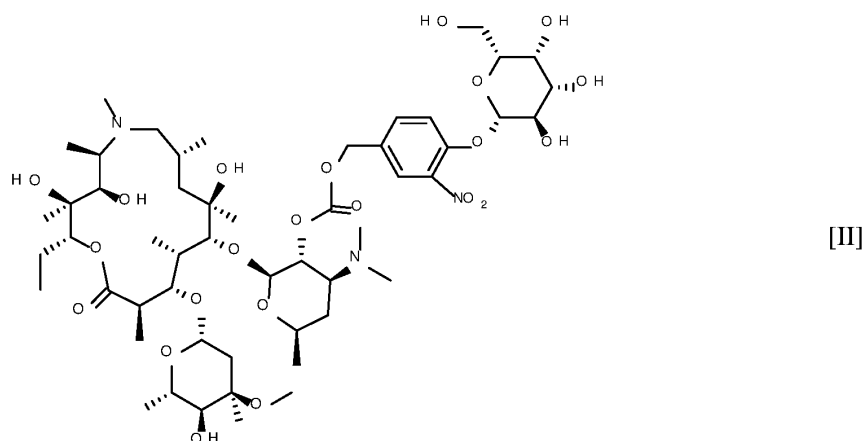
[0084] Certain azithromycin conjugates were evaluated for their improved senolytic activity, and for combination with sitagliptin. Some embodiments of the present approach take the form of a compound having the structure of Compound [I] shown below, in which 'Ac' denotes an acetyl group.



Compound [I], also referred to as AZM-Gal, has the IUPAC name [(2R,3S,4S,5R,6S)-3,4,5-triacetoxy-6-[4-[[[(2S,3R,4S,6R)-4-(dimethylamino)-2-[[[(2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-2-ethyl-3,4,10-trihydroxy-13-[(2S,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyl-tetrahydropyran-2-yl]oxy-3,5,6,8,10,12,14-heptamethyl-15-oxo-1-oxa-6-azacyclopentadec-11-yl]oxy]-6-methyl-tetrahydropyran-3-

yl]oxycarbonyloxymethyl]-2-nitro-phenoxy]tetrahydropyran-2-yl]methyl acetate. The galactose is joined via the hydroxide of the desosamine ring.

[0085] The structure shown below, Compound [II] (also referred to as de-acetylated AZM-Gal) has comparable activity to Compound [I].



[0086] The compounds disclosed herein have exhibited senolytic activity via clinical assays. For example, in one assay bromodeoxyuridine (5-bromo-2'-deoxyuridine), also known as BrdU, was used to induce senescence in a cell population. BrdU is an analog of the nucleoside thymidine commonly used to identify proliferating cells. BrdU induces controlled DNA damage, and drives cells towards senescence with high efficiency. In the BrdU assay, normal fibroblasts are subjected to prolonged culture (8-days) in the presence of BrdU at 100 μ M to induce controlled DNA-damage and senescence. In demonstrative embodiments, the inventors used two independent normal, non-immortalized, human fibroblast cell lines, MRC-5 lung cells, in the BrdU-based assay. Senolytic activity was assessed using the sulforhodamine B assay, also known in the art as the SRB assay. This assay measures the amount of protein remaining attached to the tissue-culture dishes and is a surrogate marker for cell viability.

[0087] Fig. 12 compares SRB assay results for different concentrations of azithromycin (100 μ M and 50 μ M) and Compound [I] (100 μ M and 50 μ M) of the present approach, on both control MRC-5 cells and MRC-5 cells treated with BrdU. For these data, MRC-5 cells were pre-treated with BrdU for 8 days (to induce senescence), before they were exposed to azithromycin or Compound [I] (labelled as "Azi-Gal" in Fig. 12) for another 5 days. After that, the SRB assay was performed to determine the effects of the drug on cell viability, using normal MRC-5 cells as the

control. As can be seen, azithromycin at 100 μ M had no effect on the viability of normal MRC-5 lung fibroblasts, but selectively killed senescent MRC-5 fibroblasts. However, at 50 μ M azithromycin had no effect on either the control cells or senescent cells. In contrast, Compound [I] showed exceptional selectivity towards senescent MRC-5 fibroblasts at 50 μ M, and was effective against both the control cells and the senescent cells at 100 μ M.

[0088] The xCELLigence assay was used to confirm senolytic activity for Compound [I]. MRC-5 fibroblasts were used for the assay. Fig. 13 shows xCELLigence data comparing the effect of Compound [I] of the present approach on control MRC-5 cells and MRC-5 cells treated with BrdU. The data is expressed as the final cell index, the average $\pm$ the standard error of mean, for the control cells, control cells treated with Compound [I], BrdU-treated control cells, and BrdU-treated fibroblasts subjected to Compound [I] at 50 μ M, respectively. The senescent MRC-5 cells were pretreated with BrdU for 8 days to induce senescence, before exposure to Compound [I] for another 5 days. Compared to the control, Compound [I] treatment had no effect on the viability of normal fibroblasts, but killed over 90% of the BrdU-treated fibroblasts. These data confirm that Compound [I] has exceptional senolytic activity.

[0089] Figs. 14A and 14B are images of MRC-5 fibroblasts without and with BrdU pre-treatment, respectively, treated with Compound [I] at a concentration of 50 μ M. These images show that Compound [I] had little effect on the normal MRC-5 cells, but induced cell death in senescent MRC-5 cells. The scale bar in the lower left of Figs. 14A and 14B represents 20 μ m.

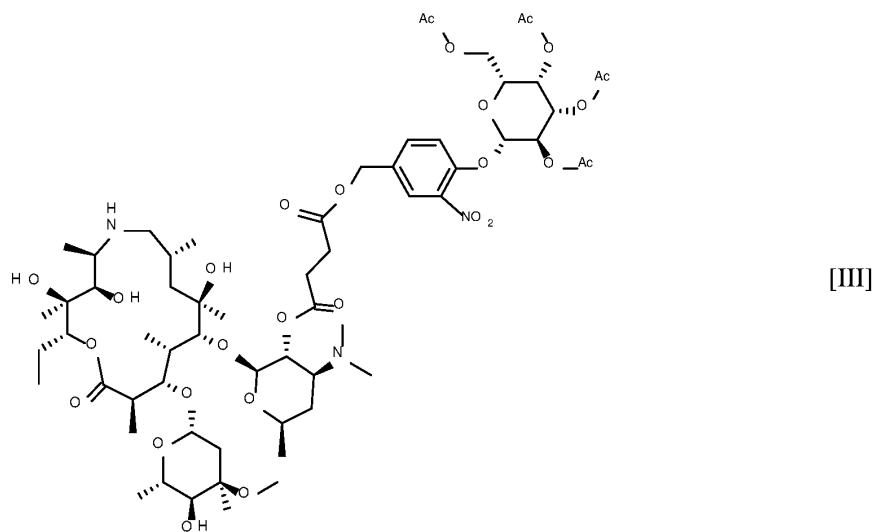
[0090] These results show that the compounds disclosed herein have senolytic activity and may be used as senolytic agents in a pharmaceutical composition. Moreover, the synergistic effect of combining azithromycin with sitagliptin in MCF-7 cells is even more pronounced with replacing azithromycin Compound [I] or Compound [II]. The result is a reduced number of senescence-escaped cells and an improved inhibition at lower concentrations of the azithromycin conjugate. The results are comparable to the difference in IC₅₀ for MCF-7 cells.

[0091] The compounds disclosed herein may also be used as therapeutic agent to selectively eradicate CSCs for treating and/or preventing tumor recurrence and/or metastasis. The data demonstrates that the compounds disclosed herein have anti-cancer activity, and are suitable for use as therapeutic agents for anti-cancer treatments, including treating and/or preventing tumor recurrence and metastasis. Data described herein demonstrates the anti-cancer activity through

inhibition of MCF-7 cells via the mammosphere formation assay. This assay measures the amount of residual protein that adheres to tissue culture dishes and is a surrogate marker of cell viability.

[0092] Fig. 15A shows mammosphere formation assay results for MCF-7 cells treated with Compound [I], and Fig. 15B shows mammosphere formation assay results for MCF-7 cells treated with Azithromycin. The data show that Compound [I] had an IC_{50} of 60 μ M, and nearly complete inhibition at a concentration of 100 μ M. Azithromycin, on the other hand, had an IC_{50} of 118 μ M, and even at a concentration of 200 μ M, inhibited only about 30% of the MCF-7 cells compared to the control. These results demonstrate the superior potency of Compound [I], at inhibiting MCF-7 propagation. Compound [II] exhibited comparable results to Compound [I].

[0093] However, modification of known senolytic compounds with a galactose moiety does not always result in improved senolytic activity. Compound [III], also referred to as AZM-Succinate or AZM-Succ, was synthesized as described herein. As can be seen, in Compound [III] the galactose is joined at the hydroxide of the desosamine, via succinate. However, as discussed below, Compound [III] showed significantly less senolytic activity compared to azithromycin and Compound [I].



[0094] Fig. 16 shows SRB assay results for Compound [III] on both control MRC5 cells and MRC5 cells treated with BrdU. The addition of the galactose moiety reduced the potency of the parent compound, Azithromycin, by about half. This demonstrates that merely conjugating the

galactose moiety does not improve the senolytic activity of the base compound. Thus, the improved senolytic behavior of Compounds [I] and [II] is unexpected.

[0095] Compounds of the present approach, Compound [I] and Compound [II], have less antibiotic activity than existing macrolide antibiotics, such as azithromycin and erythromycin. This is advantageous because use of the compounds of the present approach as therapeutic agents will have less of an impact on the development of antibiotic resistance. For example, embodiments of the present approach were screened for antibiotic activity, using the in vitro broth microdilution assay. In this assay, the Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of an agent that completely inhibits visible growth in vitro of the microorganism. The assay conditions, described by the Clinical and Laboratory Standards Institute were used for preparation of the inoculum, growth medium, and end point reading. Test substance was dissolved in 100% DMSO, suspended completely by vortexing, diluted by 2-fold serial titrations in the same vehicle, for a total of 11 test concentrations. A 4 μ L aliquot of each dilution was added to 196 μ L of broth medium seeded with the organism suspension in wells of a 96 well plate (bacterial count: 2 - 8 x 10⁵ colony forming units/mL final). The final volume was 200 μ L in each well and the final DMSO concentration was 2 percent. Test concentrations were 0.1 to 100 μ M. Following incubation, the test plates were visually examined and wells were scored for growth or complete growth inhibition to define the minimum inhibitory concentration. Each test substance was evaluated with replicates. Vehicle controls and an active reference agent were used as blank and positive controls. Results are shown in Table 1. Note that MRSA represents methicillin-resistant staphylococcus aureus, and VRE represents vancomycin resistant Enterococcus.

No.	Species	Strain ID	Strain information	MIC, μ M	
				AZM	Compound [I]
1	<i>Enterococcus faecalis</i>	ATCC 29212	QC strain	6.25	25
2	<i>Escherichia coli</i>	ATCC 25922	QC strain	6.25	12.5
3	<i>Klebsiella pneumoniae</i>	ATCC 43816	---	25	50
4	<i>Pseudomonas aeruginosa</i>	ATCC 27853	QC strain	100	>100
5	<i>Staphylococcus aureus</i>	ATCC 29213	QC strain	1.56	6.25

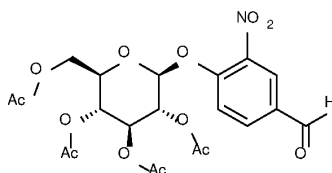
6	<i>Staphylococcus aureus</i>	ATCC 33591	MRSA	>100	>100
7	<i>Acinetobacter baumannii</i>	ATCC 17978	---	6.25	100
8	<i>Streptococcus pneumoniae</i>	ATCC 49619	QC strain	0.39	0.78
9	<i>Haemophilus influenzae</i>	ATCC 49247	QC strain	3.125	3.125
10	<i>Enterococcus faecium</i>	ATCC 700221	VanA VRE	>100	>100
11	<i>Escherichia coli</i>	JW5503	DEL-tolC	1.56	6.25

Table 1. Antimicrobial potency results.

[0096] Table 1 shows that the antimicrobial potency (expressed as MIC) of the tested embodiment was higher than the control, azithromycin. The MIC for Compound [I] AZM-Gal is considerably higher for nearly every species tested. This is demonstrative for embodiments of the present approach – the compounds disclosed herein have less antibiotic activity compared to macrolide antibiotics.

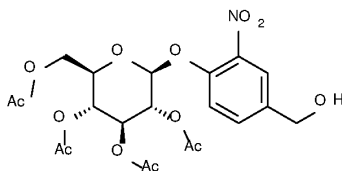
[0097] The following paragraphs provide example synthesis schemes for embodiments of the present approach. Synthesis products were confirmed using liquid chromatography and mass spectroscopy (LC-MS). The LC column was a Waters Sunfire C18 30x4.6mm, using a gradient eluent of 20-100% acetonitrile/water containing 0.05% formic acid. Time: 0-10min. The following abbreviation are used in the synthesis examples: Acetonitrile (MeCN), methanol (MeOH), dichloromethane (DCM), dichloroethane (DCE), isopropanol (IPA), sodium borohydride (NaBH₄), sodium carbonate Na₂CO₃, ammonium chloride (NH₄Cl), 4-dimethylaminopyridine (DMAP), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC HCl), triethylamine (TEA).

[0098] **Example 1** – Intermediary [(2R,3R,4S,5R,6S)-3,4,5-triacetoxy-6-(4-formyl-2-nitro-phenoxy)tetrahydropyran-2-yl]methyl acetate.



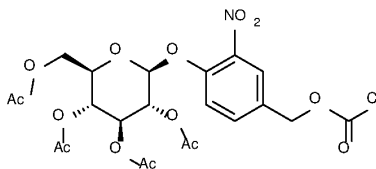
[00099] To a stirred suspension of Ag₂O (8.0g, 34.52mmol) and tetra-O-acetyl- α -D-glucopyranosyl bromide (1.26g, 7.55mmol) in MeCN (50ml) a solution of 4-hydroxy-3-nitrobenzaldehyde (3.10g, 7.55mmol) in acetonitrile (35ml) was added at room temperature. The mixture was stirred at room temperature for 4 hours, solid residue was removed by filtration and the solvent was evaporated under reduced pressure to yield a crude product. Purification on silica gel (2-4% MeOH in DCM) afforded [(2R,3R,4S,5R,6S)-3,4,5-triacetoxy-6-(4-formyl-2-nitro-phenoxy)tetrahydropyran-2-yl]methyl acetate (3.61g). LC-MS 515.2 [M+H₂O]⁺, RT 4.25min.

[00100] **Example 2** – Intermediary [(2R,3R,4S,5R,6S)-3,4,5-triacetoxy-6-[4-(hydroxymethyl)-2-nitro-phenoxy]tetrahydropyran-2-yl]methyl acetate.



[00101] To a stirred ice-cold solution of [(2R,3R,4S,5R,6S)-3,4,5-triacetoxy-6-(4-formyl-2-nitro-phenoxy)tetrahydropyran-2-yl]methyl acetate (3.00g, 6.03mmol) in a mixture of dry DCM (30ml) and dry IPA (8.5ml) under nitrogen atmosphere NaBH₄ (0.50g, 13.22mmol) was added and the mixture was stirred for 3.5 hours. A solution of saturated NH₄Cl (70ml) was added to the stirred mixture, stirring was continued for 5 minutes, the product was extracted with twice with DCM, the combined extracts was washed with brine, dried over Na₂CO₃, the solid residue was removed by filtration and the solvent was evaporated under reduced pressure to [(2R,3R,4S,5R,6S)-3,4,5-triacetoxy-6-[4-(hydroxymethyl)-2-nitro-phenoxy]tetrahydropyran-2-yl]methyl acetate (2.60g). LC-MS 517.2 [M+H₂O]⁺, RT 3.91min.

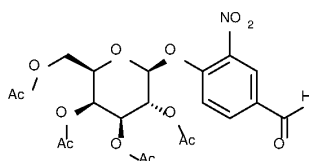
[00102] **Example 3** – Intermediary [(2R,3R,4S,5R,6S)-3,4,5-triacetoxy-6-[4-(chlorocarbonyloxymethyl)-2-nitro-phenoxy]tetrahydropyran-2-yl]methyl acetate.



[00103] To a stirred ice cold suspension of [(2R,3R,4S,5R,6S)-3,4,5-triacetoxy-6-[4-(hydroxymethyl)-2-nitro-phenoxy]tetrahydropyran-2-yl]methyl acetate (0.25g, 0.50mmol) and

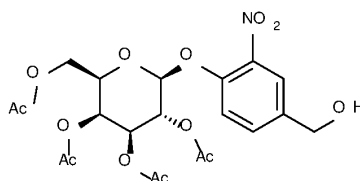
K_2CO_3 (0.44g, 3.00mmol) in dry MeCN (5ml) under nitrogen atmosphere an excess of phosgene (1.60ml, 3.00mmol) was added and the mixture was stirred at +5 °C for 2 hours. The solid residue was removed by filtration through Celite and the solvent was evaporated under reduced pressure to yield a crude product [(2R,3R,4S,5R,6S)-3,4,5-triacetoxy-6-[4-(chlorocarbonyloxymethyl)-2-nitro-phenoxy]tetrahydropyran-2-yl]methyl acetate.

[00104] **Example 4** – Intermediary [(2R,3S,4S,5R,6S)-3,4,5-triacetoxy-6-(4-formyl-2-nitro-phenoxy)tetrahydropyran-2-yl]methyl acetate.



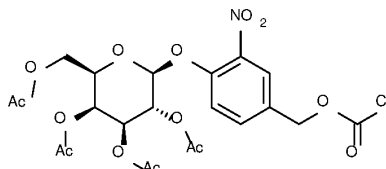
[00105] The title compound was prepared according to Ghosh Ajit, K., at al. A daunorubicin b-galactoside prodrug for use in conjunction with gene-directed enzyme prodrug therapy *Tetrahedron Lett.* 2000, 41, 4871-4874. LC-MS 515.2 $[M+H_2O]^+$, RT 4.16min.

[00106] **Example 5** – Intermediary [(2R,3S,4S,5R,6S)-3,4,5-triacetoxy-6-[4-(hydroxymethyl)-2-nitro-phenoxy]tetrahydropyran-2-yl]methyl acetate.



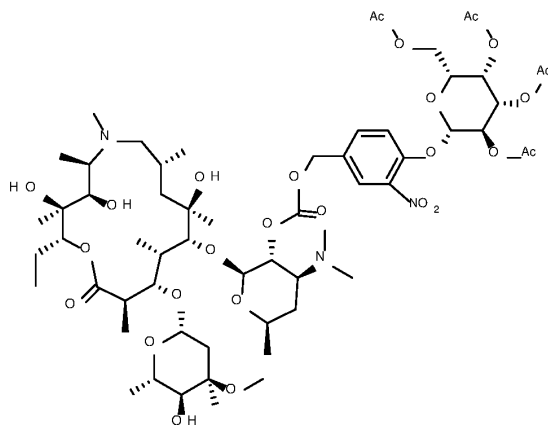
[00107] The title compound was prepared according to Ghosh Ajit, K., at al. A daunorubicin b-galactoside prodrug for use in conjunction with gene-directed enzyme prodrug therapy *Tetrahedron Lett.* 2000, 41, 4871-4874. LC-MS 517.2 $[M+H_2O]^+$, RT 3.81min.

[00108] **Example 6** – Intermediary [(2R,3S,4S,5R,6S)-3,4,5-triacetoxy-6-[4-(chlorocarbonyloxymethyl)-2-nitro-phenoxy]tetrahydropyran-2-yl]methyl acetate.



[00109] The crude title compound was prepared following the method in Example 3 above, for intermediary [(2R,3S,4S,5R,6S)-3,4,5-triacetoxy-6-[4-(hydroxymethyl)-2-nitro-phenoxy]tetrahydropyran-2-yl]methyl acetate.

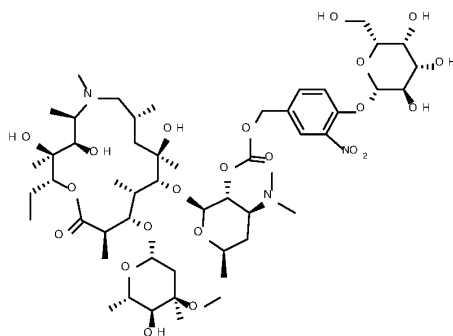
[00110] **Example 7** – Compound [I], [(2R,3S,4S,5R,6S)-3,4,5-triacetoxy-6-[4-[(2S,3R,4S,6R)-4-(dimethylamino)-2-[(2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-2-ethyl-3,4,10-trihydroxy-13-[(2S,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyl-tetrahydropyran-2-yl]oxy-3,5,6,8,10,12,14-heptamethyl-15-oxo-1-oxa-6-azacyclopentadec-11-yl]oxy]-6-methyl-tetrahydropyran-3-yl]oxycarbonyloxymethyl]-2-nitro-phenoxy]tetrahydropyran-2-yl]methyl acetate. As can be seen, the galactose moiety is joined to Compound [I] via the hydroxide on the desosamine ring.



[00111] To a stirred ice cold suspension of crude [(2R,3S,4S,5R,6S)-3,4,5-triacetoxy-6-[4-(chlorocarbonyloxymethyl)-2-nitro-phenoxy]tetrahydropyran-2-yl]methyl acetate (0.28g, 0.50mmol) and K_2CO_3 (0.14g, 1.00mmol) in dry MeCN (6ml) under nitrogen atmosphere a solution of (2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-11-[(2S,3R,4S,6R)-4-(dimethylamino)-3-hydroxy-6-methyl-tetrahydropyran-2-yl]oxy-2-ethyl-3,4,10-trihydroxy-13-[(2S,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyl-tetrahydropyran-2-yl]oxy-3,5,6,8,10,12,14-heptamethyl-1-oxa-6-azacyclopentadecan-15-one (0.23g, 0.3mmol) in dry MeCN (4ml). The reaction mixture was stirred at room temperature for 16 hours. The solid residue was removed by filtration and the solvent was evaporated under reduced pressure to yield a crude product. Purification on silica gel (2-10% MeOH in DCM) afforded [(2R,3S,4S,5R,6S)-3,4,5-triacetoxy-6-[4-[(2S,3R,4S,6R)-4-(dimethylamino)-2-[(2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-2-ethyl-3,4,10-trihydroxy-13-

[(2S,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyl-tetrahydropyran-2-yl]oxy-3,5,6,8,10,12,14-heptamethyl-15-oxo-1-oxa-6-azacyclopentadec-11-yl]oxy]-6-methyl-tetrahydropyran-3-yl]oxycarbonyloxymethyl]-2-nitro-phenoxy]tetrahydropyran-2-yl]methyl acetate (0.090g). LC-MS 638.1 [M/2+1]⁺, 1274.7 [M]⁺, RT 4.20min.

[00112] **Example** **8** - [(2S,3R,4S,6R)-4-(dimethylamino)-2-[[[(2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-2-ethyl-3,4,10-trihydroxy-13-[(2S,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyl-tetrahydropyran-2-yl]oxy-3,5,6,8,10,12,14-heptamethyl-15-oxo-1-oxa-6-azacyclopentadec-11-yl]oxy]-6-methyl-tetrahydropyran-3-yl] [3-nitro-4-[(2S,3R,4S,5R,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]oxy-phenyl]methyl carbonate.



[00113] To a stirred ice cold suspension of [(2R,3S,4S,5R,6S)-3,4,5-triacetoxy-6-[4-[[[(2S,3R,4S,6R)-4-(dimethylamino)-2-[[[(2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-2-ethyl-3,4,10-trihydroxy-13-[(2S,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyl-tetrahydropyran-2-yl]oxy-3,5,6,8,10,12,14-heptamethyl-15-oxo-1-oxa-6-azacyclopentadec-11-yl]oxy]-6-methyl-tetrahydropyran-3-yl]oxycarbonyloxymethyl]-2-nitro-phenoxy]tetrahydropyran-2-yl]methyl acetate (0.050g, 0.04mmol) in dry THF (1ml) under nitrogen atmosphere a solution 0.5M sodium methoxide solution (0.4ml, 0.20mmol) was added and the mixture was stirred at 0°C for 2.5 hours. The reaction mixture was treated with DOWEX 50WX2 (100mg) at 0°C for 0.5 hours, the solid resin was removed by filtration, washed with DCM (10ml) and the solvent was evaporated under reduced pressure to yield [(2S,3R,4S,6R)-4-(dimethylamino)-2-[[[(2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-2-ethyl-3,4,10-trihydroxy-13-[(2S,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyl-tetrahydropyran-2-yl]oxy-3,5,6,8,10,12,14-heptamethyl-15-oxo-1-oxa-6-azacyclopentadec-11-yl]oxy]-6-methyl-tetrahydropyran-3-yl] [3-nitro-4-

[(2S,3R,4S,5R,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]oxy-phenyl]methyl carbonate (0.050g). LC-MS 554.0 [M/2+1]⁺, RT 3.52min.

[00114] Cell lines and cell culturing: MCF-7, MDA-MB-231, MCF-10A and MRC-5 human cell lines were obtained commercially from the ATCC. MCF-7 and MDA-MB-231 breast cancer cells were maintained in Dulbecco's Modified Eagle Medium, High glucose (DMEM, Sigma) supplemented with 10% Heat Inactivated (HI) FBS (Gibco), 1% Glutamax (100X, Gibco), and 1% Penicillin-Streptomycin (Gibco). MCF-10A breast epithelial cells were maintained in Mammary Epithelial Cell Growth Medium (MEGM, Lonza) supplemented with 0.4% bovine pituitary extract (BPE), 0.1% insulin, 0.1% hEGF, 0.1% hydrocortisone, 0.1% GA-1000, and 100 ng/ml of cholera toxin. MRC-5 fibroblast cells were maintained in Minimum Essential Medium (MEM, Sigma) supplemented with 10% HI FBS (Gibco), 1% Glutamax (100X, Gibco), and 1% Penicillin-Streptomycin (Gibco). All the cell lines were maintained in a humidified incubator at 37°C and 5% CO₂. To seed the cells for experiments, culture medium was removed, cells were washed with sterile phosphate buffered saline (1X PBS, Gibco) and detached with Trypsin-EDTA solution (Sigma). Cells were collected by centrifugation (1200 RPM, 5 minutes) and resuspended in culture medium. Cell numbers were counted by BioRad TC20 Automated Cell Counter using 0.4%(v/v) Trypan Blue staining (Gibco) to exclude dead cells from cell counting.

[00115] Senescence-induction and treatments: Cells were seeded at an empirically determined density that allowed logarithmic growth during the senescence-inducing period, without limitation of cell growth due to reaching confluency. For senescence induction in MCF-7 and MDA-MB-231 cells, cells were treated with bromodeoxyuridine (BrdU), gemcitabine (GEM) or Palbociclib (PALBO). The concentrations and incubation times were determined empirically for both cell lines by monitoring the alteration of cell morphology, growth arrest and cell death (apoptotic cell morphology, detachment of the cells) using microscopic examination. The aim was to induce a relatively homogeneous senescence phenotype in both cell lines, avoiding the induction of substantial amount of cell death. The final senescence-inducing treatment strategies are described below. Two days after seeding, MCF-7 cells were treated with 5 µM BrdU for 7 days or 500 nM PALBO for 14 days, applying the treatment with every medium change (every 2-3 days); or MCF-7 cells were treated with 100 nM GEM for 24 hours and incubated in fresh medium without GEM for 7 days for senescence induction. One day after seeding, MDA-MB-231 cells were treated with 50 µM BrdU for 7 days, 200 nM GEM for 7 days or 1 µM PALBO for 14 days,

applying the treatment with every medium change (every 2-3 days). Senescent MCF-7/MDA-MB-231 cells were treated with azithromycin (AZI) and chloroquine (CQ) for 72 hours; or sitagliptin (SITA) for 10 days, applying the treatment with every medium change (every 2-3 days), using the concentrations indicated in each experiment. Bromodeoxyuridine, Palbociclib and azithromycin were dissolved in sterile dimethyl sulfoxide (DMSO); gemcitabine, chloroquine and sitagliptin were dissolved in sterile distilled water. All compounds were purchased from Sigma-Aldrich.

[00116] Fluorogenic SA- β -galactosidase staining: $1-2 \times 10^5$ cells/well were seeded in 6-well plates and treated with selected compounds to induce senescence or cultured without treatment (control cells). The expression of SA- β -galactosidase was detected with CellEvent™ Senescence Green Flow Cytometry Assay Kit (Thermo Fisher, Invitrogen). After senescence induction, cells were trypsinised, collected and resuspended in PBS at a concentration of $2-5 \times 10^5$ cells per sample. The collected samples were centrifuged (5 minutes at 1200 RPM) and resuspended in 100-200 μ l of 4%(v/v) paraformaldehyde (PFA, diluted in PBS) and incubated for 10 minutes at room temperature in dark. To prepare the working solution, the Senescence Green Probe (C12FDG) was diluted x1000 in the pH6 Senescence Buffer provided in the kit. After fixing the samples, the cells were washed with 3 ml of 1%(w/v) bovine serum albumin (BSA, Sigma) dissolved in PBS, resuspended in 100-200 μ l of pre-warmed (37°C) working solution and incubated for 1.5 hours at 37°C without CO₂ in dark. After incubation, working solution was removed and the cells were washed twice with 3 ml of ice-cold PBS to stop the staining reaction. 2×10^4 single cells were recorded and analysed by Attune NxT Flow Cytometer. Results were evaluated by FlowJo software.

[00117] Chromogenic SA- β -galactosidase staining: The expression of SA- β -galactosidase was detected with Senescence β -galactosidase Staining Kit (Cell Signaling). Senescent and control (untreated) cells were seeded in 96-well optical-bottom plates (Thermo Scientific) at a density of 1×10^4 cells/well. After 48 hours the medium was removed, the cells were washed twice with PBS and fixed with Fixative Solution (provided in the kit) diluted 10x in distilled water, incubated for 15 minutes at room temperature in dark. The working solution was prepared prior to use by mixing 1x Staining Solution, Solution A, Solution B and X-gal solution (20 mg of X-gal dissolved in 1 ml DMSO) according to manufacturer instructions. The pH of the solution was adjusted to pH 6 using NaOH and HCl solutions. After fixation, cells were washed twice with PBS and incubated with the working solution for 6 hours at 37°C without CO₂. After incubation, the working solution was removed, cells were washed twice with PBS and stained with 1 μ M Hoechst solution (Thermo

Fisher) diluted in PBS for 15 minutes at room temperature in dark. After Hoechst staining removal, cells were PBS washed and imaged with EVOS FL Auto Imaging System (Thermo Fisher).

[00118] Immunostaining for imaging: MCF-7/MDA-MB231 cells with or without treatment were seeded in 96-well optical-bottom plates (Thermo Scientific) at a density of 1×10^4 cells/well. Cells were incubated for 48 hours to allow them to attach well onto the plate. For staining, medium was removed, and cells were washed twice with PBS before fixing them with 4% paraformaldehyde (Thermo Fisher, PFA diluted in PBS) for 15 minutes at room temperature in the dark. After, cells were washed twice with PBS and incubated with 0.3%(v/v%) Triton-X100 (Sigma) diluted in 1%(w/v%) BSA (Sigma) for 15 minutes at room temperature to permeabilise the cells. After permeabilization, cells were incubated with 1% BSA for 30 minutes at room temperature to block the samples. For BrdU staining, an additional DNA denaturing step was added after the cells had been fixed. DNA was denatured by 20 minutes incubation with 1M HCl, at 40°C, and samples were washed twice with 0.1M borate buffer pH 8.0 (1.9 g sodium tetraborate and 5 g boric acid dissolved in water) for 10 minutes to remove the HCl and neutralise the pH. Cells were labelled with primary antibodies against Ki67 (1:250, SP6, 300 µg/ml, Thermo Fisher Invitrogen), BrdU (1:50, IIB5, 200 µg/ml, Santa Cruz) or γH2AX phospho-S139 (1:500, 9F3, 1mg/ml, Abcam) diluted in 1% BSA for 1 hour at room temperature on a microplate shaker (180 RPM). After, samples were washed three times with PBS and incubated with secondary antibodies conjugated with fluorescent compounds (1:1000, Alexa Fluor Plus 488 – anti-mouse, Alexa Fluor 594 – anti-rabbit, Alexa Fluor 594 – anti-mouse, 2 mg/ml, Thermo Fisher Invitrogen) diluted in 1% BSA for 30 minutes at room temperature in dark, on a microplate shaker (180 RPM). After incubation, the cells were washed three times with PBS and incubated with 1 µM Hoechst solution (Thermo Fisher) diluted in PBS for 15 minutes at room temperature in dark. After the removal of Hoechst staining, cells were washed twice with PBS and imaged with EVOS FL Auto Imaging System using magnification of 10X or 20X. Images were analysed with ImageJ software. Background was removed uniformly by ImageJ software.

[00119] For imaging the cells with higher magnification (40X), the cells were seeded on round coverslips (15 mm diameter) in a 24-well plate at a density of 4×10^4 cells/wells. Cells were incubated for 48 hours to allow them to attach well onto the coverslips. After staining with secondary antibodies, the coverslips were removed from the 24-well plate and mounted on glass slides using 10 µl mounting medium with DAPI staining (Vector labs). Samples were imaged the

following day with EVOS FL Auto Imaging System (Thermo Fisher) using magnification of 40X, and the images were analysed with ImageJ software.

[00120] Immunostaining for flow cytometry: Cells were trypsinized, collected and centrifuged (5 minutes, 1200 RPM) in 5 ml test tubes. Cell pellets were fixed by adding ice-cold 70% (v/v%) ethanol and the samples were kept in -20°C at least overnight. Before the staining samples were thawed, centrifuged (5 minutes, 1200 RPM) and washed with 1 ml of 1% BSA (w/v%, dissolved in PBS) to remove the remaining ethanol. Cells were incubated with primary antibodies (100 µl/2-5x10⁵ cells) against γH2AX (1:500, phospho S139, 9F3, 1mg/ml, Abcam) or Ki-67 (1:250, SP6, 300 µg/ml, Thermo Fisher Invitrogen) diluted in 1% BSA for 30 minutes at room temperature. After the incubation, samples were vortexed and centrifuged with an additional 2 ml of 1% BSA to remove the primary antibody. After the removal of primary antibodies, cells were incubated with secondary antibodies conjugated with fluorescent compounds (1:1000, Alexa Fluor Plus 488 – anti-mouse, Alexa Fluor 660 – anti-rabbit, 2 mg/ml, Thermo Fisher Invitrogen) diluted in 1% BSA for 30 minutes at room temperature in dark. After the incubation, samples were vortexed and centrifuged with an additional 2 ml of 1% BSA to remove the secondary antibody. Cells were resuspended in 1% BSA with a concentration of 5x10⁵ cells/500 µl, and 2x10⁴ single cells were recorded and analysed by Attune NxT Flow Cytometer. The results were evaluated by FlowJo software. The gates for positively stained cells were adjusted by using samples incubated only with secondary antibodies (without primary antibodies), representing the gating threshold for the positively stained cells.

[00121] Cell cycle analysis: Cell-cycle analysis was performed by flow cytometry using the double-staining of Ki-67 expression and propidium iodide (PI) nuclear DNA stain. After the immunostaining with Ki-67 antibody (described above), cells were stained with of Muse™ Cell Cycle Assay buffer (200 µl/5x10⁵ cells) containing PI stain (Luminex) for 20 minutes at room temperature, protected from light. After incubation, 300 µl/5x10⁵ cells of PBS were added to each sample. 2x10⁴ single cells were recorded and analysed by Attune NxT Flow Cytometer applying automatic compensation. Samples were manually categorized into cell-cycle stages and evaluated by FlowJo software.

[00122] DPP4/CD26 staining for flow cytometry: 1-2x10⁵ cells/well were seeded in 6 well plates and treated with selected compounds to induce senescence or cultured without senescence-inducing treatment (control cells). For the staining, cells were trypsinised, collected and

resuspended in 1% BSA dissolved in PBS with a concentration of $3\text{--}5 \times 10^5$ cells/sample. After, cells were centrifuged (5 minutes, 1200 RPM) and resuspended in 100 μl staining solution with antibodies against DPP4/CD26 conjugated with phycoerythrin CD26-PE (1:100, 2A6, 0.1 mg/ml, Invitrogen) diluted in 1% BSA and incubated for 30 minutes on ice protected from light. After incubation, samples were centrifuged (5 minutes, 1200 RPM) with 1 ml additional PBS and washed twice with PBS to remove the unbound antibodies. Cells were resuspended in 1% BSA with a concentration of 5×10^5 cells/500 μl , and 2×10^4 single cells were recorded and analysed by Attune NxT Flow Cytometer. Results were evaluated by FlowJo software. The background fluorescence intensity was determined by unstained samples and was subtracted from the fluorescence intensity of stained samples.

[00123] Sulphorhodamine B (SRB) assay: For cell viability and cell growth assays 1×10^4 cells/100 μl were seeded in 96-well plates. For growth assays, cells were incubated without changing the medium, and fixed after 24-, 48-, 72- and 96-hours. For cell viability assays, 24 hours after seeding the medium was replaced with fresh medium and the cells were treated with selected compounds, using the concentrations and incubation times indicated in each experiment. At the end of the treatment the medium was removed, and cells were washed once with PBS. After, cells were fixed with 10%(v/v%) trichloroacetic acid (TCA) for 1 hour at 4°C, stained with 0.4%(w/v%) Sulphorhodamine B (SRB, Sigma) dissolved in 1%(v/v%) acetic acid for 15 minutes, and washed three times with 1% acetic acid and dried for at least 2 hours. The incorporated dye was dissolved in 10 mM Tris-HCl pH 8.8 buffer (Trizma Base, Sigma) solution and the absorbance were read using a plate reader (Thermo Fisher, Varioskan™ LUX microplate reader) at 565 nm. Data was analysed in Excel and GraphPad Prism.

[00124] Crystal violet staining: Crystal violet staining assay was performed to assess senescence-escape. $1\text{--}2 \times 10^5$ cells/well were seeded in 6 well plates and treated with selected compounds to induce senescence. After senescence induction, cells were incubated without treatment for 10 days, allowing them to restart proliferation and form colonies of senescence-escaped cells. After, the medium was removed, and cells were fixed in 70% ethanol for 10 minutes at room temperature. After fixation, cells were stained with 0.2% (v/v%) crystal violet solution (Sigma) diluted in 70% ethanol for 30 minutes at room temperature. The staining solution was removed, and the plates were washed with water and dried overnight. The cells were imaged with

EVOS FL Auto Imaging System (Thermo Fisher), and the number of colony-forming senescence-escaped cells were quantified with ImageJ software.

[00125] Silencing of DPP4/CD26: 1.5×10^6 293Ta packaging cells (human embryonic kidney cells) were seeded in four T75 flasks 48 hours before they were transfected with lentiviral vectors encoding three different clones for DPP4 siRNA (small interfering RNA) (HSH004434-LVRV6GP-a, HSH004434-LVRV6GP-b, HSH004434-LVRV6GP-c) and a scrambled vector (HSH004434-LVRV6GP), using Lenti-PacTM HIV Expression Packaging Kit (Genecopoeia). For transfection 2.5 μ g of each lentiviral siRNA expression plasmid and 5.0 μ l of Lenti-Pac HIV mix (Genecopoeia) were diluted in 200 μ l of Opti-MEM (Gibco). After, 15 μ l of EndoFectin Lenti reagent (Genecopoeia) diluted in 200 μ l of Opti-MEM was added dropwise to the DNA solution and gently mixed. The mixture was incubated for 20 minutes at room temperature to form the DNA-EndoFectin complex, then directly added to each flask. Cells were incubated overnight (8-14 hours), and the medium was replaced with 10 ml of fresh medium supplemented with 20 μ l of TiterBoost reagent (Genecopoeia). 48 h after transfection, lentivirus-containing culture medium was collected and centrifuged (10 minutes, 500 g), and the supernatant was filtered through a 0.45 μ m syringe filter (Merck) and stored at -80°C or used immediately for the transduction. 5×10^5 MCF-7 or MDA-MB-231 cells were seeded in T25 flasks and cultured until they reached 70-80% confluence. For transduction, 3 ml of lentivirus-containing medium diluted with 3 ml of culture medium was added to the target MCF-7 or MDA-MB-231 cells in the presence of 5 μ g/ml polybrene (Santa Cruz). Mock control cells were treated only with 5 μ g/ml polybrene diluted in 6 ml culture medium. Cells were incubated overnight and incubated in fresh medium until they reached 80-90% confluence. Each of the plasmids used for the experiment encoded a puromycin-resistance gene, therefore the transduced cells were resistant to puromycin, but the mock control cells remained sensitive to it. The transduced cells were selected with a concentration of 1 μ g/ml (empirically determined concentration) of puromycin (Sigma) until mock control cells were eliminated. Cells transduced with HSH004434-LVRV6GP-a clone were selected to be used in subsequent experiments by measuring the efficacy of silencing by flow cytometer, labelled as DPP4 siRNA. Cells transduced with scrambled vector were used as experimental controls, labelled as ctrl siRNA.

[00126] Western blot analysis: $1-2 \times 10^5$ cells/well were seeded in 6 well plates and treated with selected compounds to induce senescence or cultured without senescence-inducing treatment

(control cells). After the removal of cell culture medium, cells were washed twice with cold PBS. Proteins from cells were extracted by RIPA lysis buffer (Sigma) complemented with cOmplete™ ULTRA Tablets Protease Inhibitor Cocktail (Sigma) and PhosSTOP™ Phosphatase Inhibitors (Sigma) and cell debris and proteins were collected by using a cell scraper, and incubated for 30 minutes at 4°C, with agitation. Cell debris was removed by centrifugation (10 minutes, 12000 RPM, 4°C) and the supernatant (protein extraction) was collected and stored at -20°C. Protein concentration was evaluated by Pierce™ BCA Protein Assay Kit (Thermo Fisher). 5 µl of protein samples and protein standards (known concentrations of proteins provided in the kit) were added to 200 µl of working solution/well (50:1 mixture solution A and solution B provided in the kit) in a 96 well plate, and incubated for 30 minutes at 37°C. Absorbance was measured at 562 nm, using a plate reader (Thermo Fisher, Varioskan™ LUX microplate reader), and protein concentration was evaluated in Excel, based on the standard curve generated by measuring the standard proteins with known concentrations.

[00127] 20 µg of protein samples were mixed with 8 µl of NuPAGE™ LDS Sample Buffer (4X, Invitrogen) and additional distilled water to equalize sample volumes, and incubated for 10 minutes at 98°C. After, protein samples were cooled down and subjected to electroporation (100 V, ~1 hour) through 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (50 µl wells, Bio-Rad), and electroblotted onto a nitrocellulose blotting membrane (0.2 µm) using Trans-Blot Turbo Transfer System (Bio-Rad). Premixed 10x Tris/glycine/SDS running buffer (Bio-Rad) diluted to 1x in distilled water was used as running buffer, and PageRuler™ Plus Prestained Protein Ladder (Thermo Fisher) was used as size standards. Transfer efficiency was examined by Ponceau S Staining Solution (Thermo Fisher). Membranes were washed three times with PBST (1x PBS, 0.1% (v/v%) Tween-20 detergent solution (Thermo Fisher)) and blocked with 5% (w/v%) BSA dissolved in PBS for 1 hour at room temperature. Membranes were incubated overnight at 4°C with primary antibodies against DPP4/CD26 (1:300, D6D8K, Cell Signaling), p16^{INK4a} (1:500, 1D7D2, Invitrogen) and β-actin (1:3000; Sigma). After incubation, membranes were washed three times with PBST. Membranes were incubated with HRP-linked secondary antibodies (anti-rabbit and anti-mouse IgG, 1:3000, Cell Signaling) for 1 hour at room temperature, and washed three times with PBST. Proteins were revealed by using SuperSignal West Pico chemiluminescent substrate (Thermo Fisher) and Syngene G:BOX imaging system.

[00128] CellTrace CFSE (carboxyfluorescein succinimidyl ester) staining: $0.8\text{--}1.6 \times 10^6$ cells were seeded in T75 flasks or $1\text{--}2 \times 10^5$ cells were seeded in 6 well plates, depending on the experiment, and treated with selected compounds to induce senescence or cultured without senescence-inducing treatment (control cells). After, medium was removed and cells were washed with DPBS (Dulbecco's phosphate-buffered saline with calcium and magnesium, Gibco) and incubated with 5 μM CellTrace CFSE dye (5 mM, dissolved in DMSO, Thermo Fisher) diluted in DPBS for 20 minutes in the cell culture incubator. After the incubation, the staining solution was removed, and cells were washed with culture medium and incubated in fresh medium for 10 (senescent cells) or 4 (control cells) days.

[00129] Fluorescence-activated cell sorting (FACS): After CellTrace CFSE staining, cells were collected, centrifuged, resuspended in pre-sort buffer (BD Biosciences) at a concentration of 1×10^6 cells/ml. Cells were filtered through a 40 μm cell strainer (Fisherbrand) before sorting them with Sony SH800S Cell Sorter (Sony Biotechnology). The gates were adjusted to separate senescent and senescence-escaped cells by using senescent cells stained with CellTrace CFSE. The detailed sorting strategy is described and presented in the results and supplementary figures (Figure 27-28, Supplementary Figure 11). For migration and mammosphere assays cells were sorted in tubes containing medium without FBS or mammosphere medium, collected by centrifugation (1200 RPM, 5 minutes) and resuspended in the appropriate culture medium (detailed below). For colony formation assay cells were sorted directly in the 6-well plates filled with complete medium (DMEM).

[00130] Migration assay: After sorting, MCF-7 cells were seeded in Falcon™ Cell Culture Inserts (PET membrane with 8 μm pores, for 24 well plates) at a concentration of 5×10^4 cells/500 μl medium without FBS and placed in the wells of 24-well plate filled with 500 μl medium without FBS. Cells were serum-starved for 4 hours, and then the medium in the bottom of the 24-well plates was replaced with medium containing 10% FBS as chemoattractant. The inserts were incubated for 24 hours in the incubator, allowing the cells to migrate to the other side of the membrane. MDA-MB-231 cells were seeded in Falcon™ Cell Culture Inserts at a concentration of 1×10^4 cells/500 μl medium without FBS and placed in the wells of 24-well plate filled with 500 μl medium with 10% FBS. The inserts were incubated overnight in the incubator, allowing the cells to migrate to the other side of the membrane. Before staining, medium and cells were removed from the internal side of the insert using a cotton swab. The insert was placed in

70%(v/v%) ethanol for 10 minutes to fix the migrated cell on the external side of the insert. Migrated cells were stained with 0.2%(v/v%) crystal violet solution (Sigma) diluted in 70% ethanol for 30 minutes at room temperature. The staining solution was removed, and the inserts were washed with water and dried overnight. Membranes were imaged with EVOS FL Auto Imaging System (Thermo Fisher), and images were evaluated with ImageJ software.

[00131] Mammosphere formation assay: To prepare 6-well plates for the mammosphere formation assay, 12 g of poly-HEMA was dissolved in 1 l of 95% ethanol and 2 ml of dissolved poly-HEMA was added to each well of the plates. The plates were incubated for 3 days at 50°C to create a coating on the bottom of the wells. After sorting, cells were centrifuged (5 min, 1200 RPM), resuspended in mammosphere medium (DMEM-F12 medium without phenol red (Gibco) complemented with 2%(v/v%) B-27 supplement (Gibco), 20 ng/ml EGF (Invitrogen) and 1% Penicillin-Streptomycin (Gibco)) plated at a density of 5000 cells/well in 6-well plates coated with 2-hydroxyethylmethacrylate (poly-HEMA, Sigma) with mammosphere medium. Cells seeded in the coated plates were grown for 5 days in the incubator, and mammospheres bigger than 50 μm were counted using an eye piece graticule of the microscope.

[00132] Colony formation assay: For colony formation assay, cells were seeded in 6-well plates filled with 2 ml culture medium with a density of 1000 cells/well (MCF-7) or 500 cells/well (MDA-MB-231) and incubated in the incubator for 14 days. After incubation cells were stained with crystal violet, following the fixation and staining method described before (crystal violet staining). The number of colonies were quantified with ImageJ software.

[00133] Enzyme-linked immunosorbent assay (ELISA): The secretion of IL-6, IL-8 and CXCL12A (SDF-1 α) were measured from cell culture medium using ELISA kits (Invitrogen). 1-2x10⁵ cells were seeded in 6 well plates, and treated with selected compounds to induce senescence or cultured without senescence-inducing treatments (control cells). After senescence induction, cells were incubated with fresh medium for 4 days. For collecting control samples, the medium was changed 24 hours after seeding and the cells were incubated for 4 days. After 4 days, the culture medium was collected, centrifuged (1000 g, 10 minutes, 6°C) to remove any cells/cell debris, and the supernatant was collected and used immediately or stored at -80°C in small aliquots. The cells remaining in the plates were trypsinized and cell numbers were counted by BioRad TC20 Automated Cell Counter using 0.4% Trypan Blue staining (Gibco). The levels of IL-6, IL-8 and CXCL12A (SDF-1 α) were detected by ELISA kits (Invitrogen) according to manufacturer

instructions. Briefly, standard solutions were prepared with known concentrations of each protein and experimental samples were diluted in sample dilution buffer (Invitrogen) as necessary. Standards and samples were added to the antibody-coated wells and incubated alone or together with the biotin conjugate (Invitrogen). The plate was incubated at room temperature using the incubation times indicated in each protocol (for each cytokine) and washed three times with wash buffer (Invitrogen). After, freshly diluted streptavidin-HRP solution (Invitrogen) was added to the wells and incubated at room temperature using the incubation times indicated in each protocol (for each cytokine). After incubation, wells were washed three times with wash buffer and incubated with stabilized chromogen solution (Invitrogen) for 30 minutes at room temperature in dark. After, stop solution (Invitrogen) was added to the wells to stop the reaction and the absorbance was read by a plate reader (Thermo Fisher, Varioskan™ LUX microplate reader) at 450 nm. Background signals were measured from fresh culture medium and subtracted from each value. The results were quantified by using a standard logarithmic curve and normalised by the cell numbers.

[00134] Statistical analysis: All the experiments were repeated at least 3 times with 2-3 technical replicates, or as indicated in figure legends. All data are represented as the mean + standard error of the mean (SEM) represented as error bars in the graphs, except for the analysis of the immunostaining of LC3B and p62 expression, where the mean + standard deviation is represented in the graphs. Data normality was assessed by Shapiro-Wilks test. For statistical analysis of pairwise comparisons (two groups) two-tailed unpaired T-test was used with statistical significance set at $P < 0.05$. For multiple group comparisons ($3 < \text{groups}$) one-way ANOVA test corrected with Dunnett's multiple comparison test was used, with statistical significance set at $P < 0.05$. All statistical analysis were performed by using GraphPad Prism software.

[00135] Embodiments of the present approach may also take the form of methods for preventing or reducing the likelihood of at least one of tumor recurrence and metastasis. In some embodiments, an effective amount of a composition having, as a therapeutic agent, a compound of the present approach, may be administered. In some embodiments, an effective amount of a composition having, as its therapeutic agent, an embodiment of a compound as described herein may be administered.

[00136] The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used in the description of the invention and the appended claims, the singular forms "a," "an" and

“the” are intended to include the plural forms as well, unless the context clearly indicates otherwise. The invention includes numerous alternatives, modifications, and equivalents as will become apparent from consideration of the following detailed description.

[00137] It will be understood that although the terms “first,” “second,” “third,” “a),” “b),” and “c),” etc. may be used herein to describe various elements of the invention, and the claims should not be limited by these terms. These terms are only used to distinguish one element of the invention from another. Thus, a first element discussed below could be termed an element aspect, and similarly, a third without departing from the teachings of the present invention. Thus, the terms “first,” “second,” “third,” “a),” “b),” and “c),” etc. are not intended to necessarily convey a sequence or other hierarchy to the associated elements but are used for identification purposes only. The sequence of operations (or steps) is not limited to the order presented in the claims.

[00138] Unless otherwise defined, all terms (including technical and scientific terms) used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. It will be further understood that terms, such as those defined in commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the present application and relevant art and should not be interpreted in an idealized or overly formal sense unless expressly so defined herein. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. All publications, patent applications, patents and other references mentioned herein are incorporated by reference in their entirety. In case of a conflict in terminology, the present specification is controlling.

[00139] Also, as used herein, “and/or” refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative (“or”).

[00140] The terms “decrease,” “lower,” “lessen,” and “reduce” generally refer to the ability of compositions according to the present approach to produce and/or cause a lesser physiological response (i.e., a measurable downstream effect), as compared to the response caused by either vehicle or a control molecule/composition, e.g., decreased tumor volume. A “decrease” or “reduced” response is typically a “statistically significant” response, and may include an decrease that is 1.1, 1.2, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, or more times (e.g., 500, 1000 times)

(including all integers and decimal points in between and above 1, e.g., 1.5, 1.6, 1.7, 1.8, etc.) the response produced by normal, untreated, or control-treated subject.

[00141] The phrase “treatment cycle” refers to a course of treatment, such as a dosing schedule that is repeated on a regular or pre-defined basis. A treatment cycle can comprise several days of treatment followed by several days of rest. For example only, an agent may be administered daily for two weeks, followed by two weeks of no treatment, over a 4-week treatment cycle. It should be appreciated that a treatment cycle may depend on a number of factors, such as the disease state, age, sex, and weight of the individual, as well as the particular agent(s) and/or methodologies, to elicit a desired response in the individual.

[00142] Unless the context indicates otherwise, it is specifically intended that the various features of the invention described herein can be used in any combination. Moreover, the present invention also contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be excluded or omitted. To illustrate, if the specification states that a complex comprises components A, B and C, it is specifically intended that any of A, B or C, or a combination thereof, can be omitted and disclaimed.

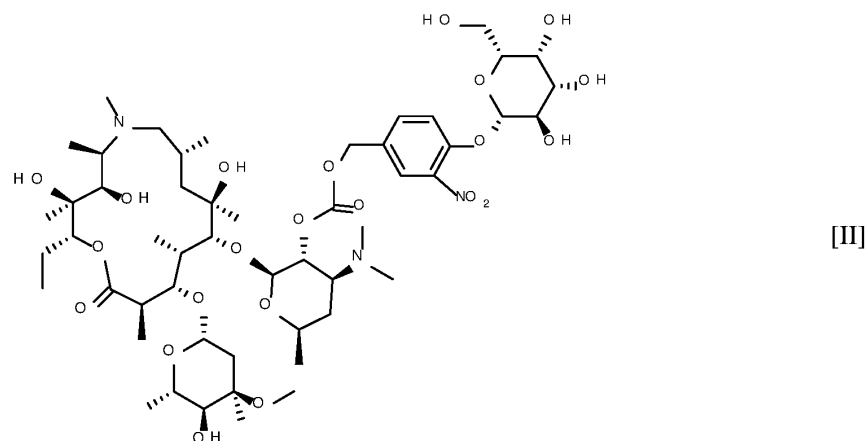
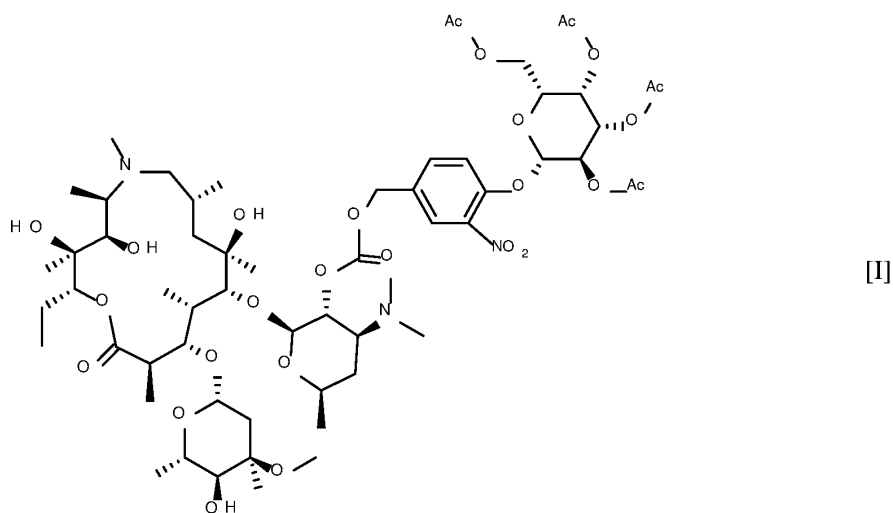
[00143] The term “about,” as used herein when referring to a measurable value, such as, for example, an amount or concentration and the like, is meant to encompass variations of $\pm 20\%$, $\pm 10\%$, $\pm 5\%$, $\pm 1\%$, $\pm 0.5\%$, or even $\pm 0.1\%$ of the specified amount. A range provided herein for a measurable value may include any other range and/or individual value therein.

[00144] Having thus described certain embodiments of the present invention, it is to be understood that the invention defined by the appended claims is not to be limited by particular details set forth in the above description as many apparent variations thereof are possible without departing from the spirit or scope thereof as hereinafter claimed.

CLAIMS

What is claimed is:

1. A composition comprising a dipeptidyl peptidase-4 (DPP-4) inhibitor and one of azithromycin, Compound [I], and Compound [II]:

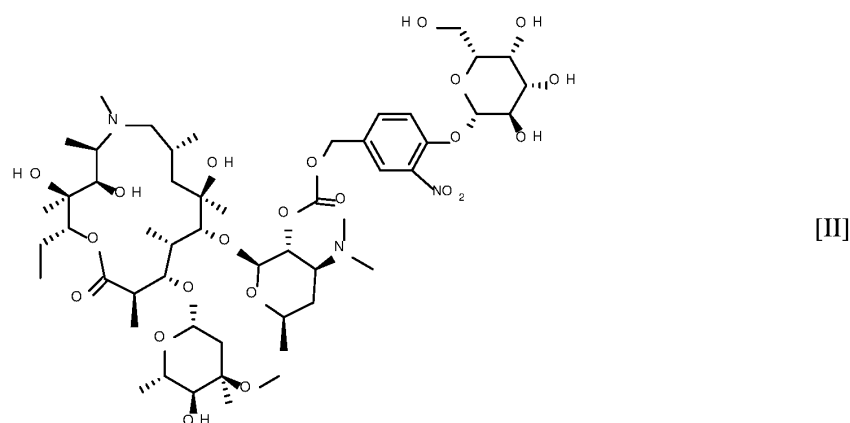
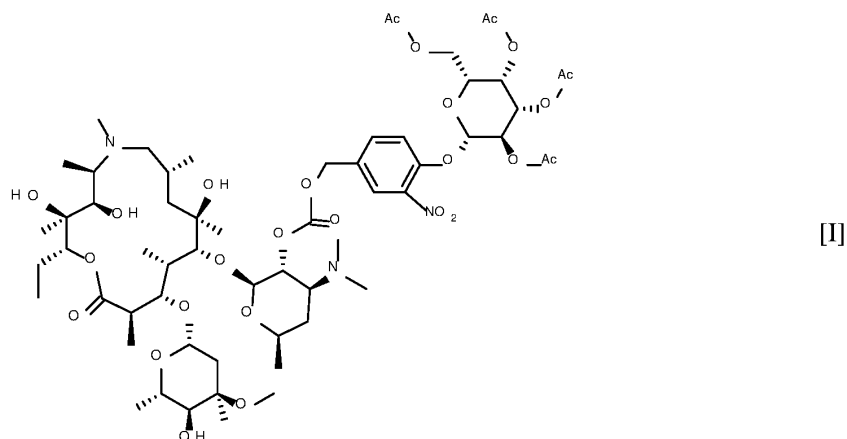


, or a pharmaceutically acceptable salt thereof.

2. The composition of claim 1, wherein the DPP-4 inhibitor is selected from the group consisting of sitagliptin, saxagliptin, linagliptin, alogliptin, and vildagliptin.

3. The composition of claim 1, wherein the DPP-4 inhibitor is sitagliptin.

4. A pharmaceutical composition comprising a pharmaceutically effective amount of a dipeptidyl peptidase-4 (DPP-4) inhibitor and one of azithromycin, Compound [I], and Compound [II]:



, or a pharmaceutically acceptable salt thereof, and at least one pharmaceutically acceptable carrier.

5. The pharmaceutical composition of claim 4, wherein the DPP-4 inhibitor is selected from the group consisting of sitagliptin, saxagliptin, linagliptin, alogliptin, and vildagliptin.

6. The pharmaceutical composition of claim 4, wherein the DPP-4 inhibitor is sitagliptin.

7. The pharmaceutical composition of any one of claims 4-6, wherein the DPP-4 inhibitor is present at $100 \text{ mg} \pm 10 \text{ mg}$, and the one of azithromycin, Compound [I], and Compound [II] is present at $500 \text{ mg} \pm 50 \text{ mg}$.

8. A method for treating or preventing tumor recurrence and/or metastasis, the method comprising administering to a patient at risk of tumor recurrence and/or metastasis a pharmaceutically effective amount of the pharmaceutical composition of any one of claims 4-7.

9. The method of claim 8, wherein the administering is performed at least one of prior to a cancer treatment, with a cancer treatment, and following a cancer treatment.

10. A method for inhibiting the propagation of cancer stem cells in a patient, the method comprising administering to the patient a pharmaceutically effective amount of the pharmaceutical composition of any one of claims 4-7.

11. The method of claim 10, wherein the administering is performed at least one of prior to a cancer treatment, with a cancer treatment, and following a cancer treatment.

12. A method for treating cancer, the method comprising: administering to a person having cancer a pharmaceutically effective amount of the pharmaceutical composition of any one of claims 4-7.

13. The method of claim 12, wherein the administering is performed at least one of prior to a cancer treatment, with a cancer treatment, and following a cancer treatment.

14. A method for eradicating senescent cells, the method comprising administering to a patient a pharmaceutically effective amount of the pharmaceutical composition of any one of claims 4-7.

15. A method for minimizing the accumulation of senescent cells in a subject, the method comprising administering to the subject a pharmaceutically effective amount of the pharmaceutical composition of any one of claims 4-7.

16. A method for delaying the onset of senescence in a subject, the method comprising administering to the subject a pharmaceutically effective amount of the pharmaceutical composition of any one of claims 4-7.

17. A method for treating the effects of aging in a subject, the method comprising administering to the subject a pharmaceutically effective amount of the pharmaceutical composition of any one of claims 4-7.

18. An anti-aging therapeutic method comprising administering to a subject a pharmaceutically effective amount of the pharmaceutical composition of any one of claims 4-7.