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(54) **Title:** BIOSYNTHESIS AND DELIVERY OF THERAPEUTIC COMPOUNDS

(57) **Abstract:** The present invention provides methods and compositions useful for treating a disease condition, e.g., neoplastic growth using microorganisms targeting to neoplastic growth sites and containing small molecules or molecules synthesized via enzymatic pathways.

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BIOSYNTHESIS AND DELIVERY OF THERAPEUTIC COMPOUNDS

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application No. 60/704,051, filed July 29, 2005, which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] One problem in the use of toxic anti-cancer therapeutics is the delivery of the toxin to the site of action at toxic doses without the concomitant delivery of a toxic dose to normal cells. Controlled or selective delivery of the therapeutic to the site of action can overcome the problem. Other potential therapeutics have pharmacokinetic problems, such as short half-lives *in vivo*, that could be overcome with a sustained delivery method. Various drug delivery techniques to preferentially deliver therapeutics to tumor sites have been described. One method is the use of preferentially replicating bacteria as tumor-targeting vectors (Pawelek et al. LANCET ONCOLOGY 4: 548-556 SEP 2003).

[0003] The mechanism that the microorganism uses to target the tumor may be known or unknown. One possible known mechanism is exemplified by the obligate anaerobe *Clostridium,* which has been used to target the hypoxic environment of the tumor. (For a review, see Minton NATURE REVIEWS MICROBIOLOGY 1(3):237-242 2003). Spores of *Clostridium* injected into tumour-bearing animals selectively germinate in tumors due to the poor vasculation of most tumors, leading to hypoxic conditions in which *Clostridium* can grow. *Clostridium* has been engineered to produce cytokines such as TNF-α such that a growth of the engineered strain in the tumor leads to increased tumor death due to the presence of the cytokine. *Clostridium* has also been engineered to produce pro-drug converting enzymes (e.g. as cytosine deaminase) that convert a pro-drug (e.g. 5-fluorocytosine), delivered through traditional methods, to an active drug (e.g. 5-fluoruracil) at the site of the tumor.

[0004] Another example is the use of attenuated *Salmonella* to deliver proteins, antigens, or pro-drug converting enzymes to the site of tumors (Sznol et al. JOURNAL OF CLINICAL INVESTIGATION 105 (8): 1027-1030 APR 2000, Low et al. NATURE BIOTECHNOLOGY 17 (1): 37-41 JAN 1999). These *Salmonella* strains, when injected intravenously, preferentially localize and replicate within tumors, achieving as much as three orders of magnitude higher cell densities within those regions of tumors than in normal tissue. A strain of *Salmonella typhimurium* was repeatedly mutagenized and selected for its ability to preferentially infect cancerous cells (as opposed to normal cells) *in vitro*, to chemotacticly respond to secretory products of cancer cells *in vitro*, and to preferentially infect cancerous cells *in vivo*. In this way, a "super-infective, tumor-targeting" strain was created that would more readily infect tumor cells. (Pawelek et al. U.S. Patent # 6,685,935). These strains were further modified to reduce their virulence versus normal cells by modifying the *msbB* gene altering the lipid A produced by the strain. (Low et al. NATURE BIOTECHNOLOGY 17 (1): 37-41 JAN 1999). *Salmonella* has been engineered, in a similar manner to *Clostridium,* to produce cytokines such as TNF-α, protein toxins, such as Colicin E3, and pro-drug converting enzymes such as cytosine deaminase, and thymidine kinase.

[0005] Many natural products that can be extracted from various organisms have been used or have potential for use as chemotherapeutic drugs for the treatment of cancer. Examples include, but are not limited to, isoprenoids such as perillyl alcohol, limonene, taxol, and artemisinin, alkaloids such as vincristine and vinblastine, polyketides such as the epothilones and deoxytedanolide, and flavonoids such as silibinin

(Singh, et al. CURRENT CANCER DRUG TARGETS 4 (1): 1-11 FEB 2004). The biosynthesis of many of these compounds in recombinant microorganisms such as *E. coli* has been reported, including isoprenoids (see Reiling et al. BIOTECHNOLOGY AND BIOENGINEERING 87 (2): 200-212 JUL 20 2004, Martin et al. NATURE BIOTECHNOLOGY 21 (7): 796-802 JUL 2003); alkaloids (see Hailes, et al. ENZYME ENGINEERING XIII ANNALS OF THE NEW YORK ACADEMY OF SCIENCES 799: 391-396 1996); polyketides (see Kennedy et al., BIOCHEMISTRY 42 (48): 14342-14348 DEC 9 2003, Murli et al. JOURNAL OF INDUSTRIAL MICROBIOLOGY & BIOTECHNOLOGY 30 (8): 500-509 AUG 2003, Dayem et al. BIOCHEMISTRY 41 (16): 5193-5201 APR 23 2002, Pfeifer et al. SCIENCE 291 (5509): 1790-1792 MAR 2 2001); and flavonoids (see Kaneko et al. JOURNAL OF INDUSTRIAL MICROBIOLOGY & BIOTECHNOLOGY 30 (8): 456-461 AUG 2003, Hwang et al APPLIED AND ENVIRONMENTAL MICROBIOLOGY 69 (5): 2699-2706 MAY 2003).

[0006] There remains a need in the art to provide methods and compositions useful for making and delivering such compounds for their effective use as therapeutic agents, particularly for the treatment of cancer.

SUMMARY OF THE INVENTION

[0007] The present invention is based, in part, on the discovery that certain molecules especially small molecules or molecules synthesized via enzymatic pathways can be made in microorganisms preferentially targeted to desired tissues or cells, especially neoplastic tissues or cells. Accordingly present invention provides methods and compositions useful for treating a disease condition, *e.g.*, neoplastic growth using such microorganisms containing small molecules or molecules synthesized via enzymatic pathways.

[0008] In one embodiment, the present invention provides a pharmaceutical composition comprising a bacteria and a pharmaceutically acceptable carrier, wherein the bacteria comprises a heterologous nucleic acid molecule, wherein the nucleic acid molecule encodes a pathway for producing an agent and wherein the bacteria preferentially targets a site with neoplastic growth. In one embodiment, the agent is an isoprenoid compound.

[0009] In another embodiment, the present invention provides a method for treating a subject. The method comprises administering to a subject in need of such treatment an effective amount of the bacteria provided by the present invention. In one embodiment, the subject is a mammal. In one embodiment, the mammal is a human.

[0010] In another embodiment, the present invention provides a method for delivery an agent to a subject. The method comprises administering to a subject a bacteria comprising a heterologous nucleic acid molecule, wherein the nucleic acid molecule encodes a pathway for producing the agent. In one embodiment, the agent is an isoprenoid. In one embodiment, the bacteria is attenuated.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0011] The present invention is based, in part, on the discovery that certain molecules especially small molecules or molecules synthesized via enzymatic pathways can be made in microorganisms preferentially targeted to desired tissues or cells, especially neoplastic tissues or cells. Accordingly the present invention provides targeting microorganisms containing one or more heterologous nucleic acids encoding one or more pathways that produce such molecules and pharmaceutical compositions containing such microorganisms.

In addition, the present invention provides methods of using such targeting microorganisms for delivery of therapeutic agents and/or treating a disease condition, *e.g.*, neoplastic growth.

[0012] The microorganism provided by the present invention can be any microorganism that preferentially targets a desired site or location, *e.g.*, neoplastic site, including without any limitation tumor or cancer. For example, the microorganism of the present invention can preferentially replicate at a desired site, attach to, infect, or remain viable at a desired site or have an activity, *e.g.*, transcription or translation, at a higher level at a desired site than at an undesired site. Such microorganism can be a naturally existing microorganism or a modified version of any naturally existing microorganism.

[0013] In one embodiment, the microorganism of the present invention is a facultative aerobe or facultative anaerobe. Alternatively, the microorganism of the present invention is any microorganism that preferentially localizes or grows in hypoxic environment, *e.g.*, a condition usually associated with poor vasculation of neoplastic growth. In another embodiment, the microorganism of the present invention is *Escherichia coli,* including without any limitation enteroinvasive *Escherichia coli, Salmonella spp., Shigella spp., Yersinia enterocohtica, Listeria monocytogenies, Mycoplasma hominis,* and *Streptococcus spp.* In yet another embodiment, the microorganism of the present invention is *Salmonella,* including any species of *Salmonella, e.g., Salmonella typhi, Salmonella choleraesuis,* and *Salmonella enteritidis,* and any serotype of *Salmonella, e.g., typhimurium,* a subgroup of *Salmonella enteritidis* commonly referred to as *Salmonella typhimurium.* In still another embodiment the microorganism of the present invention is an obligate anaerobe such as *Bifidobacterium spp.* or *Clostridium spp.*

[0014] In still yet another embodiment, the microorganism of the present invention is attenuated so that it is less pathogenic than the original microorganism. For example, the microorganism of the present invention can be modified to reduce its toxicity or inflammatory effect. Alternatively, the microorganism of the present invention is modified to cause minimum or non-substantial harm or pathological effect to a host, *e.g.*, human or animal. Any known or later discovered means to attenuate a microorganism can be used. For example, lipopolysaccharide (LPS) or endotoxin is primarily responsible for the pathological effect of bacterial sepsis. One of the key components of LPS is lipid A (LA). Mutations to the LPS biosynthetic pathway can be used to produce modified LA that has less toxic effects. See also Roy and Coleman, 1994, J. Bacteriol. 176: 1639-1646.

[0015] According to the present invention, agents produced or carried by the microorganism of the present invention can be any agent, *e.g.*, small molecules or molecules synthesized via one or more enzymatic pathways. Typically, the agent produced by the microorganism of the present invention is a molecule with a molecular weight less than a protein or polypeptide, *e.g.*, a molecular weight that is less than or equal to about 1 kDa. Alternatively agents produced by the microorganism of the present invention can be larger; any molecule that can be produced by a pathway, *e.g.*, an enzymatic pathway for the production of a compound such as an isoprenoid.

[0016] Agents produced by the microorganism of the present invention have a desired effect or function. For example, agents produced by the microorganism can have a therapeutic effect such as an anti-neoplastic effect, *e.g.*, are capable of inhibiting neoplastic growth or causing cell death in a cancer cell in a solid tumor or other cancer. Agents produced by the microorganism of the present invention can have other beneficial effects as well, *e.g.*, agents for imaging tissues or cells associated with neoplastic growth can be produced and administered in accordance with the methods of the invention. In one embodiment, agents produced by the microorganism of the present invention are useful for treating or identifying neoplastic growth when

combined with another agent or treatment. For example, agents produced by the microorganism of the present invention can be any agent or molecule that can be sensitized or activated to produce a desired effect, *e.g.,* cytotoxicity or generating detectable signal upon radiation, laser treatment, etc. In another embodiment, the agent is directly toxic or otherwise growth inhibitory to the cancer or tumor cell, and is administered alone or in combination with another anti-cancer or tumor agent to treat cancer or tumor. In yet another embodiment, the agent produced by the microorganism of the present invention does not include antibodies or enzymes that activate pro-drugs.

[0017] In still another embodiment, agents produced by the microorganism of the present invention are naturally existing compounds, *e.g.,* compounds naturally produced in animals, plants, yeast, etc. Examples of such naturally existing compounds include but are not limited to 1) isoprenoids, *e.g.,* steroids, cholesterol, geraniol, farnesol, monoterpenes such as perillyl alcohol and limonene, sesquiterpenes such as artemisinin, diterpenes such as taxol, and carotenoids, 2) alkaloids, *e.g.,* vincristine and vinblastine, 3) polyketides, *e.g.,* epothilones (epothilone A, B, C, and D), deoxytedanolide, and geldanamycin, 4) flavonoids, *e.g.,* quercetin or silibinin and 5) phenylpropanoids such as honokiol and magnolol. Such agents produced by the microorganism of the present invention are produced by a recombinant, in part or wholly, enzymatic pathway, *e.g.,* the mevalonate pathway for converting acetyl-CoA to form the precursor to all isoprenoids, isopentenyl phyophosphate, through a mevalonate intermediate, the non-mevalonate pathway for converting pyruvate and glyceraldehyde-3-phosphate to form the isoprenoid precursor isopentenyl pyrophosphate, the acyl-CoA biosynthesis pathways for polyketide precursor synthesis, the phenylpropanoid biosynthesis pathway for flavonoid and phenylpropanoid precursor (coumarate) biosynthesis, and the tropane-, isoquinoline- and purine-alkaloid payhways for alkaloid precursor biosynthesis.

[0018] Agents produced by the microorganisms of the present invention are made by pathways encoded in whole or in part by one or more heterologous nucleic acids in the microorganisms. According to the present invention, heterologous nucleic acids of the present invention include any polymer of nucleic acids wherein: (a) the sequence of nucleic acids, either in part or whole, is foreign to (i.e., not naturally found in) a given host microorganism; (b) the sequence is naturally found in a given host microorganism, but in an unnatural (e.g., greater than expected) amount or location; or (c) the sequence of nucleic acids comprises two or more subsequences not found in the same relationship to each other in nature. For example, a heterologous nucleic acid sequence that is recombinantly produced can have two or more sequences from genes or sequences not naturally adjoined together, *e.g.,* arranged to make a new functional nucleic acid. In one embodiment, the present invention provides an expression vector for a host microorganism, *e.g.,* which vector can either integrate into the genome of a host microorganism or exist in a host microorganism extrachromosomally, wherein the expression vector contains a nucleic acid sequence coding for an enzyme that is not normally found in a host microorganism. With reference to the host microorganism's genome, then, in that embodiment, the nucleic acid sequence that codes for the enzyme is heterologous.

[0019] According to the present invention, the recombinant pathway in the microorganisms of the present invention can be any pathway suitable for making the desired agent. Usually, the pathway or one or more enzymes of which it is comprised is exogenous to the host microorganism employed in the therapy. Thus, in one embodiment, the pathway does not naturally exist in the host microorganism. In another embodiment, the pathway is a modified version of a naturally existing pathway in the host microorganism, *e.g.,* a host pathway in which one or more genes for one or more enzymes in the pathway is regulated by a

promoter not naturally adjoined to the gene or a gene with one or more mutations or alterations to change the function or output of the corresponding enzyme in the pathway. In one embodiment, the pathway of the present invention is an enzymatic pathway including at least one, two, three, four, or five enzymes necessary or useful for making a desired agent. In another embodiment, the pathway of the present invention is a mevalonate pathway. In another embodiment, the pathway is a combination of one or more enzymatic pathways provided in one or more heterologous nucleic acids. Alternatively, the pathway of the present invention in some embodiments includes one or more enzymatic pathways and one or more other molecules, *e.g.,* proteins or polypeptides capable of interacting directly or indirectly with the product(s) produced by the enzymatic pathway(s).

[0020] In another embodiment, the pathway is a combination of a mevalonate pathway and a pathway for converting isopentenyl pyrophosphate (IPP) to perillyl alcohol via geranyl pyrophosphate (GPP) and limonene.

[0021] In one specific embodiment, a tumor-targeting *Salmonella* is engineered to produce the therapeutic isoprenoid perillyl alcohol, as detailed in Example 1. Genes encompassing a mevalonate pathway are introduced into an attenuated, tumor-targeting *Salmonella*, whose enzyme products produce the isoprenoid precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). A gene encoding geranyl pyrophosphate synthase is also introduced into the *Salmonella*. The GPP synthase enzyme converts IPP and DMAPP into the molecule geranyl pyrophosphate (GPP). A gene encoding limonene synthase is also introduced in the *Salmonella*. Limonene synthase is responsible for the conversion of GPP to limonene. Limonene has been shown to have anticancer properties both *in vitro* and in animal models. In the example, the *Salmonella* strain is further engineered to express the genes encoding a limonene hydroxylase and its accessory ferredoxin and ferredoxin reductase. This limonene hydroxylase is responsible for the conversion of limonene to perillyl alcohol, which has also been shown to have anticancer properties *in vitro* and in animal models.

[0022] Similar methods could be used to produce other isoprenoids. Depending on the type of isoprenoid compound, the GPP synthase may be replaced by a farnesyl pyrophosphate synthase (for sesquiterpenoids), a geranylgeranyl pyrophosphate synthase (for diterpenoids), or other prenyltransferase. Likewise, the limonene synthase may be replaced by another terpene synthase that is responsible for making the carbon backbone of the isoprenoid of interest. Likewise, the limonene hydroxylase and associated enzymes may be replaced by one or more of a variety of different modifying enzymes that are responsible for the biosynthesis of the isoprenoid compound.

[0023] In a second specific embodiment a tumor-targeting *Salmonella* is engineered to produce the therapeutic polyketides Epothilones A and B, as detailed in Example 4. Genes encoding enzymes responsible for the production of the polyketide precursors malonyl-CoA and methylmalonyl-CoA from commonly occurring metabolites are expressed in the *Salmonella* strain. Specifically, the genes encoding the four subunits of acetyl-CoA transferase, responsible for the production of malonyl-CoA from bicarbonate and acetyl-CoA, and methylmalonyl-CoA mutase and methylmalonyl-CoA epimerase, the enzymes responsible for the production of (2S)-methylmalonyl-CoA from succinyl-CoA, are expressed in the *Salmonella* strain. Additionally, the epothilone polyketide synthase gene cluster can be expressed in the *Salmonella* strain. This polyketide synthase is responsible for the production of Epothilones C and D from malonyl-CoA and methylmalonyl-CoA, and cysteine. Additionally, a phosphopantetheinyl transferase must be expressed in the *Salmonella* strain which is responsible for the posttranslational modification of the polyketide synthase

such that it is active. Optionally, the *epoK* gene can be expressed along with appropriate P450 reductase auxiliary enzymes to convert Epothilones C and D to Epothilones A and B.

[0024] Similar methods could be used to produce other polyketides. Additional polyketide precursor pathways may be necessary to provide the precursors such as propionyl-CoA. Alternate polyketide synthases and modifying enzymes may be required and could be substituted for the Epothilone polyketide synthase and *epoK*.

[0025] According to the present invention, the pathway of the present invention can be regulated by including a regulatory sequence, such as a promoter, that affects the transcription of one or more genes for one or more enzymes in the pathway. The promoter can be either constitutive or inducible for the regulation of the expression of some or all of the enzymes of the pathway in the microorganism of the present invention. In one embodiment, one or more of the enzymes of the pathway is encoded by a gene that contains a constitutive promoter. A constitutive promoter can be selected such that the appropriate amount of the desired agent is delivered to a target site without inducing genetic instability of the microorganism of the present invention, or delivering therapeutically ineffective large or small quantities of the agent.

[0026] In another embodiment, one or more of the enzymes of the pathway is encoded by a gene that contains an inducible promoter, *e.g.*, a promoter regulated by an endogenous or exogenous molecule or condition. In one embodiment, the promoter is inducible by anaerobic conditions. Such a promoter includes the focAp1 promoter in *E. coli.*, which is particularly suitable for delivering the agents produced by the pathway of the present invention at sites with neoplastic growth that contain necrotic hypoxic areas. In other embodiments, promoters from genes, known or later discovered, that are up-regulated in a neoplastic growth environment are employed. In another embodiment, a quorum sensing promoter is used to express the enzyme(s) in proportion to the presence of other colonizing bacteria.

[0027] Any suitable means can be used to introduce one or more enzymes, pathways, or genetic control regions encoded by one or more heterologous nucleic acids to a host microorganism to prepare a recombinant host cell useful in the therapeutic methods of the present invention. For example, a variety of methods are well known in the art to introduce heterologous nucleic acids by transforming a microorganism with one or more expression vectors. Heterologous nucleic acids encoding members of the pathway of the present invention can be expressed in a single expression vector, or as a series of expression vectors, any one or more of which can integrate into the host chromosome. The expression vector(s) can optionally contain one or more selection markers, *e.g.*, genes conferring various selectable traits to the host microorganism, including without limitation, traits for antibiotic resistance on the host microorganism. Alternatively or in addition, genes that confer plasmid maintenance can be used; such genes include the *hok/sok* (Gerdes et al. PNAS 83: 3116-3120 May 1986), *ccd/sop* (Boe et al. JOURNAL OF BACTERIOLOGY 169 (10): 4646-4650 May 1987), and *parDE* (Easter et al. JOURNAL OF BACTERIOLOGY 179 (20): 6472-6479 OCT 1997) genes. The disclosure of US 2003/0148479, the entirety of which is incorporated herein by reference, provides detailed teaching and guidance with respect to introducing exogenous enzymatic pathways into microorganisms, *e.g.*, including methods for introducing a mevalonate pathway into *E. coli.*

[0028] Microorganisms of the present invention can additionally express any other desired molecules, *e.g.*, proteins or polypeptides not produced by the pathway that produces the therapeutic agent, but encoded by one or more heterologous, non-pathway-related nucleic acids. For example, microorganisms of the present invention can contain one or more pathway-produced agents and one or more additional molecules, *e.g.*, molecules that are compatible with or capable of enhancing, stimulating, or promoting the effect of

pathway-produced agents or molecules that confer some other benefit. For example, the microorganism of the present invention can additionally express an anti-neoplastic effector.

[0029] Examples of anti-neoplastic effectors include members of the TNF family or functional fragments thereof, *e.g.*, TNF-α, TNF-β, TNF-α-related apoptosis-inducing ligand (TRAIL), TNF-α-related activation-induced cytokine (TRANCE), TNF-α-related weak inducer of apoptosis (TWEAK), CD40 ligand (CD40L), LT-α (lymphotoxin alpha), LT-β (lymphotoxin beta), OX40L (OX40 ligand), FasL, CD27L (CD27 ligand), CD30L (CD30 ligand), 4-1BBL, APRIL (a proliferation-inducing ligand), LIGHT (a 29 kDa type II transmembrane protein produced by activated T cells), TL1 (a tumor necrosis factor-like cytokine), TNFSF16, TNFSF17, and AITR-L (ligand of the activation-inducible TNFR family member).

[0030] Other examples of anti-neoplastic effectors include 1) anti-angiogenic factors or functional fragments thereof, *e.g.*, endostatin, angiostatin, apomigren, anti-angiogenic antithrombin III, the 29 kDa N-terminal and a 40 kDa C-terminal proteolytic fragments of fibronectin, a uPA receptor antagonist, the 16 kDa proteolytic fragment of prolactin, the 7.8 kDa proteolytic fragment of platelet factor-4, the anti-angiogenic 24 amino acid fragment of platelet factor-4, the anti-angiogenic factor designated 13.40, the anti-angiogenic 22 amino acid peptide fragment of thrombospondin I, the anti-angiogenic 20 amino acid peptide fragment of SPARC, RGD and NGR containing peptides, the small anti-angiogenic peptides of laminin, fibronectin, procollagen and EGF, and peptide antagonists of integrin $\alpha_v\beta_3$ and the VEGF receptor, 2) cytotoxic polypeptides or peptides or functional fragments thereof, *e.g.*, members of the bacteriocin family, verotoxin, cytotoxic necrotic factor 1 (CNF1), cytotoxic necrotic factor 2 (CNF2), *Pasteurella multiocida* toxin (PMT), *Pseudomonas* endotoxin, hemolysin, CAAX tetrapeptides which are potent competitive inhibitors of farnesyltransferase, cyclin inhibitors, Raf kinase inhibitors, CDC kinase inhibitors, caspases, p53, p16, and p21, and 3) tumor inhibitory enzymes or functional fragments thereof, *e.g.*, methionase, asparaginase, lipase, phospholipase, protease, ribonuclease, DNAase, and glycosidase.

[0031] The present invention also provides pharmaceutical compositions of the microorganisms of the present invention that include one or more other non-active ingredients, *e.g.*, ingredients that do not interfere with the function of the agents produced or carried by the microorganism of the present invention but aid in the manufacture or use of the microorganism in therapy. For examples, the composition of the present invention can include a suitable carrier or another therapeutic agent.

[0032] Suitable carriers include aqueous carriers including any safe and effective material for use in the compositions of the present invention. In one embodiment, an aqueous carrier is used for compositions of the present invention suitable for oral administration. Suitable carriers also include, without limitation, thickening materials, humectants, water, buffering agents, abrasive polishing materials, surfactants, titanium dioxide, flavor system, sweetening agents, coloring agents, and mixtures thereof. A suitable carrier can also be a pharmaceutically acceptable carrier that is well known to those in the art. Such carriers include, without limitation, large, slowly metabolized macromolecules, *e.g.*, proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Pharmaceutically acceptable salts can also be used in the composition, for example, mineral salts such as sodium or stannous fluorides, or sulfates, as well as the salts of organic acids such as acetates, proprionates, carbonates, malonates, or benzoates. The composition can also contain liquids, *e.g.*, water, saline, glycerol, and ethanol, as well as substances, *e.g.*, wetting agents, emulsifying agents, or pH buffering agents.

[0033] According to another aspect of the present invention, microorganisms of the present invention are used to deliver one or more desired agents, *e.g.,* therapeutic agents, and/or treat a disease condition. In general, microorganisms of the present invention, *e.g.,* in a pharmaceutical composition, can be used to treat or identify neoplastic growth. Neoplastic growth includes any abnormal growth, *e.g.,* such as tumors or cancer. In one embodiment, neoplastic growth includes growth in sarcomas, carcinomas, lymphomas, germ line tumors, tumors of the central nervous system, breast cancer, prostate cancer, cervical cancer, uterine cancer, lung cancer, ovarian cancer, testicular cancer, thyroid cancer, astrocytoma, glioma, pancreatic cancer, stomach cancer, liver cancer, colon cancer, melanoma, renal cancer, bladder cancer, and mesothelioma.

[0034] In general, microorganisms useful in the therapeutic methods of the present invention can be used alone or in combination with other therapeutics or treatments, prior to, concurrently, or subsequently. According to the present invention, an effective amount of the composition of the present invention to be administered to a subject can be determined generally for large groups (for example, one dose for adults and another for children) or on a case-by-case basis. Factors to be considered usually include the total surface area of the patient to be treated, and/or the patient's age, body weight, disease stage, other disease conditions, and/or response to the initial treatment, and/or the duration of the treatment. For example, intratumoral injection can be at about $3x10^6$ CFU/m^2, systemic intravenous injection can be up to 10^9 CFU/m^2. Typically, the dosage range is from about 10^4 CFU/m^2 to about 10^9 CFU/m^2 with different preferential dosages depending on method of delivery, *e.g.,* from about 10^4 CFU/m^2 to about 10^7 CFU/m^2 for intratumoral while from about 10^4 CFU/m^2 to about $3x10^8$ CFU/m^2 for intravenous.

[0035] In one embodiment, the compositions used in the therapeutic methods of the present invention are prepared for administration as an injectable agent, such as, for example, as a suspension of cells. The composition can also be formulated into an enteric-coated gel capsule according to known methods in the art. The agents or compositions of the present invention can, however, be administered in any medically acceptable manner, which may depend on the condition or patient being treated. Alternate administration routes include injections, by parenteral routes such as intravascular, intravenous, intraepidural or others, as well as oral, nasal, ophthalmic, rectal, vaginal, topical, or pulmonary, *e.g.,* by inhalation, administration. The compositions can also be directly applied to tissue surfaces. Sustained release, pH dependent release, and other specific chemical or environmental condition mediated release administration methods are also provided by the invention. The invention also provides methods and materials for administration of the therapeutic microorganisms, by depot injections and erodible implants.

EXAMPLES

[0036] The following examples are intended to illustrate and not to limit the invention in any manner, explicitly or implicitly. While the reagents and conditions described in the examples are illustrative of those that might be used, other reagents, procedures, methodologies, or techniques known to those skilled in the art may alternatively be used in view of this disclosure.

Example I. Engineering of an attenuated *Salmonella* to produce perillyl alcohol

[0037] To produce perillyl alcohol in sufficient amounts to be effective intratumorally, the microbial strain must be modified such that it has the ability to produce large amounts of the isoprenoid precursor isopentanyl pyrophosphate (IPP). Currently, the best method for doing so is the expression of the mevalonate pathway (USPTO publication #20030148479). From here, two molecules of IPP can be condensed to form geranyl pyrophosphate (GPP) using the enzyme geranylpyrophosphate synthase. GPP synthases are ubiquitous in

nature and have been cloned from many organisms as well as engineered via mutation of farnesyl pyrophosphate synthase (Reiling et al. BIOTECHNOLOGY AND BIOENGINEERING 87 (2): 200-212 JUL 20 2004). Geranyl pyrophosphate can then be cyclized by limonene synthase to form limonene. Limonene synthase can be found in citrus (limonene is the major constituent in orange peel oil) as well as mint, lavender and many other plants.

[0038] Limonene can then be converted to perillyl alcohol through the use of a cytochrome P450 that hydroxylates limonene at the 7 position. For example van Beilen, et al (APPLIED AND ENVIRONMENTAL MICROBIOLOGY 71 (4)1737-1744 APR 2005) have discovered a cytochrome p450 from *Mycobacterium* that oxidizes limonene to perillyl alcohol. Alternatively, a novel p450 could be discovered from a perillyl alcohol producing plant such as *Lavendula angustifolia* (lavender), peppermint, cherry, sage, perilla or various other plants. As an aside, each of these plants will also contain limonene synthase. A cytochrome P450 could also be evolved from existing P450 such as P450cam or P450BM3 (Sowden et al. ORGANIC & BIOMOLECULAR CHEMISTRY 3 (1): 57-64 2005). Alternatively, a P450 with existing limonene hydroxylase activity at the 7 position, but lacking the strict regiospecificity, could be evolved to obtain the strict regiospecificity, performing all or the vast majority of the hydroxylation at the 7 position.

[0039] Specifically perillyl alcohol can be produced in an attenuated, tumor-targeting *Salmonella,* such as *Salmonella* typhimurium strain VNP20009, via genetic engineering. One skilled in the art will recognize that similar techniques and constructs to those described below can be used to can be used to engineer tumor targeting bacteria such as other tumor-targeting strains of *Salmonella, Clostridium* and *Bifidobacterium* (Pawelek et al. LANCET ONCOLOGY 4: 548-556 2003), liver targeting bacteria such as *Listeria monocytogenes,* or other tumor targeting bacteria.

[0040] For the production of perillyl alcohol in any strain of *Salmonella typhimurium*, the following steps must be taken.

1. Introduction of the top half of the mevalonate pathway to covert acetyl-coA to mevalonate

[0041] The top half of mevalonate pathway (*atoB, hmgs, hmgr*) can be introduced into VNP20009 on plasmid pMevT (Martin et al. NATURE BIOTECHNOLOGY 21 (7): 796-802 JUL 2003) using standard electroporation methods.

2. Introduction of the bottom half of the mevalonate pathway and GPP synthase to convert mevalonate to geranylpyrophosphate

[0042] The bottom half of the pathway to be introduced into the perillyl alcohol producing bacterium will be substantially similar to the construct pMBIS (Martin et al. NATURE BIOTECHNOLOGY 21 (7): 796-802 JUL 2003). The only significant modification of this construct would be to swap the FPP synthase for a GPP synthase. This can be done through mutation or through cloning of a GPP synthase from another organism.

[0043] *E. coli* FPP synthase, such as the one in pMBIS, can be converted to GPP synthase through a single amino acid modification – the mutation of Ser81 to Phe (Reiling et al. BIOTECHNOLOGY AND BIOENGINEERING 87 (2): 200-212 JUL 20 2004). In a similar manner, PCR-mediated site-directed mutations within pMBIS can be performed using the Strategene Quick-Change kit and primers S81F-for and S81F-rev (see Reiling et al.) to form pMBIS'.

[0044] Alternatively, GPP synthase could be cloned from *Arabidopsis thaliana* (Bouvier et al. PLANT JOURNAL 24 (2): 241-252 OCT 2000), or another organism known or hypothesized to contain a limonene synthase

(e.g., any monoterpene-producing plant), using PCR primers with a SacI upstream end and a SacII downstream end. After digestion this SacI/SacII fragment could be ligated into the larger of the two SacI/SacII fragments of pMBIS (excising the FPP synthase), to form pMBIS(AtGPP).

3. Cloning of limonene synthase into an expression plasmid

[0045] Limonene synthase can be cloned into a vector such as pTrc99A. Using PCR primers with flanking NcoI sites, the limonene synthase can be amplified from *Cirus limon* (Lueker et al. EUROPEAN JOURNAL OF BIOCHEMISTRY 269 (13) 3160-3171 2002.), or another organism known or hypothesized to contain a limonene synthase (e.g., any citrus or mint species). After digestion this can then be cloned into the NcoI site in pTrc99A to form pTrcLS. Alternatively, analogous to the synthesis of the amorphadiene synthase gene in Martin, et al., a codon optimized limonene synthase could be synthesized from overlapping oligonucleotides through PCR based methods.

4. Cloning of perillyl alcohol synthetic genes into an expression plasmid

[0046] The CYP153 operon containing the perillyl alcohol P450, a ferredoxin, and a ferredoxin reductase from *Mycobacterium sp.* strain HXN-1500 can be cloned with primers HXN-1500-op-FW2 and –RV2 as in van Beilen, et al (APPLIED AND ENVIRONMENTAL MICROBIOLOGY 71 (4)1737-1744 APR 2005). After digestion with MunI and HinDIII, these can then be cloned between the EcoRI and HinDIII sites in pTrcLS to form pTrcPOH. This places the entire biosynthetic operon responsible for the conversion of FPP to perillyl alcohol under control of the lactose-inducible Trc promoter. Alternatively. analogous to the synthesis of the amorphadiene synthase gene in Martin, et al., a codon optimized CYP153 operon could be synthesized from overlapping oligonucleotides through PCR based methods.

5. Swapping of the lactose inducible promoter in each of the plasmids for a constitutive or anaerobic inducible promoter

[0047] pMevT, pMBIS', and pTrcPOH each have lactose inducible promoters. This promoter may not be ideal for the production of compounds intra-tumorally as delivery of lactose to the site of the tumor may be difficult. Elimination of the lac operator in each of these constructs would remove the requirement of lactose induction and leave the promoter constitutively expressing the genes under its control. This elimination could occur by mutating the existing operator such that the lac repressor no longer binds the operator, or by eliminating the operator sequence completely. The site of the lac operator is generally approximately -84 base pairs upstream of the transcription start site and the consensus sequence in *E. coli* is GGCAGTGAGCGCAACGCAA. Modifications to this or a related site in each of the plasmids can be performed using the Strategene Quick-change kit. One could also eliminate the operator site via PCR, amplifying the region of the plasmid from the end of the operator sequence to the beginning of the operator sequence using PCR primers with the same restriction sites. After digestion and re-ligation, the resulting plasmid would have the operator site removed. The resulting plasmids would be called pMevTΔlac, pMBIS'Δlac, and pTrcPOHΔlac.

[0048] Alternatively, a different constitutive promoter could be cloned upstream of each of the operons. In this way, one could regulate the strength of the expression of each of the operons and the rate of production of the small molecule by introducing stronger or weaker promoters. One could also clone a relevant inducible promoter upstream of each of the operons, for example the focAp1 promoter from *E. coli*.

[0049] This mutation/elimination/replacement of promoters could occur after the cloning of the genes to be expressed into the vector, or it could occur prior to the cloning of the genes into the vector. For example,

one could modify the lac operator pTrcPOH such that lactose induction was no longer necessary, or one could modify the lac operator in pTrc99A prior to the cloning of the limonene synthase.

[0050] Finally, antibiotic resistance markers on pMevTΔlac, pMBIS'Δlac, and pTrcPOHΔlac can be replaced with *hok/sok*, *parDE*, and *ccd/sop*, respectively to make the plasmids pMevTΔlacStab, pMBIS'ΔlacStab, and pTrcPOHΔlacStab.

Example 2. Determination of appropriate level of production of perillyl alcohol from the engineered VNP20009.

[0051] Preclinical *in vitro* experiments using perillyl alcohol have shown effectiveness at levels of approximately 0.5 to 1 mM (Xu et al. TOXICOLOGY AND APPLIED PHARMACOLOGY 195 (2): 232-246 MAR 1 2004). In order for the engineered strain to have the desired effectiveness, similar or higher concentrations are desired to be delivered to the site of the tumor. U.S. patent number 6190657 shows approximately $1x10^9$ cells per g of tumorous tissue in C57B6 mice 96 hours after inoculation with VNP20007. Since a gram of tumorous tissue occupies the volume of approximately 1 mL, productivity of $1x10^{-6}$ moles of perillyl alcohol per 10^9 cells is desired. Since 10^9 cells are found in approximately 2.5 ml of culture at an optical density of 1 when measured at 600nm, the appropriate level of production may be around $4x10^{-4}$ moles/L OD_{600}, or approximately 60 mg/L/OD.

[0052] VNP20009 with pMevTΔlacStab, pMBIS'ΔlacStab, and pTrcPOHΔlacStab can be inoculated to an OD of 0.05 and grown in anaerobic conditions, similar to those seen intratumorally, in Luria Broth. Growth can be tracked by measuring optical density and the concentration of perillyl alcohol can be measured using GC-MS or LC-MS. If, after 48 hours, the levels of perillyl alcohol are not 60 mg/L/OD, the rate of production of the compound must be increased. This can be done through the manipulation of promoter strengths controlling the genes in the pathway.

Example 3. Treatment of cancers using VNP20009 containing pMevTΔlacStab, pMBIS'ΔlacStab, and pTrcPOHΔlacStab.

[0053] VNP20009 containing pMevTΔlacStab, pMBIS'ΔlacStab, and pTrcPOHΔlacStab (perillyl alcohol-producing tumor-targeting attenuated *Salmonella*) can be grown in Luria Broth at 30 C in fermenters or shake flasks and collected during exponential phase growth at an OD_{600} of 0.5. This culture can be centrifuged and the supernatant can be discarded. The cell pellet can be resuspended in a 15% glycerol solution and centrifuged, discarding the supernatant. This wash procedure can be repeated two additional times. The cell pellet can then be resuspended in a volume of glycerol that is $1/10^{th}$ of the original volume of media and stored at -80C. These cultures will be approximately $2x10^9$ cells/ml.

[0054] As per the treatment course design in Neumanaitis et al. (CANCER GENE THERAPY 10: 737-744 2003), two hours prior to the administration of the strain, patients can begin a regimen of acetaminophen and indomethacin to control fever. Patients can then receive injections of strains at a total volume of 10% of the tumoral volume. Dilutions of the culture can be performed in preservative-free normal saline (0.9%). Previous studies have shown the effective intratumoral administration of VNP20009 at levels between 3×10^6 and 3×10^8 CFU/m^2.

Example 4. Engineering of an attenuated salmonella to produce Epothilones

[0055] In this example, an attenuated tumor-targeting bacterium is engineered to produce the potential chemotherapeutics epothilone A and B. This strain can then be delivered intravenously or injected into the site of a tumor.

[0056] Engineering of precursor pathway to produce malonyl-CoA and methylmalonyl-CoA

[0057] The production of the epothilones requires the polyketide precursors malonyl-CoA and methylmalonyl-CoA. Production of amounts of these precursors sufficient for the production intratumorally can be engineered into a tumor-targeting attenuated *Salmonella*.

[0058] *AccA* (carboxyltransferase subunit α), *AccD* (carboxyltransferase subunit β), *AccC* (biotin carboxylase) and *AccB* (biotin carboxyl carrier protein) can be cloned from *E. coli* and placed, as an operon, under the control of an inducible or constitutive promoter. This operon could be expressed from a plasmid vector or integrated into the chromosome. The four enzymes encoded by these genes form four subunits in acetyl-CoA carboxylase which is responsible for the transfer of a carboxyl from bicarbonate to acetyl-CoA to form malonyl-CoA.

[0059] *sbm* (methylmalonyl-CoA mutase *aka* sleeping beauty mutase) from *E. coli* (Haller et al. BIOCHEMISTRY 39: 4622-4629 2000) and methylmalonyl-CoA epimerase from *Streptomyces coelicolor* (Murli et al. JOURNAL OF INDUSTRIAL MICROBIOLOGY & BIOTECHNOLOGY 30 (8): 500-509 AUG 2003) encode proteins that are responsible for the conversion of succinyl-CoA via (2R)-methylmalonyl-CoA to (2S)-methylmalonyl-CoA. These genes can be cloned into an operon and placed under control of the same or a different inducible or constitutive promoter as the acetyl-coA carboxylase operon, and could be expressed from a plasmid vector or on the chromosome.

[0060] While *sbm* is a B12-dependent enzyme, *Salmonella typhimurium* has been shown to produce vitamin B12 *de novo* in anaerobic conditions. Since the sites of tumors are often, at least in part, anaerobic, the natural ability of *S. typhimurium* should be sufficient to produce the B12 requirements for the anaerobic areas as well as the aerobic areas surrounding the anaerobic areas of the tumor. Alternatively, the patient could be given supplements of vitamin B12, or the strain could be engineered to constitutively produce B12 (Raux et al. JOURNAL OF BACTERIOLOGY 178 (3): 753-767 FEB 1996).

[0061] 2. Expression of the polyketide posttranslational modification machinery and elimination of precursor utilization pathways

[0062] Polyketide synthases need to be postranslationally modified with a phosphopantetheinyl transferase (PPT) for activity. In the past, this has been successfully performed using a *Bacillus subtilis* PPT, the product of the gene *sfp* (Pfeifer et al. SCIENCE 291: 1790-1792 MAR 2001). In addition, *E. coli* has native methylmalonyl-CoA decarboxlase activity, responsible for the conversion of methylmalonyl-CoA to propionyl-CoA, encoded for by *ygfG*. This would be a deleterious activity in the epothilone production strain. *sfp* can be cloned under the control of the T7 promoter and integrated into the *ygfG* gene through homologous recombination, thus introducing a constitutatively expressed *sfp* and eliminating the expression of *ygfG*.

[0063] 3. Expression of the epothilone polyketide synthase gene cluster

[0064] The 56-kb nine-module epothilone biosynthetic gene cluster can then be synthesized from overlapping oligonucleotides using *E. coli* codon optimization and cloned into an expression vector under the control of an appropriate constitutive or an inducible promoter to engineer the strain to produce Epothilones C and D. The additional expression of the *epoK* gene, along with the appropriate P450 ferredoxin and ferredoxin reductase would allow the production of Epothilones A and B. Determination of the appropriate production level and administration of the epothilone-producing tumor-targeting attenuated *Salmonella* can be performed similar to examples 2 and 3.

[0065] Although the invention has been described with reference to the presently preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

CLAIMS

WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising a bacteria and a pharmaceutically acceptable carrier, wherein the bacteria comprises one or more heterologous nucleic acid molecules, wherein the nucleic acid molecule encodes a pathway for producing an agent and wherein the bacteria preferentially targets a site with neoplastic growth.

2. The pharmaceutical composition of claim 1, wherein the pathway is an enzymatic pathway.

3. The pharmaceutical composition of claim 1, wherein the pathway is exogenous to the bacteria.

4. The pharmaceutical composition of claim 1, wherein the pathway comprises at least two enzymes.

5. The pharmaceutical composition of claim 1, wherein the pathway is selected from the group consisting of mevalonate pathway, non-mevalonate pathway, acyl-CoA biosynthesis pathway, phenylpropanoid biosynthesis pathway, tropane pathway, isoquinoline pathway, and purine-alkaloid pathway.

6. The pharmaceutical composition of claim 1, wherein the pathway is regulated by a promoter operably linked to the heterologous nucleic acid molecule.

7. The pharmaceutical composition of claim 1, wherein the pathway is regulated by a promoter operably linked to the heterologous nucleic acid molecule.

8. The pharmaceutical composition of claim 1, wherein the agent is a small molecule and inhibits neoplastic growth.

9. The pharmaceutical composition of claim 1, wherein the agent is a naturally produced molecule.

10. The pharmaceutical composition of claim 1, wherein the agent is selected from the group consisting of isoprenoids, alkaloids, polyketides, flavonoids, and macrocycle.

11. The pharmaceutical composition of claim 1, wherein the agent is perillyl alcohol.

12. The pharmaceutical composition of claim 1, wherein the bacteria is attenuated.

13. The pharmaceutical composition of claim 1, wherein the bacteria is a facultative aerobe or facultative anaerobe.

14. The pharmaceutical composition of claim 1, wherein the bacteria is a member of the *Salmonella* genus.

15. The pharmaceutical composition of claim 1, wherein the bacteria is an obligate anaerobe.

16. The pharmaceutical composition of claim 1, wherein the bacteria is a member of the *Clostridium* genus.

17. The pharmaceutical composition of claim 1, wherein the site with neoplastic growth is a tumor or cancer site.

18. The pharmaceutical composition of claim 1, wherein the bacteria comprises a second heterologous nucleic acid encoding an anti-neoplastic effector.

19. A method for treating a subject comprising administering to a subject in need of such treatment an effective amount of the pharmaceutical composition of claim 12.

20. A method for delivery an agent to a subject comprising administering to a subject a bacteria comprising one or more heterologous nucleic acid molecules, wherein the nucleic acid molecule encodes a pathway for producing the agent and wherein the bacteria is attenuated.

21. The method of claim 18, wherein the bacteria preferentially targets a site with neoplastic growth.