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(54) Title: INTRACELLULAR DELIVERY OF THERAPEUTIC CARGOS AND VIRAL RNAs BY ENGINEERED *SALMONELLA*

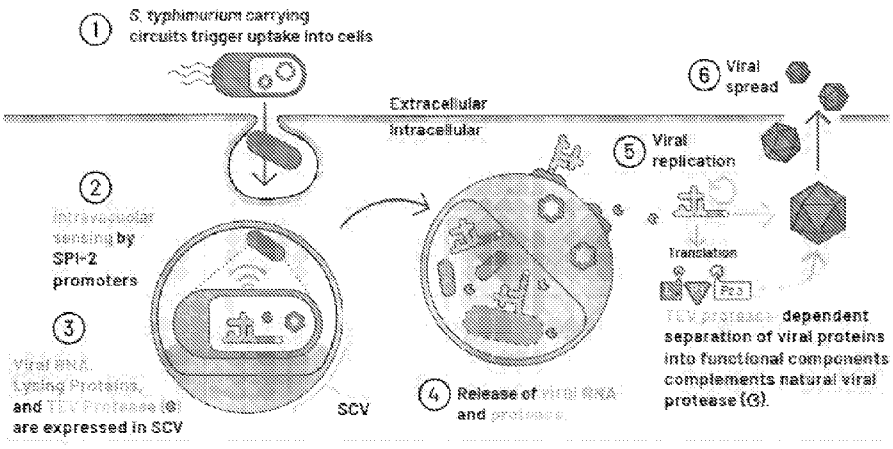

FIGURE 1

(57) Abstract: The disclosure provides an engineered Salmonella typhimurium comprising a first heterologous nucleic acid encoding a first polypeptide that causes bacterial lysis, a second heterologous nucleic acid encoding a second polypeptide that causes vacuolar lysis, and a third heterologous nucleic acid encoding a virus, wherein the virus comprising a picornavirus. Further disclosed is a method of using the engineered bacteria for treating a tumor in a subject.

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[Continued on next page]

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A. CLASSIFICATION OF SUBJECT MATTER

IPC - INV. A61K 48/00, A01N 63/00, C12N 1/20, A61K 35/74, A61P 35/00 (2024.01)
ADD. C07H 21/04 (2024.01)

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ADD. C07H 21/04, A61K 2039/5256

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.
Y	US 2021/0308195 A1 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 07 October 2021 (07.10.2021), Abstract, [0008], [0017], [0026], [0047], [0048], [0067], [0099], [0101], [0116], [0147], [0247], and [0270]	1-10
Y	US 2008/0193479 A1 (AU et al.) 14 August 2008 (14.08.2008), Abstract	1-10
Y	FERNANDES et al., A virulent and pathogenic infectious clone of Senecavirus A. J Gen Virol. 2021, Vol. 102(8): 001643. PDF File: pg 1-12. Abstract	1-10
Y	XU et al., Efficacy of Intracellular Activated Promoters for Generation of Salmonella-Based Vaccines. Infect Immun. 2010, Vol. 78(11), p. 4828-4838. Abstract; pg 4824, col 2, top para and last para; pg 4829, col 1, top para; and pg 4831, Fig 1	3, 7
Y	US 2022/0062234 A1 (TRUSTEES OF BOSTON UNIVERSITY) 03 March 2022 (03.03.2022), Abstract, para [0031], [0058], [0063], [0091], [0300], [0303], [0454], [0457], and [0463]	6-10
Y	GUENTHER et al., Protease-Activatable Adeno-Associated Virus Vector for Gene Delivery to Damaged Heart Tissue. Mol Ther. 2019, Vol. 27(3), p. 611–622. Abstract; pg 611, col 2, middle para and last para; pg 612, col 1, last para; and pg 617, col 2, last para	9-10
A	GRAY et al., Activation of Specific Apoptotic Caspases with an Engineered Small Molecule-Activated Protease. Cell. 2010, Vol. 142(4), p. 637-646. PDF File: pg 1-19. Entire documentation especially Abstract; pg 2, last para; and pg 14, Fig 1	1-10

☒ 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
"D" document cited by the applicant in the international application
"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
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INTERNATIONAL SEARCH REPORT

International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LIU et al., Innate sensing of picornavirus infection involves cGAS-STING-mediated antiviral responses triggered by mitochondrial DNA release. PLoS Pathog. 2023 Feb, Vol. 19(2): e1011132. PDF File: pg 1-35. Entire documentation especially Abstract	1-10
A	WO 2018/069782 A2 (KEMIJSKI INSTITUT et al). 19 April 2018 (19.04.2018), Abstract	1-10
A	US 2021/0169941 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 10 June 2021 (10.06.2021), Abstract, para [0004], [0006], [0007], [0013], and [0014]	1-10
A	US 2018/0195056 A1 (MAX-PLANCK-GESELLSCHAFT ZUR FOERDERUNG DER WISSENSCHAFTEN E.V.) 12 July 2018 (12.07.2018), Abstract, and Fig 1	1-10
A	DIN et al., Synchronized cycles of bacterial lysis for in vivo delivery. Nature. 2016, Vol. 536(7614), p. 81-85. PDF File: pg 1-18. Entire documentation especially Abstract; pg 2, para 1; pg 14, Fig 1; and pg 17, Fig 4	1-10
A	RICHARDS et al., Behind Closed Membranes: The Secret Lives of Picornaviruses? PLoS Pathog. 2013, Vol. 9(5): e1003262. PDF File: pg 1-3. Entire documentation especially Abstract; and pg 2, Fig 1	1-10
P;A	NGUYEN et al., Bioengineering of bacteria for cancer immunotherapy. Nat Commun. 2023, Vol. 14: 3553. Epub 2023 Jun 15. PDF File: pg 1-5. Entire documentation especially Abstract; and pg 3, Fig 1	1-10

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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. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-10, directed to an engineered Salmonella typhimurium bacterium.

Group II, claims 11-20, directed to a method of treating a tumor in a subject.

The inventions listed as Groups I-II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

**********Continued in the extra sheet**********

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.:
1-10

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 24/32961

Continuation of:
Box No III (unity of invention is lacking)

Special Technical Feature
Groups I-II are related as a product (Group I) and a method of using the product (Group II).

Group II includes the special technical feature of treating a tumor in a subject, not required by Group I.

Common Technical Features
The inventions of Groups I-II share the technical features of an engineered Salmonella typhimurium bacterium, comprising: (a) a lysis circuit comprising a first heterologous nucleic acid encoding a first polypeptide that causes bacterial lysis and a second heterologous nucleic acid encoding a second polypeptide that causes vacuolar lysis; and (b) a third heterologous nucleic acid encoding a virus.

However, these shared technical features do not represent a contribution over prior art as being obvious over over US 2021/0308195 A1 to THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK (hereinafter 'Columbia_U'), in view of US 2008/0193479 A1 to GOUGH GEOFFREY AU et al. (hereinafter 'Au'), and further in view of an article entitled 'A virulent and pathogenic infectious clone of Senecavirus A' by FERNANDES et al. (hereinafter 'Fernandes'; J Gen Virol. 2021, Vol. 102(8): 001643. PDF File: pg 1-12) as follows:

Columbia_U discloses an engineered Salmonella typhimurium bacterium (Abstract - 'programmable bacteria comprise at least one synchronized lysis circuit', wherein 'programmable bacteria' are 'engineered bacteria' which comprising 'engineered Salmonella typhimurium bacterium'; para [0099] - 'the programmable bacteria are ... bacteria include...Salmonella typhimurium'; para [0008] - 'programmable bacteria comprising at least one synchronized lysis circuit contained in a single operon which can be further engineered to produce a therapeutic agent'), comprising:

(a) a lysis circuit comprising a first heterologous nucleic acid encoding a first polypeptide that causes bacterial lysis (Abstract - 'programmable bacteria comprise at least one synchronized lysis circuit contained in a single operon and at least one plasmid producing a therapeutic agent, i.e., at least one plasmid comprising a nucleic acid sequence which encodes a therapeutic agent', wherein 'a nucleic acid sequence which encodes a therapeutic agent' can only be 'heterologous nucleic acid', and wherein 'synchronized lysis circuit contained in a single operon and at least one plasmid ...comprising a nucleic acid sequence which encodes a therapeutic agent' comprising 'a lysis circuit comprising a first heterologous nucleic acid encoding a first polypeptide that causes bacterial lysis', see the quotations that follow; para [0147] - 'the therapeutic agent is a toxin... toxins would include ...Hemolysin E... anti-microbial peptides'; para [0047] - 'DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide'; para [0026] - 'bacteria encoding a synchronized lysis circuit (SLC)...single plasmid synchronized lysis circuit;... E. coli with SLC reaching a quorum and inducing the phage lysis protein ?X174E, leading to bacterial lysis'; para [0099] - 'the programmable bacteria are ... Exemplary bacteria include...Salmonella typhimurium') and

--a second heterologous nucleic acid encoding a second polypeptide that causes a targeted effect (Abstract - 'the programmable bacteria comprise at least one synchronized lysis circuit contained in a single operon which are capable of being further engineered to cyclically produce anti-cancer therapeutic agents including ... to nanobodies against immune checkpoint inhibitors and over-expressed markers in cancers, toxins, tumor antigens, cytokines, and chemokines'; para [0026] - 'bacteria encoding a synchronized lysis circuit (SLC)...single plasmid synchronized lysis circuit;...with SLC reaching a quorum and inducing the phage lysis protein ?X174E, leading to bacterial lysis and release of a constitutively produced, anti-CD47 blocking nanobody which binds to CD47 on the tumor cell surface').

Although Columbia_U does not specifically disclose wherein the targeted effect is 'vacuolar lysis', this limitation is encompassed in the disclosure of Columbia_U, because Columbia_U discloses wherein the engineered Salmonella typhimurium bacterium comprising at least one synchronized lysis circuit are capable of being further engineered to produce anti-cancer therapeutic agents including toxins Hemolysin E (Abstract - 'the programmable bacteria comprise at least one synchronized lysis circuit contained in a single operon which are capable of being further engineered to cyclically produce anti-cancer therapeutic agents including ... to nanobodies against immune checkpoint inhibitors and over-expressed markers in cancers, toxins, tumor antigens, cytokines, and chemokines'; para [0147] - 'the therapeutic agent is a toxin... toxins would include ...Hemolysin E... anti-microbial peptides'; para [0099] - 'the programmable bacteria ...include...Salmonella typhimurium'), which can be 'the second polypeptide is Hemolysin E' (see Dependent claim 2), and therefore inherently resulted in 'a second polypeptide that causes vacuolar lysis'.

Columbia_U further discloses the engineered bacterium comprising at least one synchronized lysis circuit are capable of being further engineered to produce anti-cancer therapeutic agents (Abstract), and a method of administering the engineered bacteria for treating cancer (para [0017] - 'programmable bacteria are administered in conjunction with conventional cancer treatment or therapy').

Columbia_U does not specifically teach the engineered bacterium further comprising (b) a third heterologous nucleic acid encoding a virus. Au discloses a method of using picornaviruses for treating cancer (Abstract - 'oncolytic Picornaviruses and methods and compositions for treating subjects having hematologic cancers').

Au also does not specifically teach the engineered bacterium further comprising (b) a third heterologous nucleic acid encoding a virus. Fernandes discloses a method of characterization of a full-length cDNA genome expressed picornavirus, a recombinant senecavirus A (SVA) strain, which has comparable infection dynamics in comparison with its wild-type virus (Abstract - 'Senecavirus A (SVA) is a picornavirus ...full-length cDNA genome of SVA was cloned into a plasmid under a T7 RNA polymerase promoter. Following in vitro transcription, the genomic viral RNA was transfected into BHK-21 cells and rescue of infectious virus (rSVA SD15-26) was shown by inoculation of highly susceptible H1299 cells. In vitro characterization of the rSVA SD15-26 showed similar replication properties and protein expression levels as the wt SVA SD15-26...data demonstrates that the rSVA SD5-26 clone is fully virulent and pathogenic in pigs, presenting comparable pathogenesis and infection dynamics to the wt SVA SD15-26 strain').

**********Continued in the next extra sheet**********

Continuation of:
The previous extra sheet - Box No III (unity of invention is lacking)

It would have been obvious to one of ordinary skill in the art to combine the teachings of Columbia_U, Au, and Fernandes, to obtain an engineered Salmonella typhimurium bacterium, comprising: (a) a lysis circuit comprising a first heterologous nucleic acid encoding a first polypeptide that causes bacterial lysis and a second heterologous nucleic acid encoding a second polypeptide that causes vacuolar lysis, based on the teaching of Columbia_U, and further the engineered bacterium further comprising (b) a third heterologous nucleic acid encoding a virus, based on the combination of Au, Fernandes, and Columbia_U, in order to combine methods, technologies, virus genetic information, and knowledge available in the art for facilitating obtaining an engineered Salmonella typhimurium bacterium with desired features for treating targeted diseases with desired effects with an expressed success and without undue experimentation.

Without a shared special technical feature, the inventions lack unity with one another.

Accordingly, the inventions listed as Groups I-II above lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature providing contribution over prior art.