(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 June 2018 (14.06.2018)

WIPO | PCT

(10) International Publication Number
WO 2018/106754 A1

(51) International Patent Classification:
A01N 63/00 (2006.01) *A01N 65/00* (2009.01)

(21) International Application Number:
PCT/US2017/064813

(22) International Filing Date:
06 December 2017 (06.12.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/430,962 07 December 2016 (07.12.2016) US

(71) Applicant: **MD BIOSCIENCES, INC.** [US/US]; 1 Imation Way, Oakdale, Minnesota 55128 (US).

(72) Inventor: **SALTZMAN, Daniel A.**; 1921 Glenhill Road, Mendota Heights, Minnesota 55118 (US).

(74) Agent: **RAHLIN, Katherine J.** et al.; Fredrikson & Byron, P.A., 200 South Sixth Street, Suite 4000, Minneapolis, Minnesota 55402-1425 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: METHODS OF SYNERGISTIC TREATMENT OF CANCER

(57) Abstract: A method of treating cancer includes administering a dose of a chemotherapy agent in combination with a dose of a composition consisting essentially of attenuated *Salmonella typhimurium*. The dose of the chemotherapy agent is lower than a maximum effective dose of the chemotherapy agent. The combination provides a synergistic reduction in tumor burden when compared to the reduction in tumor burden provided by administration of an equivalent dose of the chemotherapy agent without the composition consisting essentially of attenuated *Salmonella typhimurium*.

WO 2018/106754 A1

METHODS OF SYNERGISTIC TREATMENT OF CANCER

RELATED APPLICATIONS

[0001] This application claims priority to U.S. Patent Application No. 62/430,962, filed December 7, 2016, the entire contents of which are incorporated herein by reference.

TECHNICAL FIELD

[0002] This disclosure relates to methods of treating cancer, and more particularly, to methods of treating cancer with attenuated *Salmonella typhimurium*.

BACKGROUND

[0003] Despite continuing efforts of fighting cancer, nearly 600,000 cancer-related deaths still occur in the United States each year, and over 8 million cancer-related deaths occur worldwide. Thus, according to the World Health Organization, cancer continues to be a leading cause of morbidity and mortality worldwide. In addition, it is expected that annual cancer cases will rise from 14 million in 2012 to 22 million within the next two decades. The mainstay of cancer treatment is chemotherapy with the aim of curing or controlling this disease with the maximum tolerated dose (MTD) or the highest dose of a drug with tolerable side effects. Strategies employed to decrease the side effects include, for example, varying the combination of anti-neoplastic agents, metronomic dosing, and delivery of the chemotherapeutic agent directly to the affected organ. In the last several years, advancements have been made with immunotherapy for cancer treatment and many immunologic agents have demonstrated promise in this field. However, significant toxicities and tumor resistance limit this treatment strategy.

SUMMARY

[0004] In general, this disclosure relates to methods of treating cancer with *Salmonella typhimurium*. A dose of a chemotherapy agent is administered in combination with a dose of attenuated *Salmonella typhimurium*. The dose of the chemotherapy agent is lower than a maximum effective dose of the chemotherapy agent. The attenuated *Salmonella typhimurium* can include a truncated interleukin-2 gene. The combination provides a synergistic effect that provides a greater reduction in tumor burden than the administration of an equivalent dose of the chemotherapy agent alone. As a result, a lower and less toxic dose of the chemotherapy

agent can be used, which provides effective treatment while minimizing side effects caused by the toxicity of the chemotherapy agent.

[0005] In one embodiment, a method of treating cancer includes administering a combination of a dose of a chemotherapy agent and a dose of a composition consisting essentially of attenuated *Salmonella typhimurium*. The dose of the chemotherapy agent is lower than a maximum effective dose of the chemotherapy agent. The combination provides a synergistic reduction in tumor burden when compared to the reduction in tumor burden provided by administration of an equivalent dose of the chemotherapy agent without the composition consisting essentially of attenuated *Salmonella typhimurium*. The toxicity of the combination is lower than the toxicity of the maximum effective dose of the chemotherapy agent.

[0006] In another embodiment, a method of treating cancer includes administering a combination of a dose of a chemotherapy agent and a dose of attenuated *Salmonella typhimurium* containing a plasmid carrying a coding sequence encoding a truncated human interleukin-2, wherein the truncated human interleukin-2 consists of the amino acid sequence shown in SEQ ID NO: 2. The dose of the chemotherapy agent is lower than a maximum effective dose of the chemotherapy agent. The combination provides a synergistic reduction in tumor burden when compared to the reduction in tumor burden provided by administration of an equivalent dose of the chemotherapy agent without the attenuated *Salmonella typhimurium* containing the plasmid carrying the coding sequence encoding the truncated human interleukin-2. The toxicity of the combination is lower than the toxicity of the maximum effective dose of the chemotherapy agent.

[0007] In another embodiment, an anti-tumor agent for use in a method of treating cancer includes a combination of a dose of a chemotherapy agent and a dose of a composition consisting essentially of attenuated *Salmonella typhimurium*. The method includes administering the combination of the dose of the chemotherapy agent and the dose of the composition consisting essentially of attenuated *Salmonella typhimurium*. The dose of the chemotherapy agent is lower than a maximum effective dose of the chemotherapy agent. The combination provides a synergistic reduction in tumor burden when compared to the reduction in tumor burden provided by administration of an equivalent dose of the chemotherapy agent without the composition consisting essentially of attenuated *Salmonella typhimurium*. The toxicity of the combination is lower than the toxicity of the maximum effective dose of the chemotherapy agent.

[0008] In another embodiment, an anti-tumor agent for use in a method of treating cancer includes a combination of a dose of a chemotherapy agent and a dose of attenuated

Salmonella typhimurium containing a plasmid carrying a coding sequence encoding a truncated human interleukin-2, wherein the truncated human interleukin-2 consists of the amino acid sequence shown in SEQ ID NO: 2. The method includes administering the dose of the chemotherapy agent in combination with the dose of attenuated *Salmonella typhimurium* containing the plasmid carrying the coding sequence encoding the truncated human interleukin-2. The dose of the chemotherapy agent is lower than a maximum effective dose of the chemotherapy agent. The combination provides a synergistic reduction in tumor burden when compared to the reduction in tumor burden provided by administration of an equivalent dose of the chemotherapy agent without the attenuated *Salmonella typhimurium* containing the plasmid carrying the coding sequence encoding the truncated human interleukin-2. The toxicity of the combination is lower than the toxicity of the maximum effective dose of the chemotherapy agent.

BRIEF DESCRIPTION OF DRAWINGS

[0009] FIG. 1A shows the pIL2 plasmid containing the coding sequence encoding the human interleukin-2 protein, used to construct SalpIL2, attenuated *S. typhimurium* with the IL-2 gene.

[0010] FIG. 1B shows the pNG.1 plasmid without the coding sequence encoding the human interleukin-2 protein, used to construct SalpNG.1, attenuated *S. typhimurium* without the IL-2 gene.

[0011] FIG. 2 is a flow diagram of a method of treating cancer with a combination of attenuated *S. typhimurium* and a chemotherapy agent according to various embodiments.

[0012] FIG. 3 is a line graph of single tumor burden versus days post-treatment in mice treated with combinations of attenuated *S. typhimurium* and doxorubicin compared to control groups.

[0013] FIG. 4 is a line graph of single tumor burden versus days post-treatment in mice treated with a combination of SalpIL2 and doxorubicin compared to control groups.

[0014] FIG. 5 is a line graph of single tumor burden versus days post-treatment in mice treated with combinations of SalpNG.1 and doxorubicin compared to control groups.

[0015] FIG. 6 is a line graph of percent weight change versus days post-treatment in mice treated with combinations of attenuated *S. typhimurium* and doxorubicin compared to control groups.

DETAILED DESCRIPTION

[0016] The following detailed description is exemplary in nature and is not intended to limit the scope, applicability, or configuration of the disclosure in any way. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as those commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein may be used in the invention or testing, suitable methods and materials are described herein. The materials, methods and examples are illustrative only, and are not intended to be limiting. Those skilled in the art will recognize that many of the noted examples have a variety of suitable alternatives. The nomenclatures utilized in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well-known and commonly used in the art. Standard techniques may be used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients.

[0017] As used in the description herein and throughout the claims that follow, the meaning of "a," "an," and "the" includes plural reference unless the context clearly dictates otherwise.

[0018] "Attenuated," as used herein, means bacteria selected or altered to greatly diminish its capacity to cause disease, but still able to retain its ability to colonize the gut associated lymphoid tissue.

[0019] "Coding sequence" and "coding region," as used herein, are used interchangeably and refer to a polynucleotide that encodes a protein and, when placed under the control of appropriate regulatory sequences, expresses the encoded protein. The boundaries of a coding region are generally determined by a translation start codon at its 5' end and a translation stop codon at its 3' end.

[0020] "IL-2," as used herein, means the protein human interleukin-2.

[0021] "NK" or "NK cell," as used herein, means natural killer cell.

[0022] "Operably linked," as used herein, refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A regulator sequence is operably linked to a coding region when it is joined in such a way that expression of the coding region is achieved under conditions compatible with the regulatory sequence.

[0023] "Regulatory Sequence," as used herein, refers to a nucleotide sequence that regulates expression of a coding region to which it is operably linked. Non-limiting examples of regulatory sequences include promoters, transcription initiation sites, translation start sites, translation stop sites and terminators.

[0024] Attenuated *Salmonella typhimurium* has been developed as a vector to deliver therapeutic agents to tumors. The potential of *S. typhimurium* is largely due to its reported propensity to accumulate at greater than 1,000-fold higher concentration in tumors relative to healthy tissues. In addition, the genetic manipulability of *S. typhimurium* allows for the expression of foreign recombinant proteins, making these bacteria an effective delivery system for proteins that may be toxic when administered systemically.

[0025] Interleukin-2 (IL-2) is a protein naturally produced by the human body which promotes lymphocyte proliferation and enhances the cytolytic function of T cells and natural killer (NK) cells. It is thus able to stimulate the immune system to produce cancer-destroying white blood cells. IL-2 based immunotherapy in certain types of cancer has been studied for years with limited success. The amino acid sequence (SEQ ID NO: 3) of the normal human IL-2 protein encoded by SEQ ID NO: 4 (the DNA sequence encoding normal human IL-2) is shown in FIG. 3 of U.S. patent application Ser. No. 13/524,503 now U.S. Pat. No. 8,647,618, which is a continuation of U.S. patent application Ser. No. 12/425,927, filed Apr. 17, 2009 now U.S. Pat. No. 8,221,739, which is a continuation in part of and claims priority of U.S. patent application Ser. No. 10/834,587, filed Apr. 29, 2004 now abandoned, the contents of which applications are hereby incorporated by reference in their entirety.

[0026] While IL-2 is naturally produced by the human body, its maximum effectiveness requires a higher concentration and more specific delivery vector to the disease site. However, high doses of IL-2 are found to result in severe toxicity in many patients. A solution to this problem is using a live bacterial strain of *Salmonella typhimurium* which was attenuated to greatly diminish its capacity to cause disease. *S. typhimurium* is used due to its natural ability to colonize the gut associated lymphoid tissue (GALT), liver and spleen. Colonization of the liver by the attenuated *S. typhimurium* further initiates a generalized cellular response against the bacteria or can persist as a carrier state. The χ4550 strain of *S. typhimurium* used in the present disclosure contains a gene deletion constructed by transposon mutagenesis with Tn10 followed by selection for furasic acid resistance. This method of genetic alteration leads to deletional loss of Tn10 and adjacent DNA sequences to produce a deletion of aspartate semialdehyde dehydrogenase (asd). This mutation imposes a requirement for diaminopimelic acid. The lack of the asd enzyme in these bacteria leads to

the inability to construct a stable cell wall causing lethal lysis of the *S. typhimurium*. Thus, to insure stable expression of a desired protein, a plasmid (pYA292) was constructed which carries the asd gene.

[0027] FIG. 1A shows the pIL2 plasmid containing the coding sequence encoding the human interleukin-2 protein, used to construct SalpIL2, attenuated *S. typhimurium* with the IL-2 gene. In order to insure avirulence of the *S. typhimurium* strain, standard P22 phage transduction of the mouse virulent *S. typhimurium* SR-11 strain χ3306 was employed to construct the χ4550 strain that lacks the ability to synthesize adenylate cyclase and the cAMP receptor protein (CRP). Cyclic AMP and cAMP receptor protein are necessary for the transcription of many genes and operons concerned with the transport and breakdown of catabolites. Although cAMP is found in mammalian tissue and theoretically could be used by the bacteria to increase the potential for virulence, the lack of a cAMP receptor protein should abolish any benefit that could occur by the uptake of cAMP by these mutant bacteria.

[0028] A synthetic cDNA (SEQ ID NO: 5), coding for a truncated human IL-2 protein, optimized for expression in *Escherichia coli* was inserted into plasmid pYA292 using well known methods. The truncated cDNA (SEQ ID NO: 1) is a part of the synthetic IL-2 nucleotide sequence (SEQ ID NO: 5). This sequence is one nucleotide short of the sequence that was intended to code for a full-length mature human IL-2 protein. As used herein, "mature" means a protein lacking the beginning (N-terminal) 20 amino acid signal sequence that is cleaved off as the molecule is secreted from the a human cell. The mutation that occurred is a deletion of a "t" nucleotide between the "a" at position 272 and the "g" at position 273. This resulted in an in-frame taa stop codon at position 274 that truncated the resultant IL-2 protein. The resulting DNA nucleotide sequence is SEQ ID NO: 1 and the expressed protein is SEQ ID NO: 2.

[0029] Both the aspartate semialdehyde dehydrogenase (asd+) vector and the synthetic truncated human IL-2 cDNA were digested to completion with restriction enzymes EcoRI (Promega, Madison, Wis.) and HindIII (New England Biolabs, Beverly, Mass.). The about ~3.4 kb linearized vector fragment of pYA292 and the EcoRI-HindIII fragment of the IL-2 gene were isolated following agarose gel electrophoresis using the PrepaGene Kit (BioRad, Hercules, Calif.). The IL-2 gene fragment was ligated into the pYA292 vector using T4 DNA ligase (Promega, Madison, Wis.) with a 3:1 molar excess of insert and incubating for 4 hours at 16 °C. The ligation mix was then electroporated into the χ4550 strain of attenuated *S. typhimurium*. *S. typhimurium*, Δcya-1 Δcrp-1 ΔasdA1 strain χ4550 was grown in Luria Broth (Sigma, St. Louis, Mo.) containing 50 mg/ml diaminopimelic acid (DAP).

[0030] Cultures were grown to an absorbance of 0.200 at OD_{600} (approximately 10^8 colony forming units (cfu)/ml broth) and the cells were prepared for electroporation. Plasmid vector pYA292 and the ligation mix were electroporated into χ4550 utilizing an electroporation device (BioRad) with 0.2 cm disposable cuvettes. Cells were pulsed at 2.5 kV and 25 μF with a pulse controller at 200 ohms. Cells were then subsequently plated on Luria agar without DAP and recombinant clones were identified using the Magic Mini-Prep DNA Purification System (Promega), and restriction enzyme digestion with EcoRI and HindIII and gel electrophoresis with 1.2 agarose. The restriction enzyme mapping revealed a plasmid corresponding to that expected for an insert of the IL-2 fragment in pYA292 and the plasmid was renamed pIL2. The new transformant was renamed χ4550 (pIL2), also referred to herein as "SalpIL2."

[0031] Transforming an asd deleted strain with the plasmid (pIL2) allows for the stable expression of IL-2. As discussed above, stability of this vector is maintained because the particular strain of *S. typhimurium* used here (χ4550) lacks the enzyme aspartate semialdehyde dehydrogenase (asd), which, conversely, the plasmid containing the IL-2 gene (pIL2) contains. Bacteria lacking asd cannot make diaminopimelic acid (DAP), an essential component of the bacterial cell wall and, thus, would not long survive. Thus, if the attenuated *S. typhimurium* were to attempt to revert to its wild-type strain and lose the plasmid, it would die a "DAP-less" death. Because the loss of the IL-2 containing plasmid would also result in the loss of the plasmid encoded asd, stable expression of the IL-2 gene is achieved.

[0032] FIG. 1B shows the pNG.1 plasmid without the coding sequence encoding the human interleukin-2 protein, which is used to construct SalpNG.1, attenuated *S. typhimurium* without the IL-2 gene. SalpNG.1 was constructed by transforming χ4550 with pNG.1, a plasmid containing cDNA coding for aspartate semialdehyde dehydrogenase to complement the χ4550 requirement for diaminopimelic acid. To construct plasmid pNG.1, plasmid pYA292 was cut with EcoRI and HindIII, the ends filled in, and the plasmid recircularized to eliminate the LacZ(alpha) coding sequence. Overnight cultures of SalpNG.1 were grown in lysogeny broth (LB) and flash frozen with liquid nitrogen in 15% glycerol in LB and stored at -80°C. Before treatment, bacteria were thawed at 37°C and appropriately diluted in phosphate-buffered normal saline (PBS). The difference between SalpNG.1 and SalpIL2 is the presence of the truncated human IL-2 gene.

[0033] FIG. 2 is a flow diagram of method 200. Method 200 is a method of treating cancer with a combination of attenuated *S. typhimurium* and a chemotherapy agent according to

various embodiments. Method 200 includes administering a first dose of attenuated *S. typhimurium* and a first dose of a chemotherapy agent (201), administering a second dose of attenuated *S. typhimurium* (202), administering a second dose of a chemotherapy agent (203), administering a third dose of attenuated *S. typhimurium* (204), and administering a third dose of a chemotherapy agent (205). Method 200 need not include all of the steps shown in FIG. 2. For example, in some embodiments, method 120 may exclude the steps of administering a second dose of attenuated *S. typhimurium* (202) and administering a third dose of attenuated *S. typhimurium* (204). Additionally, method 200 can include additional steps, such as administering a fourth dose of attenuated *S. typhimurium* and/or administering a fourth dose of the chemotherapy agent. The number of doses of attenuated *S. typhimurium* and the chemotherapy agent in method 200 can be varied depending on the organism and the type of cancer being treated.

[0034] In one embodiment, method 200 includes administering a first dose of attenuated *S. typhimurium* and a first dose of a chemotherapy agent (201) on a first day, administering a second dose of a chemotherapy agent (203) on a second day a week after the first day, and administering a third dose of a chemotherapy agent (205) on a third day a week after the second day. In this embodiment, a single dose of attenuated *S. typhimurium* is administered throughout the entire treatment period. In another embodiment, method 200 includes administering a first dose of attenuated *S. typhimurium* and a first dose of a chemotherapy agent (201) on a first day, administering a second dose of attenuated *S. typhimurium* (202) on a second day a week after the first day, administering a second dose of a chemotherapy agent (203) on the second day a week after the first day, and administering a third dose of a chemotherapy agent (205) on a third day a week after the second day. In this embodiment, two doses of attenuated *S. typhimurium* are administered throughout the entire treatment period.

[0035] In another embodiment, method 200 includes administering a first dose of attenuated *S. typhimurium* and a first dose of a chemotherapy agent (201) on a first day, administering a second dose of attenuated *S. typhimurium* (202) on a second day three weeks after the first day, administering a second dose of a chemotherapy agent (203) on the second day three weeks after the first day, administering a third dose of attenuated *S. typhimurium* (204) on a third day three weeks after the second day, and administering a third dose of a chemotherapy agent (205) on the third day three weeks after the second day. This embodiment further includes administering a fourth dose of attenuated *S. typhimurium* and a fourth dose of a chemotherapy agent on a fourth day three weeks after the third day, administering a fifth dose

of attenuated *S. typhimurium* and a fifth dose of a chemotherapy agent on a fifth day three weeks after the fourth day, and administering a sixth dose of attenuated *S. typhimurium* and a sixth dose of a chemotherapy agent on a sixth day three weeks after the fifth day. In this embodiment, six doses of attenuated *S. typhimurium* are administered throughout the entire treatment period.

[0036] In some embodiments, administering the first dose of attenuated *S. typhimurium* (201) includes orally or intravenously administering the attenuated *S. typhimurium*. In one embodiment, administering a first dose of attenuated *S. typhimurium* (201) includes administering a oral dose of SalpIL2. In some embodiments, the dose is about 1×10^9 cfu. In another embodiment, administering a first dose of attenuated *S. typhimurium* (201) includes administering a intravenous (IV) dose of SalpNG.1. In some embodiments, the dose is about 2×10^6 cfu.

[0037] In some embodiments, administering a first dose of a chemotherapy agent (201) includes administering a IV dose of doxorubicin. In one embodiment, the dose of doxorubicin is 1.25 mg/kg. In another embodiment, the dose of doxorubicin is 2.5 mg/kg. In other embodiments, the chemotherapy agent can be carboplatin, cisplatin, cyclophosphamide, daunorubicin, oxaliplatin, 5-fluorouracil, gemcitabine, or any other appropriate chemotherapy agent.

[0038] Method 200 is advantageous, because the combination provides a synergistic effect that provides a greater reduction in tumor burden than the administration of the chemotherapy agent alone. As a result, a lower and less toxic dose of the chemotherapy agent can be used, which provides effective treatment while minimizing side effects caused by the toxicity of the chemotherapy agent.

EXAMPLES

BALB-neuT Tumor Treatment Model

[0039] The BALB-neuT model is a genetically engineered mouse model in which mammary tumor development is driven by expression of a constitutively activated rat homolog of human epidermal growth factor receptor 2. In this model, autochthonous tumors develop over several months and are palpable in the mammary pads of female mice around 16 weeks of age. The tumors closely resemble the aggressive Her2-driven cancer found in human patients.

[0040] BALB-neuT mice were maintained in specific pathogen free conditions and fed standard mouse chow (Harlan). Animals were cared for by the University of Minnesota's

Research Animal Resources, and all animal use was approved by the University's Institutional Animal Care and Use Facility. Genotyping for the *neu* transgene was performed by Transnetyx on male and female pups. Breeding pairs consisted of heterozygous males and homozygous negative females. Female mice that were positive for the *neu* transgene were monitored for tumor development.

Methods

[0041] Female BALB-neu-T mice spontaneously developed palpable mammary fat pad tumors around 16 weeks of age (approximately 50-60mm^3). At this time (day 0), the mice typically had 1-3 palpable tumors. For each experiment, individual tumors were measured by caliper, and their volume was calculated. Individual tumor volumes were calculated as spheroid (L x W^2 x 0.52) and combined to give a total tumor burden measurement for each mouse. Tumor burden data was gathered weekly from day 0 to day 35. Additionally, percent weight change data was gathered weekly for each mouse from day 0 to day 35. The percent weight change was calculated based on the baseline weight of each mouse.

[0042] Various embodiments of method 200, described above with respect to FIG. 2, were used to treat tumors in the BALB-neuT model using combinations of attenuated *S. typhimurium* and the chemotherapy agent doxorubicin. A prescribed amount per cfu of the appropriate *S. typhimurium* strain (SalpIL2 or SalpNG.1) was administered via intravenous injection or gavage orally in 100 μL of PBS. The doxorubicin was administered intravenously via tail vein. Additionally, using the same methods of administration, a number of control groups received PBS alone, doxorubicin alone, SalpIL2 alone, or SalpNG.1 alone.

[0043] One control group (301) received PBS alone. For this control group, the tumors in the mice enlarged over time, and new tumors appeared on the remaining fat pads, usually until each mammary pad developed a tumor. When left untreated, average total tumor burden per mouse reached 5.66 cm^3 by day 35, at which point the mice were moribund and euthanized.

[0044] Three control groups received doxorubicin alone. The first control group (302) received the maximum tolerated dose (MTD) of intravenous (IV) 5 mg/kg doxorubicin on days 0, 7, and 14. The second control group (303) received IV 2.5 mg/kg (50% reduction in MTD) doxorubicin on days 0, 7 and 14. The third control group (304) received IV 1.25 mg/kg (75% reduction in MTD) doxorubicin on days 0, 7, and 14.

[0045] One control group (305) of mice received SalpIL2 alone. This control group received an oral dose of $1x10^9$ cfu SalpIL2 on day 0 and did not receive any additional SalpIL2 or doxorubicin treatments. Another control group (306) received Salp NG.1 alone. This control group received an IV dose of $2x10^6$ cfu SalpNG.1 on day 0 and did not receive any additional SalpNG.1 or doxorubicin treatments.

[0046] For combination therapy treatment with SalpIL2, one group of mice (307) received an oral dose of $1x10^9$ cfu SalpIL2 as well as IV 1.25 mg/kg doxorubicin on day 0. Two additional doses of IV 1.25 mg/kg doxorubicin were administered on days 7 and 14. For combination therapy treatment with SalpNG.1, a first group of mice (308) received an IV dose of $2x10^6$ cfu SalpNG.1 as well as IV 1.25 mg/kg doxorubicin on day 0. Two additional doses of IV 1.25 mg/kg doxorubicin were administered on days 7 and 14. A second group of mice (309) received an IV dose of $2x10^6$ cfu SalpNG.1 as well as IV 2.5 mg/kg doxorubicin on day 0. Two additional doses of IV 2.5 mg/kg doxorubicin were administered on days 7 and 14.

Results

[0047] FIGS. 3-6 show the results of tumor treatment in the BALB-neuT model using combinations of attenuated *S. typhimurium* and the chemotherapy agent doxorubicin as compared to a number of control groups that received PBS alone, doxorubicin alone, SalpIL2 alone, or SalpNG.1 alone. The data shown includes day 0, on which the first treatment was administered, day 14 on which a second treatment was administered, and day 21, on which a third treatment was administered. Subsequent data points were taken post-treatment on days 28 and 35.

[0048] FIG. 3 is a line graph of single tumor burden versus days post-treatment in mice treated with combinations of attenuated *S. typhimurium* and doxorubicin compared to control groups. In the PBS control group (301), the mice did not survive or were moribund and euthanized by day 30 or 35. In the group that received the MTD of 5 mg/kg of doxorubicin (302), the tumor burden was the lowest and remained around 100 mm^3 at day 35. In the group that received 25% of the MTD of doxorubicin, i.e. 1.25 mg/kg of doxorubicin (304), the tumor burden was almost double that of the group that received the MTD of doxorubicin (302) by day 21, and almost 5 times as high by day 35. The groups that received a single oral dose of SalpIL2 (305) and a single IV dose of SalpNG.1 (306) showed a similar trend to that of the group that received 1.25 mg/kg of doxorubicin (304).

[0049] The group that received a combination of oral SalpIL2 and 1.25 mg/kg doxorubicin (307) and the group that received a combination of IV SalpNG.1 and 1.25 mg/kg doxorubicin (308) surprisingly showed a synergistic effect in tumor treatment. A statistically significant reduction in tumor burden is shown. The tumor burden by day 21 was less than double that of the group that received the MTD of doxorubicin (302), and was only slightly more than double by day 35. Unexpectedly, there was no difference in the combination treatment with the oral SalpIL2 and the combination treatment with the IV SalpNG.1. Thus, the combination treatments of 1.25 mg/kg of doxorubicin with either SalpIL2 or SalpNG.1 are nearly as effective as treatment with the MTD of doxorubicin alone and significantly more effective than treatment with 1.25 mg/kg of doxorubicin alone. The dose of doxorubicin in the combination treatments is only 25% of the MTD, which significantly reduces the toxic effects of the doxorubicin (explained in greater detail below with respect to FIG. 6).

[0050] FIG. 4 is a line graph of single tumor burden versus days post-treatment in mice treated with a combination of SalpIL2 and doxorubicin compared to control groups. As explained above with respect to FIG. 3, in the PBS control group (301), the mice did not survive or were moribund and euthanized by day 30 or 35. A statistically significant reduction in tumor burden is shown in the group that received a combination of a single oral dose of SalpIL2 and 1.25 mg/kg doxorubicin (307) as compared to the control group that received only a single oral dose of SalpIL2. The tumor burden of the control group that received a single oral dose of SalpIL2 (305) was double that of the group that received the combination of SalpIL2 and 1.25 mg/kg doxorubicin (307) by day 35. This further demonstrates the synergistic effect of the combination treatment of oral SalpIL2 and 1.25 mg/kg doxorubicin.

[0051] FIG. 5 is a line graph of single tumor burden versus days post-treatment in mice treated with combinations of SalpNG.1 and doxorubicin compared to control groups. As explained above with respect to FIG. 3, in the PBS control group (301), the mice did not survive or were moribund and euthanized by day 30 or 35. In the control group that received the MTD of 5 mg/kg of doxorubicin (302), the tumor burden was the lowest and remained around 100 mm^3 at day 35. In the control group that received 25% of the MTD of doxorubicin, i.e. 1.25 mg/kg of doxorubicin (304), the tumor burden was almost double that of the group that received the MTD of doxorubicin by day 21, and almost 5 times as high by day 35. In the control group that received 50% of the MTD of doxorubicin, i.e. 2.5 mg/kg of doxorubicin (303), as expected, the tumor burden was in between that of the control group

that received the MTD of doxorubicin (302) and the group that received 1.25 mg/kg of doxorubicin (304).

[0052] The group that received a combination of IV SalpNG.1 and 1.25 mg/kg doxorubicin (308) showed a synergistic effect in tumor treatment. A statistically significant reduction in tumor burden is shown. The tumor burden by day 21 was less than double that of the group that received the MTD of doxorubicin (302), and was only slightly more than double by day 35. The group that received a combination of IV SalpNG.1 and 2.5 mg/kg doxorubicin (309) also showed a synergistic effect in tumor treatment. A statistically significant reduction in tumor burden is also shown for this group. The tumor burden by day 21 was even less than the tumor burden in the group that received a combination of SalpNG.1 and 1.25 mg/kg, and by day 35 was barely double the tumor burden of the group that received the MTD of doxorubicin (302).

[0053] Thus, the combination treatments of SalpNG.1 with 1.25 mg/kg of doxorubicin or 2.5mg/kg doxorubicin are nearly as effective as treatment with the MTD of doxorubicin alone and significantly more effective than treatment with 1.25 mg/kg of doxorubicin alone or 2.5 mg/kg of doxorubicin alone. This further demonstrates the synergistic effect of the combination treatment of IV SalpNG.1 and 1.25 mg/kg doxorubicin, as well as demonstrates the synergistic effect of the combination treatment of IV SalpNG.1 and 2.5 mg/kg of doxorubicin.

[0054] FIG. 6 is a line graph of percent weight change versus days post-treatment in mice treated with combinations of attenuated *S. typhimurium* and doxorubicin compared to control groups. Weight change in mice is an accurate measure of the degree of toxicity of a chemotherapy agent. Significant weight loss translates to significant toxicity. While the control group that received PBS (301) saw a greater than 25% increase in weight by day 30, the mice in that group died or were moribund and euthanized due to tumor growth. The control group that received the MTD dose of 5 mg/kg of doxorubicin (302) showed a significant reduction in weight, with a loss of greater than 20% by day 14 and almost 10% by day 35. This indicates significant toxicity of the MTD dose of doxorubicin. The group that received 25% of the MTD dose of doxorubicin, i.e. 1.25 mg/kg of doxorubicin (304), did not show any weight loss by day 14 and showed less than a 10% weight gain by day 35, and thus was deemed non-toxic. The group that received a single oral dose of SalpIL2 (305) showed a slight weight gain by day 14 and almost a 15% weight gain by day 35. The control group that received a single IV dose of SalpNG.1 (306) showed no weight loss by day 14 and about a 10% weight gain by day 35.

[0055] The group that received a combination treatment of oral SalpIL2 and 1.25 mg/kg of doxorubicin (307) did not show any weight loss by day 14 and showed about a 10% weight gain by day 35. The group that received a combination treatment of IV SalpNG.1 and 1.25 mg/kg of doxorubicin (308) showed a slight weight loss by day 14 and greater than a 5% weight gain by day 35. This data further supports the synergistic effect of combination treatments of doxorubicin with attenuated *S. typhimurium* with minimal or no toxicity.

[0056] As explained with respect to FIGS. 3-5, treatments with 1.25 mg/kg of doxorubicin in combination with oral SalpIL2 or IV SalpNG.1 are nearly as effective as treatment with the MTD of doxorubicin alone and significantly more effective than treatment with 1.25 mg/kg of doxorubicin alone. The dose of doxorubicin in the combination treatments is only 25% of the MTD, which significantly reduces the toxic effects of the doxorubicin, as evidenced by the statistically significant reduction in weight loss shown in FIG. 6. Therefore, the synergistic effect of combination treatments of a significantly less toxic dose of doxorubicin combined with an appropriate attenuated *S. typhimurium* strain provides an effective tumor treatment while minimizing the negative side effects, such as weight loss, of chemotherapy agents.

CLAIMS

1. A method of treating cancer, the method comprising:

administering a combination of a dose of a chemotherapy agent and a dose of a composition consisting essentially of attenuated *Salmonella typhimurium*;

wherein the dose of the chemotherapy agent is lower than a maximum effective dose of the chemotherapy agent;

wherein the combination provides a synergistic reduction in tumor burden when compared to the reduction in tumor burden provided by administration of an equivalent dose of the chemotherapy agent without the composition consisting essentially of attenuated *Salmonella typhimurium*; and

wherein the toxicity of the combination is lower than the toxicity of the maximum effective dose of the chemotherapy agent.

2. The method of claim 1, wherein the dose of the chemotherapy agent is less than or equal to about 1.25 mg/kg.

3. The method of claim 1, wherein the dose of the chemotherapy agent is 25% of the maximum effective dose of the chemotherapy agent and the combination.

4. The method of claim 1, wherein the dose of attenuated *Salmonella typhimurium* is about 2×10^6 colony forming units.

5. The method of claim 1, wherein the attenuated *Salmonella typhimurium* is administered intravenously.

6. The method of claim 1, wherein a first dose of the chemotherapy agent and the dose of the attenuated *Salmonella typhimurium* are administered on a first day.

7. The method of claim 6, wherein a second dose of the chemotherapy agent is administered on a second day a week after the first day.

8. The method of claim 7, wherein a third dose of the chemotherapy agent is administered on a third day a week after the second day.

9. The method of claim 6, wherein a plurality of doses of the chemotherapy agent and a plurality of doses of the attenuated *Salmonella typhimurium* are administered on a plurality of days after the first day.

10. The method of claim 1, wherein the chemotherapy agent is selected from the group consisting of doxorubicin, carboplatin, cisplatin, cyclophosphamide, daunorubicin, oxaliplatin, 5-fluorouracil, and gemcitabine.

11. A method of treating cancer, the method comprising:

administering a combination of a dose of a chemotherapy agent and a dose of attenuated *Salmonella typhimurium* containing a plasmid carrying a coding sequence encoding a truncated human interleukin-2, wherein the truncated human interleukin-2 consists of the amino acid sequence shown in SEQ ID NO: 2;

wherein the dose of the chemotherapy agent is lower than a maximum effective dose of the chemotherapy agent;

wherein the combination provides a synergistic reduction in tumor burden when compared to the reduction in tumor burden provided by administration of an equivalent dose of the chemotherapy agent without the attenuated *Salmonella typhimurium* containing the plasmid carrying the coding sequence encoding the truncated human interleukin-2;and

wherein the toxicity of the combination is lower than the toxicity of the maximum effective dose of the chemotherapy agent.

12. The method of claim 11, wherein the dose of the chemotherapy agent is less than or equal to 1.25 mg/kg.

13. The method of claim 11, wherein the dose of the chemotherapy agent is 25% of the maximum effective dose of the chemotherapy agent.

14. The method of claim 9, wherein the dose of attenuated *Salmonella typhimurium* containing the plasmid carrying the coding sequence encoding the truncated human interleukin-2 is about 1×10^9 colony forming units.

15. The method of claim 9, wherein the attenuated *Salmonella typhimurium* containing the plasmid carrying the coding sequence encoding the truncated human interleukin-2 is administered orally.

16. The method of claim 11, wherein a first dose of the chemotherapy agent and the dose of the attenuated *Salmonella typhimurium* containing the plasmid carrying the coding sequence encoding the truncated human interleukin-2 are administered on a first day.

17. The method of claim 16, wherein a second dose of the chemotherapy agent is administered on a second day a week after the first day.

18. The method of claim 17, wherein a third dose of the chemotherapy agent is administered on a third day a week after the second day.

19. The method of claim 16, wherein a plurality of doses of the chemotherapy agent and a plurality of doses of the attenuated *Salmonella typhimurium* are administered on a plurality of days after the first day.

20. The method of claim 9, wherein the chemotherapy agent is selected from the group consisting of doxorubicin, carboplatin, cisplatin, cyclophosphamide, daunorubicin, oxaliplatin, 5-fluorouracil, and gemcitabine.

21. An anti-tumor agent for use in a method of treating cancer, the anti-tumor agent comprising a combination of a dose of a chemotherapy agent and a dose of a composition consisting essentially of attenuated *Salmonella typhimurium* for use in a method of treating cancer, the method comprising:

administering the combination of the dose of the chemotherapy agent and the dose of the composition consisting essentially of attenuated *Salmonella typhimurium*;

wherein the dose of the chemotherapy agent is lower than a maximum effective dose of the chemotherapy agent;

wherein the combination provides a synergistic reduction in tumor burden when compared to the reduction in tumor burden provided by administration of an equivalent dose of the chemotherapy agent without the composition consisting essentially of attenuated *Salmonella typhimurium*; and

wherein the toxicity of the combination is lower than the toxicity of the maximum effective dose of the chemotherapy agent.

22. The anti-tumor agent of claim 21, wherein the dose of the chemotherapy agent is less than or equal to about 1.25 mg/kg.

23. The method of claim 21, wherein the dose of the chemotherapy agent is 25% of the maximum effective dose of the chemotherapy agent.

24. The anti-tumor agent of claim 21, wherein the dose of attenuated *Salmonella typhimurium* is about 2 x 10^6 colony forming units.

25. The anti-tumor agent of claim 21, wherein the attenuated *Salmonella typhimurium* is administered intravenously.

26. The anti-tumor agent of claim 21, wherein a first dose of the chemotherapy agent and the dose of the attenuated *Salmonella typhimurium* are administered on a first day.

27. The anti-tumor agent of claim 26, wherein a second dose of the chemotherapy agent is administered on a second day a week after the first day.

28. The anti-tumor agent of claim 27, wherein a third dose of the chemotherapy agent is administered on a third day a week after the second day.

29. The method of claim 26, wherein a plurality of doses of the chemotherapy agent and a plurality of doses of the attenuated *Salmonella typhimurium* are administered on a plurality of days after the first day.

30. The anti-tumor agent of claim 21, wherein the chemotherapy agent is selected from the group consisting of doxorubicin, carboplatin, cisplatin, cyclophosphamide, daunorubicin, oxaliplatin, 5-fluorouracil, and gemcitabine.

31. An anti-tumor agent for use in a method of treating cancer, the anti-tumor agent comprising a combination of a dose of a chemotherapy agent and a dose of attenuated

Salmonella typhimurium containing a plasmid carrying a coding sequence encoding a truncated human interleukin-2, wherein the truncated human interleukin-2 consists of the amino acid sequence shown in SEQ ID NO: 2, the method comprising:

administering the combination of the dose of the chemotherapy agent and the dose of attenuated *Salmonella typhimurium* containing the plasmid carrying the coding sequence encoding the truncated human interleukin-2;

wherein the dose of the chemotherapy agent is lower than a maximum effective dose of the chemotherapy agent;

wherein the combination provides a synergistic reduction in tumor burden when compared to the reduction in tumor burden provided by administration of an equivalent dose of the chemotherapy agent without the attenuated *Salmonella typhimurium* containing the plasmid carrying the coding sequence encoding the truncated human interleukin-2; and

wherein the toxicity of the combination is lower than the toxicity of the maximum effective dose of the chemotherapy agent.

32. The anti-tumor agent of claim 31, wherein the dose of the chemotherapy agent is less than or equal to about 1.25 mg/kg.

33. The method of claim 31, wherein the dose of the chemotherapy agent is 25% of the maximum effective dose of the chemotherapy agent.

34. The anti-tumor agent of claim 31, wherein the dose of attenuated *Salmonella typhimurium* containing the plasmid carrying the coding sequence encoding the truncated human interleukin-2 is about 1×10^9 colony forming units.

35. The anti-tumor agent of claim 31, wherein the attenuated *Salmonella typhimurium* containing the plasmid carrying the coding sequence encoding the truncated human interleukin-2 is administered orally.

36. The anti-tumor agent of claim 31, wherein a first dose of the chemotherapy agent and the dose of the attenuated *Salmonella typhimurium* containing the plasmid carrying the coding sequence encoding the truncated human interleukin-2 are administered on a first day.

37. The anti-tumor agent of claim 36, wherein a second dose of the chemotherapy agent is administered on a second day a week after the first day.

38. The anti-tumor agent of claim 37, wherein a third dose of the chemotherapy agent is administered on a third day a week after the second day.

39. The method of claim 36, wherein a plurality of doses of the chemotherapy agent and a plurality of doses of the attenuated *Salmonella typhimurium* are administered on a plurality of days after the first day.

40. The anti-tumor agent of claim 31, wherein the chemotherapy agent is selected from the group consisting of doxorubicin, carboplatin, cisplatin, cyclophosphamide, daunorubicin, oxaliplatin, 5-fluorouracil, and gemcitabine.

SEQUENCES

DNA sequence for truncated interleukin-2 (homo sapiens) (SEQ ID NO: 1)

```
ATGGCTCCTA CTAGCTCGAG CACTAAGAAA ACTCAACTGC AATTGGAGCA TCTGCTGCTG       60

GATCTGCAGA TGATTCTGAA TGGCATCAAT AACTACAAGA ACCCTAAGCT GACTCGCATG       120

CTGACTTTCA AATTCTACAT GCCGAAAAAG GCTACCGAGC TCAAACATCT CCAGTGCCTG       180

GAAGAGGAAC TGAAGCCGCT GGAGGAAGTA CTTAACCTGG CACAGTCTAA GAACTTCCAC       240

CTGCGTCCGC GTGACCTGAT CTCCAACATC AAGTAA                                  276
```

Protein sequence for truncated interleukin-2 (homo sapiens) (SEQ ID NO: 2)

```
Met Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr Gln Leu Gln Leu Glu
1               5                  10                  15

His Leu Leu Leu Asp Leu Gln Met Ile Leu Asn Gly Ile Asn Asn Tyr
            20                  25                  30

Lys Asn Pro Lys Leu Thr Arg Met Leu Thr Phe Lys Phe Tyr Met Pro
        35                  40                  45

Lys Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu Glu Glu Leu
    50                  55                  60

Lys Pro Leu Glu Glu Val Leu Asn Leu Ala Gln Ser Lys Asn Phe His
65                  70                  75                  80

Leu Arg Pro Arg Asp Leu Ile Ser Asn Ile Lys
                85                  90
```

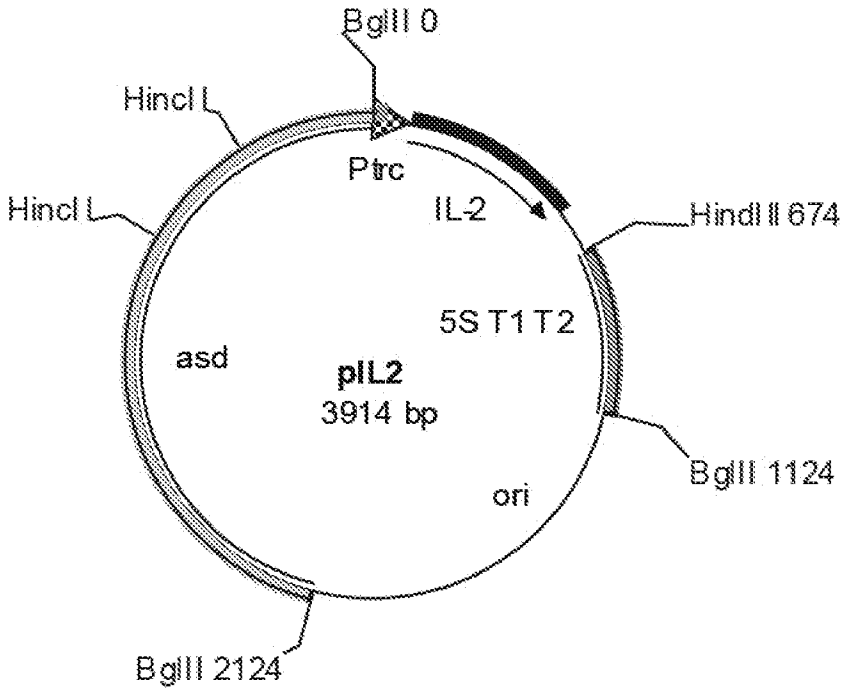
BglII 0
HincI L
Ptrc
HincI L
IL-2
HindIII 674
5S T1 T2
asd
pIL2
3914 bp
BglII 1124
ori
BglII 2124

FIG. 1A

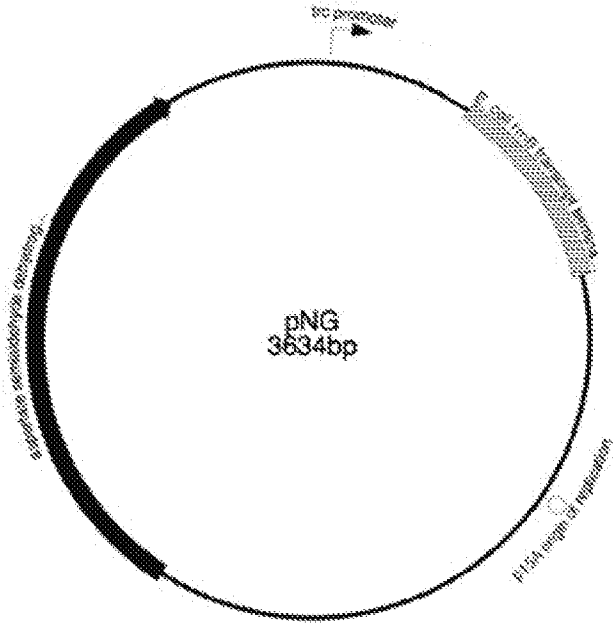
pNG
3634bp

FIG. 1B

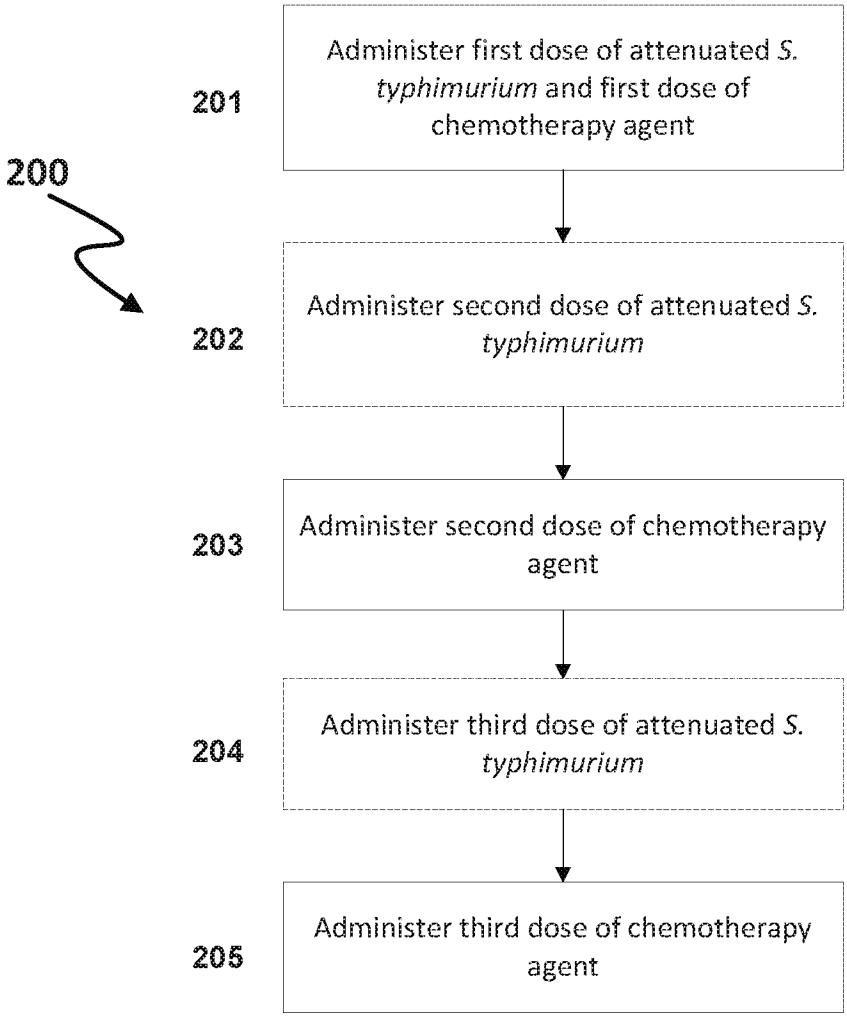
200
201
Administer first dose of attenuated S. typhimurium and first dose of chemotherapy agent
202
Administer second dose of attenuated S. typhimurium
203
Administer second dose of chemotherapy agent
204
Administer third dose of attenuated S. typhimurium
205
Administer third dose of chemotherapy agent

FIG. 2

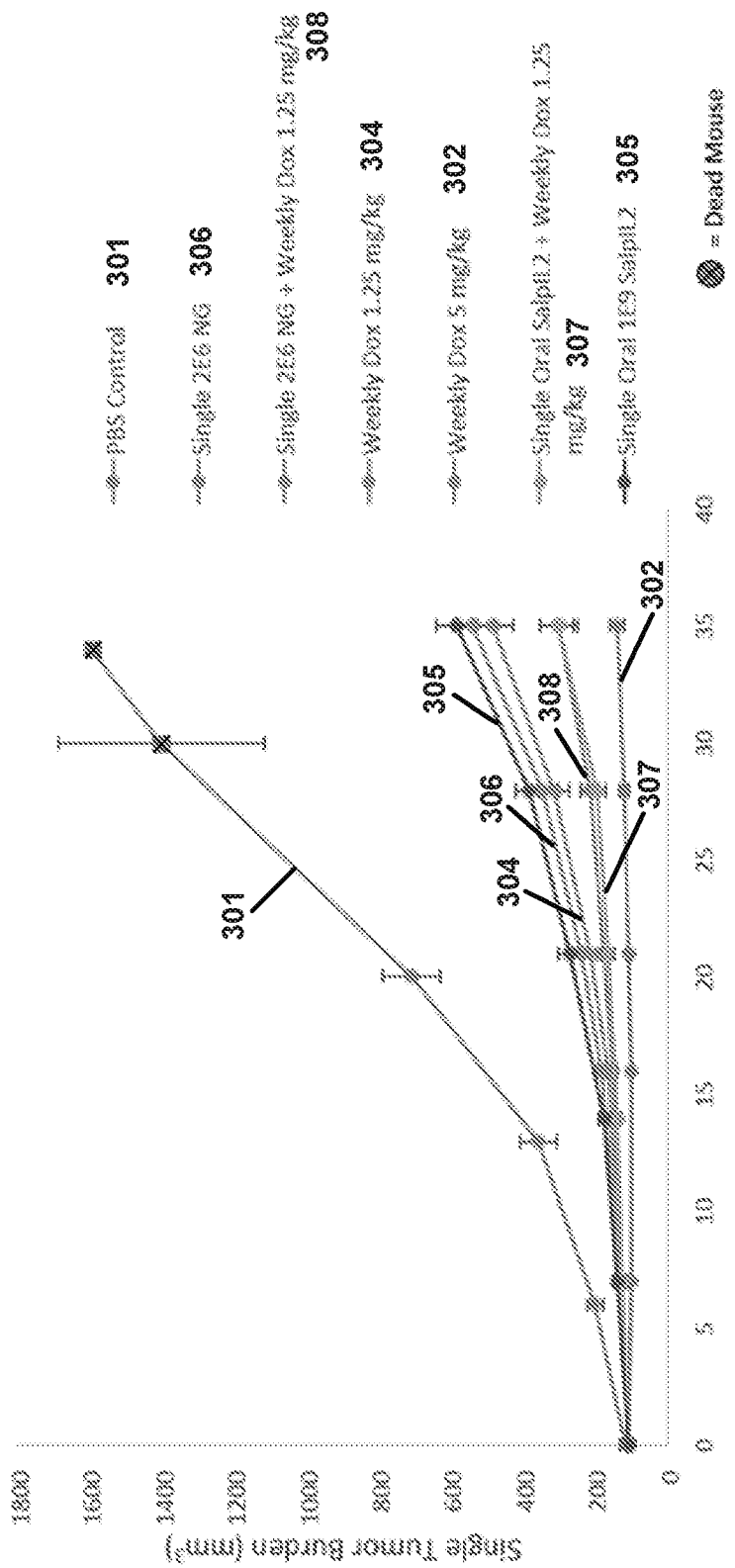
PBS Control 301
Single 2E6 NG 306
Single 2E6 NG + Weekly Dox 1.25 mg/kg 308
Weekly Dox 1.25 mg/kg 304
Weekly Dox 5 mg/kg 302
Single Oral Salpil2 + Weekly Dox 1.25 mg/kg 307
Single Oral 1E9 Salpil2 305
= Dead Mouse
Single Tumor Burden (mm³)
Days Post-Treatment
1800
1600
1400
1200
1000
800
600
400
200
0
0
5
10
15
20
25
30
35
40
301
304
306
305
308
307
302

FIG. 3

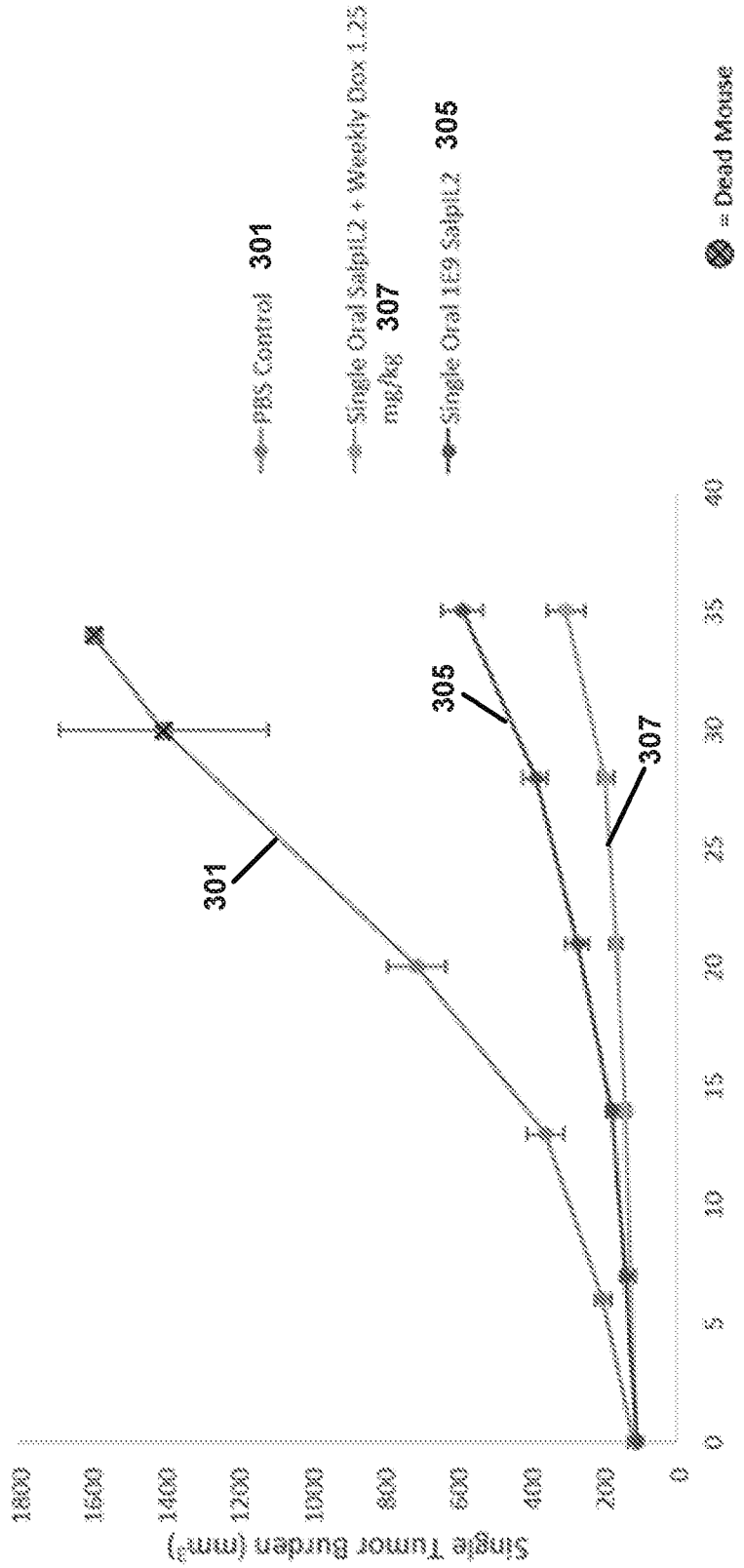
PBS Control 301
Single Oral SolpIL2 + Weekly Dox 1.25 mg/kg 307
Single Oral IE9 SolpIL2 305
= Dead Mouse
Single Tumor Burden (mm³)
Days Post-Treatment
0
200
400
600
800
1000
1200
1400
1600
1800
0
5
10
15
20
25
30
35
40
301
305
307

FIG. 4

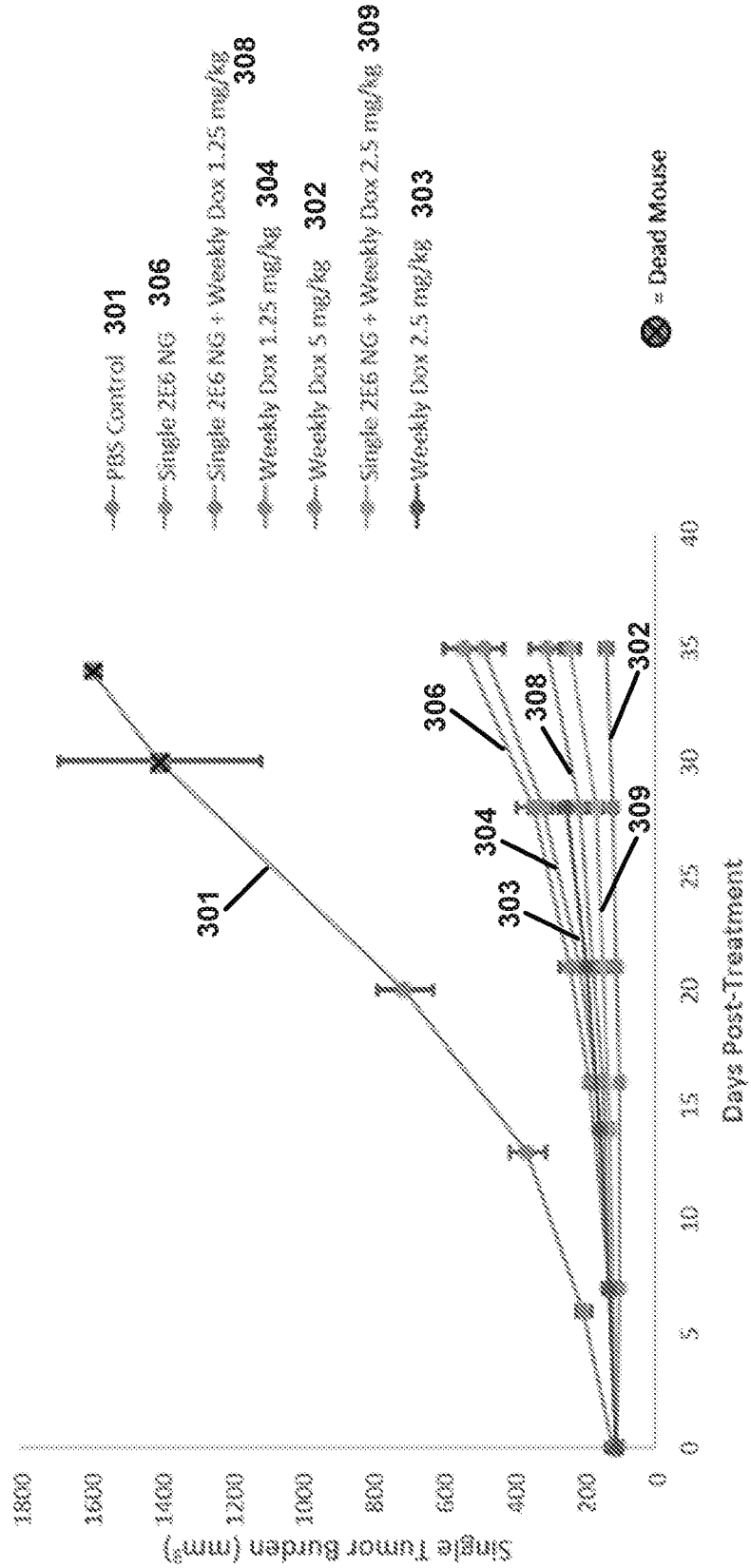
PBS Control 301
Single 2E6 NG 306
Single 2E6 NG + Weekly Dox 1.25 mg/kg 308
Weekly Dox 1.25 mg/kg 304
Weekly Dox 5 mg/kg 302
Single 2E6 NG + Weekly Dox 2.5 mg/kg 309
Weekly Dox 2.5 mg/kg 303
= Dead Mouse
Single Tumor Burden (mm³)
Days Post-Treatment
0
200
400
600
800
1000
1200
1400
1600
1800
0
5
10
15
20
25
30
35
40
301
306
308
304
303
309
302

FIG. 5

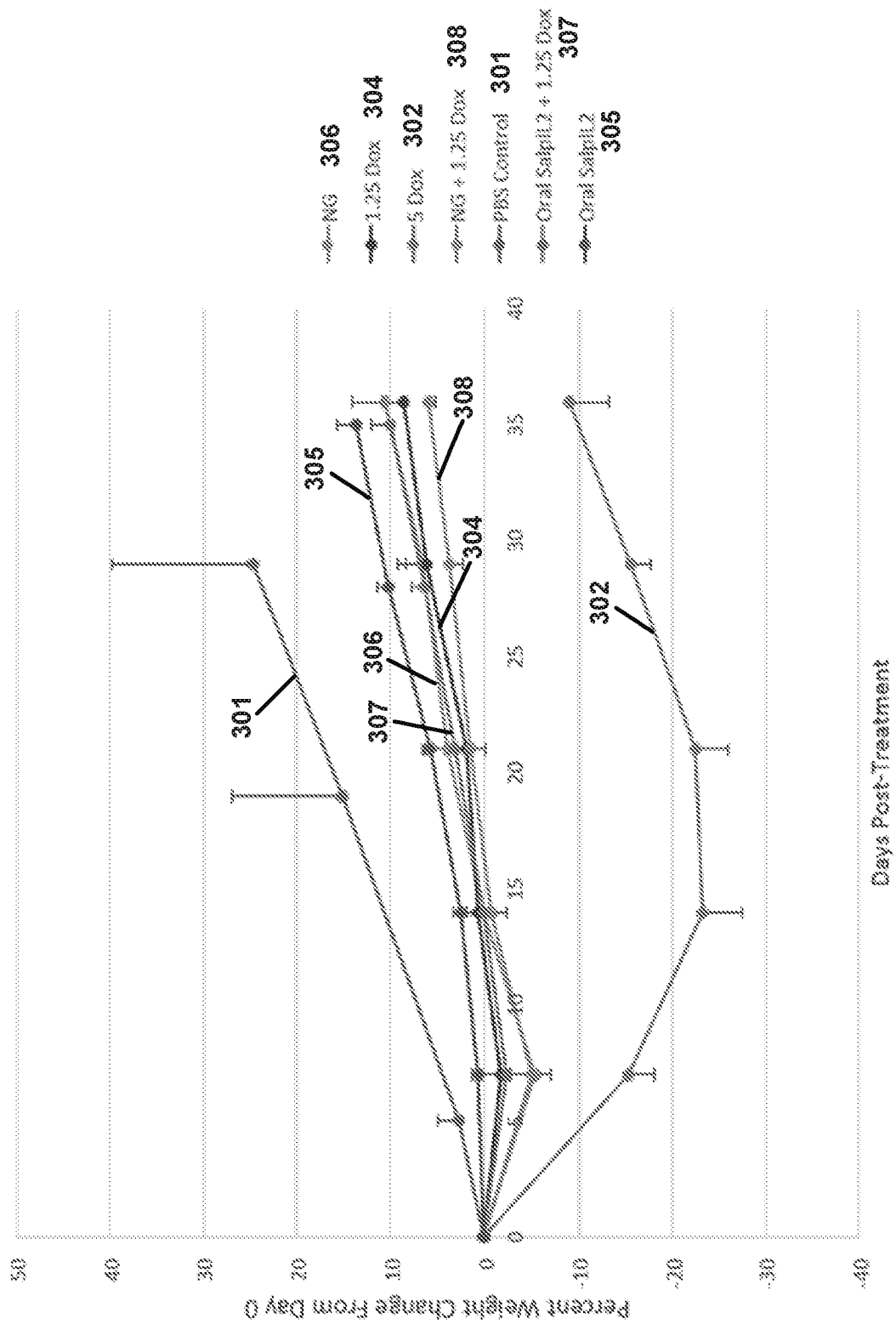
NG 306
1.25 Dox 304
5 Dox 302
NG + 1.25 Dox 308
PBS Control 301
Oral Satpil.2 + 1.25 Dox 307
Oral Satpil.2 305
Percent Weight Change From Day 0
Days Post-Treatment
50
40
30
20
10
0
-10
-20
-30
-40
0
5
10
15
20
25
30
35
40
301
305
308
304
306
307
302

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/64813

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A01N 63/00, A01N 65/00 (2018.01)
CPC - A61K 36/73, A61K 38/2013, C07K 14/55, A61K 2035/11, A61K 2300/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Murakami, et al. Tumor-targeting Salmonella typhimurium A1-R in combination with doxorubicin eradicate soft tissue sarcoma in a patient-derived orthotopic xenograft (PDOX) model. Oncotarget 15 March 2016 (15.03.2016), 7(11):12783-90; Abstract, pg 12784, Table 1; pg 12785, Fig 1 and its legend; pg 12786, Fig 3 and its legend; pg 12787, col 2	1-10, 21-30
Y	Fritz, et al. A phase I clinical study to evaluate safety of orally administered, genetically engineered Salmonella enterica serovar Typhimurium for canine osteosarcoma. Vet Med Sci. 06 June 2016 (06.06.2016), 2(3):179-190; Abstract, pg 181, col 1; pg 182; pg 183, col 1; pg 188, col 1	11-20, 31-40
Y	US 2013/045525 A1 (LEONARD, et al.) 21 February 2013 (21.02.2013) claim 17, SEQ ID NO: 2; para [0025], [0131], [0132],	11-20, 31-40

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 March 2018	18 MAY 2018
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/64813

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I+, claims 1-40, directed to a method of treating cancer by administering a combination of a chemotherapy agent and Salmonella typhimurium, and an anti-tumor agent comprising said combination. The method and the anti-tumor agent will be searched to the extent that the chemotherapy agent encompasses doxorubicin. It is believed that claims 1-40 encompass this first named invention, and thus these claims will be searched without fee to the extent that the chemotherapy agent encompasses doxorubicin. Additional chemotherapy agent(s) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected chemotherapy agent(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be a method of treating cancer by administering a combination of a dose of 5-fluorouracil and a dose of Salmonella typhimurium, and an anti-tumor agent comprising said combination, i.e., claims 1-40. ********** See Supplemental Sheet to continue **********

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-40, restricted to doxorubicin

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/64813

In Continuation of Box III. Observations where unity of invention is lacking:

The inventions listed as Group I+ do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of some inventions of Group I+ is a specific chemotherapy agent recited therein.

The inventions of Group I+ share the technical features of a method of claim 1 or an anti-tumor agent of claim 21. However, these shared technical features do not represent an improvement over prior art as being obvious over a paper titled "Tumor-targeting Salmonella typhimurium A1-R in combination with doxorubicin eradicate soft tissue sarcoma in a patient-derived orthotopic xenograft (PDOX) model" by Murakami, et al. (Oncotarget. 2016 Mar 15;7(11):12783-90) (hereinafter "Murakami").

Murakami discloses a method of treating cancer (Abstract, "combining S. typhimurium A1-R with chemotherapy such as DOX for soft tissue sarcoma patients"), the method comprising:

administering a combination of a dose of a chemotherapy agent and a dose of a composition consisting essentially of attenuated Salmonella typhimurium (pg 12785, Figure 1, "Treatment model and protocol. A. Soft tissue sarcoma PDOX mouse model... B. Treatment protocol. G1: untreated control; G2: treated with S. typhimurium A1-R (A1-R)-alone (i.t...., weekly, 4 weeks) G3: treated with doxorubicin (DOX)-alone (i.p., 3 mg/kg, weekly, 4 weeks); G4: treated with S. typhimurium A1-R (i.t...., weekly, 2 weeks) followed by DOX (i.p., 3 mg/kg, weekly, 2 weeks)"; pg 12784, col 1, "The tumor-targeting S. typhimurium A1-R strain... has ... attenuation mutations. S. typhimurium A1-R is auxotrophic for Leu-Arg, which prevents it from mounting a continuous");

wherein the dose of the chemotherapy agent is lower than a maximum effective dose of the chemotherapy agent (pg 12785, Fig 1, and its legend, compare G3 regime (DOX-alone, 3 mg/kg, weekly, 4 weeks) versus G4 regime (DOX 3 mg/kg, weekly, 2 weeks);

wherein the combination provides a synergistic reduction in tumor burden when compared to the reduction in tumor burden provided by administration of an equivalent dose of the chemotherapy agent without the composition consisting essentially of attenuated Salmonella typhimurium (pg 12784, Table 1: Treatment response between groups, compare efficiency of G4 regime (S. typhimurium A1-R followed by DOX) (Grade I- 0; Grade IIA- 0; Grade IIB- 0; Grade III- 2; Grade IV-0; CR- 3) versus efficiency of G3 regime (DOX-alone) (Grade I-1; Grade IIA- 1; Grade IIB- 0; Grade III- 0; Grade IV ? 1; CR - 1); pg 12785, Figure 2: S. typhimurium and A1-R doxorubicin or their combination significantly inhibited tumor growth in a soft tissue sarcoma PDOX model... On day 25 from initial treatment, tumor volume in ... G3 (DOX-alone) (165.5 +/-247.7 mm3, $p < 0.05$); and G4 (S. typhimurium A1-R followed by DOX) (138.4 +/- 209.3 mm3, $p < 0.01$); pg 12786, Figure 3: Histological response... One of the tumors treated with doxorubicin (DOX) (G3) showed no necrosis (E. Grade I), while another tumor showed complete tumor necrosis (F. Grade IV). Tumors treated with the combination of S. typhimurium A1-R and DOX were destroyed and replaced with inflammatory-type cells (G. H. Grade III)).

Murakami does not specifically disclose that the toxicity of the combination is lower than the toxicity of the maximum effective dose of the chemotherapy agent. However, said limitation would have been obvious to one of ordinary skill in the art, because Murakami discloses that total amount of DOX administered in G3 (DOX-alone) (i.p., 3 mg/kg, weekly, 4 weeks) was twice larger than total amount of DOX administered in G4: (S. typhimurium A1-R followed by DOX (i.p., 3 mg/kg, weekly, 2 weeks).

Murakami discloses that S. typhimurium is administered intratumorally (i.t.), while DOX is administered intraperitoneally (i.p.), 3 mg/kg, weekly, 2 weeks), but does not specifically disclose that S. typhimurium A1-R and DOX are combined to form a single anti-tumor agent. However, one of ordinary skill in the art would have been motivated to formulate the G4 therapeutic regime as a single anti-tumor agent, to simplify the anticancer disclosed by Murakami.
As the technical features would have been obvious to one of ordinary skill in the art at the time of the invention, these cannot be considered special technical features that would otherwise unify the inventions.

Some inventions of Group I+ share the technical features of a method of claim 11 or an anti-tumor agent of claim 31. However, these shared technical features do not represent an improvement over prior art as being obvious over US 2013/0045525 A1 to LEONARD et al. (21 February 2013) (hereinafter "Leonard") in view of a paper titled "A phase I clinical study to evaluate safety of orally administered, genetically engineered Salmonella enterica serovar Typhimurium for canine osteosarcoma" by Fritz, et al. (Vet Med Sci. 06 June 2016, 2(3):179-190) (hereinafter "Fritz").

Leonard discloses a method of treating cancer (para [0025], "FIG. 22 is a bar graph representing attenuated Salmonella-IL2 suppression in vivo tumor metastases, whereby the number of hepatic metastases is significantly reduced in mice orally administered Salmonella-IL2 vs. saline (control) or SaS Salmonella-no-IL2"), the method comprising:

administering a combination of a dose of an antioxidant agent and a dose of attenuated Salmonella typhimurium containing a plasmid carrying a coding sequence encoding a truncated human interleukin-2 (para [0132], "Prior to the oral delivery of the S. typhimurium .chi.4550pIL2 and anti-oxidant oil alkalization of the patient's stomach is necessary to neutralize gastric acid to prevent the acid induced destruction of the S. typhimurium .chi.4550pIL2. This is accomplished by orally administering 30 ml of Bicitra.RTM. 15 minutes prior to administering the S. typhimurium .chi.4550pIL2 with anti-oxidant oil. A dose containing approximately 10.sup.6 to 10.sup.8 S. typhimurium .chi.4550pIL2 is administered to a human patient once, at the initiation of treatment. Approximately one-half teaspoon of cold pressed black raspberry oil is administered to the patient twice a day, throughout the treatment period"),

wherein the truncated human interleukin-2 consists of the amino acid sequence shown in SEQ ID NO: 2 (claim 17, "An anti-tumor agent comprising an effective amount of attenuated Salmonella typhimurium containing a plasmid carrying a coding sequence encoding a truncated human interleukin-2, wherein the truncated human interleukin 2 consists of SEQ ID NO:2", such that said SEQ ID NO: 2 has 100% identity to the claimed SEQ ID NO: 2).

********** See the Following Supplemental Sheet to continue **********

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/64813

In Continuation of Box III. Observations where unity of invention is lacking and the Preceding Supplemental Sheet:

Leonard discloses that "the administration of S. typhimurium .chi.4550pIL2 concurrently with highly potent antioxidant oils will also be effective in treating infectious disease, other cancerous tumors... An additional use of the present invention would be administering it to cancer patients between periods of chemotherapy and/or radiation treatments" (para [0131]), but does not specifically disclose a combination therapy comprising S. typhimurium .chi.4550pIL2 and chemotherapy.

Fritz discloses that a combination therapy comprising attenuated S. typhimurium and doxorubicin might be efficient for treament non-metastatic appendicular osteosarcoma (pg 188, col 1, "Our data indicate that SalpIL2 in combination with amputation and adjuvant doxorubicin is safe and well tolerated, and it might provide clinical benefit for dogs with non-metastatic appendicular osteosarcoma"; Abstract, "We conducted a prospective phase I study to evaluate safety of an orally administered Salmonella encoding IL-2 (SalpIL2) in combination with amputation and adjuvant doxorubicin for canine appendicular osteosarcoma. Efficacy was assessed as a secondary measure. The first dose of SalpIL2 was administered to 19 dogs on Day 0; amputation was done after 10 days with chemotherapy following 2 weeks later. SalpIL2 was administered concurrent with chemotherapy, for a total of five doses of doxorubicin and six doses of SalpIL2... Dogs receiving SalpIL2 had significantly longer disease-free interval (DFI) than a comparison group of dogs treated with doxorubicin alone... The data indicate that SalpIL2 is safe and well tolerated, which supports additional testing to establish the potential for SalpIL2 as a novel form of adjuvant therapy for dogs with osteosarcoma").
It would have been obvious to one of ordinary skill in the art to combine, in the course of routine experimentation and with a reasonable expectation of success, Leonard and Fritz by administering a combination therapy comprising S. typhimurium .chi.4550pIL2 disclosed by Leonard concurrently with doxorubicin chemotherapy, as disclosed by Fritz, to improve efficiency of the anti-cancer therapy disclosed by Leonard.

As to claim 31, Leonard in view of Fritz discloses a concurrent oral administration of an attenuated S. typhimurium and i.v. administration of doxorubicin (Fritz, Abstract, "We conducted a prospective phase I study to evaluate safety of an orally administered Salmonella encoding IL-2 (SalpIL2) in combination with amputation and adjuvant doxorubicin for canine appendicular osteosarcoma... SalpIL2 was administered concurrent with chemotherapy", pg 181, col 1, "These dogs were ... had appendicular osteosarcoma that was staged, evaluated, and treated with five doses of doxorubicin alone (30 mg/m2, i.v.)"), but does not specifically disclose that S. typhimurium and DOX are combined to form a single anti-tumor agent.
However, one of ordinary skill in the art would have been motivated to combine S. typhimurium and DOX into a single anti-tumor agent, to simplify the anticancer therapy disclosed by Leonard in view of Fritz. As the technical features would have been obvious to one of ordinary skill in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the inventions.

The inventions of Group I-I therefore lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature