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(54) Title: METHOD FOR TREATING CANCER BY DISABILING BRACA1/FANCM INTERACTION

(57) Abstract: The method involves an approach of inhibiting replication of dysfunctional cells which adopt alternative lengthening of telomeres, or "ALT." The methodology involves inhibition of Fanconi anemia complementation group M (FANCM), and one or both of breast cancer 1 (BRCA1) and Bloom syndrome protein (BLM). These molecules are the keys to the elimination of replication stress caused by ALT. In other words, they permit the dysfunctional cells to proliferate. Inhibition of these molecules inhibits proliferation.



METHOD FOR TREATING CANCER BY DISABILING BRCA1/FANCM INTERACTION

FIELD OF THE INVENTION

[001] This invention relates to a method for alleviating a condition characterized by dysfunctional cells, where the dysfunction itself is characterized by cells using the alternative lengthening of telomeres, or “ALT”, as the way to maintain their telomeres. More specifically, it relates to inhibiting replication stress response or destruction of, said cells by inhibiting expression of FANCM, and one or both of BRCA1 and BLM, all of which are involved in replication stress response in ALT cells.

BACKGROUND AND PRIOR ART

[002] Faithful replicating of the genome is vital for mammalian cells. The “replisome” (the DNA replication machinery) of the cell frequently encounters impediments during replication. The term “replication stress” is used to describe temporary or transient stalling of the replication fork (see, e.g., Berti, et al., Nat. Struct. Mol. Biol., 23(2):103-109 (2016); Zeman, et al., Nat. Cell Biol., 16(1):2-9 (2004)).

[003] One of the endogenous regions of DNA which poses challenges to the replisome is the telomere. This structure consists of tandem repeats of the DNA sequence (TTAGGG)_n located at the end of every linear chromosome. Telomerase catalyzes the additional of this sequence to the end of the chromosomes. The telomeres vary in length from 10-15 Kb in humans, and 25-50 Kb in mice.

[004] With few exceptions, somatic cells do not express telomerase, and undergo progressive telomere shortening with each cell cycle, eventually leading to senescence, and cell death. See, e.g., Lopez-Ortin, et al., Cell, 153(6):1194-1217 (2013).

[005] Dysfunction of cells, like cancer cells, is sometimes characterized by replicative immortality. See, e.g., Hanahan, et al., Cell, 144(5):646-674 (2011). This “replicative immortality” is achieved by overcoming telomere shortening. A percentage of these dysfunctional cells do so by utilizing homologous recombination based lengthening of telomeres. See, Dilley, et al., Trends in Cancer, 1(2):145-156 (2015); Cesare, et al., Nat. Rev. Genet., 11(5):319-330 (2010). Cells using this “alternative lengthening of telomeres” (“ALT” hereafter) are prone to susceptibility to replicative

stress. Nonetheless, ALT cells, i.e., cells which maintain their telomere length via the above referenced method, do undergo replication, as is evident from, e.g., cancer cells which acquire ALT. (It should be noted that various methodologies are well known for determining if a cell is an ALT cell).

[006] Clearly, there is a mechanism for overcoming the replicative stress experienced by ALT cells, which enables their continued replication, notwithstanding the defect, or other defects associated with the ALT cells.

[007] FANCM is a recognized member of the Fanconi Anemia (“FA”) family of genes. The members of this family are recognized, *inter alia*, as being involved in repairing interstrand cross linking lesions. See, e.g., Caccaldi, et al., Nat. Rev. Mol. Cell Biol., 17(6):337-349 (2016); and Michl, et al., EMBO J., 35(9):909-923 (2016), both of which are incorporated by reference. Information on FANCM and its three obligatory binding partners (FAAP24, MHFI, and MHF2), can be found at, e.g., Meetei, et al., Nat. Genet., 37(9):958-963 (2005); Mosedale, et al., Nat. Struct. Mol. Biol., 12(9):763-771 (2005); Ceccia, et al., Mol. Cell, 25(3):331-343 (2007); Yam, et al., Mol. Cell, 37(6):854-878 (2010); Singh, et al., Mol. Cell, 37(6):879-886 (2010); Collis, et al., Mol. Cell, 32(3):313-324 (2008); Huang, et al., Mol. Cell, 52(3):434-446 (2013); Huang, et al., Mol. Cell, 39(2):259-268 (2010); and Wang, et al., Mol. Cell, 49(5):997-1009 (2013). Also see, Schwab, et al., EMBO J., 29(4):806-818 (2010); Luke-Glaser, et al., EMBO J., 29(4):795-805 (2010); Singh, et al., Blood, 114(1):174-180 (2009); and Blackford, et al., Hum. Mol. Genet., 21(9):2005-2016 (2012).

[008] Collis, et al., *supra*, have observed that acute depletion of FANCM in unperturbed human cancer cells causes increased formation of γ -H2AX foci, a common marker for DNA damage. Schwab, et al., *supra*, observed this in chicken DT40 cells as well.

[009] Neither of Collis nor Schwab pinpointed the cause or nature of DNA damage in these cells, nor have other investigators.

[0010] It has now been found that depletion of FANCM, or one of its obligate binding partners induces replication stress in ALT cells, primarily taken place at the telomeres. Deletion of FANCM was shown to dramatically reduce replication efficiency at these ALT telomeres.

[0011] Further, it was found that BLM and BRCA1, both of which are critical proteins for homologous recombination, are recruited to the site of replication stress and help to resolve it by stimulating DNA end resection and promoting homologous recombination.

[0012] Codepletion of FANCM and one of BLM or BRCA1 dramatically increases micronuclei formation and increases synthetic lethality.

[0013] Hence, it is an object of the invention to develop a method for reducing the number of defective ALT cells in a subject by depleting or reducing expression of FANCM, and one or both of BLM and BRCA1.

[0014] How this and other aspects of the invention are achieved will be seen in the disclosure which follows:

EXAMPLE 1

[0015] This first set of experiments were designed to confirm that DNA damage seen in FANCM deficient cells was in fact due to replication stress, in contrast to double strand DNA breaks.

[0016] Each of these activates a different pathway, i.e., replication stress activates the ATR-DNA-PKcs-Chk1 pathway, while double strand breaks activate the ATM-Chk2 pathway. See, e.g., Marichal, et al., Cold Spring Harb. Perspect Biol., 5(9) (2013); and Ceccia, et al., Mol. Cell, 40(2):179-204 (2010).

[0017] Further, Ser 345 of Chk1 (“ChKpS345”) and Ser33 of RPA (“RPA32-pS33”) are phosphorylated by ATR, while Ser4 and Ser8 of RPA32 (“RPA32-pS4pS8 or pRPA32”) are phosphorylated by DNA-PK.

[0018] U2-OS and SaOS-2 cells, available from the American Type Culture Collection, are human ALT cells. HeLa and MG63 cells discussed *infra*, are not. All cells were cultured in DMEM supplemented with 10% FBS, penicillin and streptomycin, at 37°C in a humidified incubator with 5% CO₂.

[0019] The cells were depleted of FANCM using siRNA. To elaborate, cells were transfected with siRNA targeting luciferase as a negative control, or two different siRNA molecules which target FANCM. These siRNA molecules are commercially available and were purchased from Dharmacon. Human cells do not express luciferase, and hence the added siRNA should not deplete any genes. Hence, the negative control becomes normal conditions for the experiments which follow. See *supra* for the standard cultivation conditions. These cells were then contacted with antibodies labeled with a green dye which recognize γ H2AX, ChK1- pS345, RPA32-pS4S8, or BLM. All cells were also stained with DAPI which is blue. All antibodies were either purchased, or provided by other investigators. Over 200 cells were counted in each sample.

[0020] The results indicated that the luciferase control cells exhibit less than 1% ChK1-pS345 and RPA32-pS4pS8 foci, as compared to controls.

[0021] In contrast, when cells were depleted of FANCM with siRNA, approximately 15% of the cells exhibited bright foci containing the above referenced markers. In addition to the increase in RPA-pS4pS8 foci, immunoblotting studies showed a dramatic increase in both RPA32-pS33 and RPA-pS4pS8, indicating that the cells were under replicative stress.

[0022] These results suggest that DNA damage in FANCM deficient cells, is mainly due to DNA replication stress.

[0023] The results demonstrate that 20-30% of the cells stained positive for BLM. BLM is a DNA helicase which is involved in several crucial steps of the homologous recombination discussed *supra*.

EXAMPLE 2

[0024] These experiments were designed to determine if the replication stress observed in Example 1 was taking place at the telomeres.

[0025] Cells depleted of FANCM as discussed *supra* were probed with three telomere specific markers as were control cells also as described *supra*. TelC, a peptide nucleic acid probe, TRF1 and TRF2, both of which are key components of the Shelterin complex.

[0026] Cells were co-stained with the same ChK1-pS345 antibody used *supra* together with the DNA probe, or an antibody specific to TRF1, or one specific to TRF2.

[0027] In parallel experiments, the ChK1-pS345 antibody was substituted by one specific for BLM.

[0028] The results indicated that the foci resulting from staining all colocalized with the telomeric markers, and they were bigger and brighter than normal telomeric staining, suggesting clustering at telomeres experiencing replicative stress.

[0029] When these experiments were repeated in ALT cell SaOS-2, similar results were obtained. In contrast, when telomerase positive cells HeLa and MG63 were tested, the results did not correlate.

[0030] These data, collectively, suggest that FANCM functions to alleviate replicative stress at the telomeres of ALT cells.

[0031] Further, telomeric foci which colocalize with the other foci tend to be larger and brighter than normal foci, suggesting replication stress. This pattern was repeated in a second set of experiments, with cells of cell line Saos-2, also purchased from the ATCC.

EXAMPLE 3

[0032] As noted *supra*, various functions of FANCM require three obligatory binding partners: FAAP24, MHF1, and MHF2. Also, noted *supra*, FANCM is part of a family of FA proteins.

[0033] To review their functions, FAAP24 binds the C terminus of FANCM, and works as part of the FA core complex, to promote monoubiquitination of FANCD2 and FANCI (ID2). MHF1 and MHF2 are both histone fold proteins and form stable, stoichiometric heterodimers, referred to as “MHF1/2.” The dimer binds the N-terminus of FANCM, and also functions as in the manner of FAAP24. Experiments were carried out to determine if these molecules or other members of the FA family acted similarly to FANCM.

[0034] U2-OS cells were transfected, with siRNA that either targeted luciferase, or with two siRNAs, “FAAP24-1 and -2”. Transfected cells were costained with an antibody recognizing ChK1-pS345, or BLM, as described *supra*, together with either a PNA probe recognizing the G rich strand of telomeres, or a TRF1 specific antibody. Cells were counted as in the prior examples.

[0035] These two siRNA molecules deplete the same gene, but two were used in order to eliminate the so-called “off target” effect. In brief, although siRNA (generally 19-27 nucleotides long) is chosen to be specific to only one gene, there are situations where the siRNA depletes a gene in addition to the one targeted. Using the two molecules serves to minimize the off-target effect.

[0036] The results show that depletion of FAAP24 induced a dramatic increase in ChK1-pS345 and BLM foci. Further, deletion of one or both of MHF1 or MHF2 had the same effect. Deletion of MHF1/2, however, induced fewer relevant foci, suggesting that FAAP24 has the more important role in relieving replication stress. Depletion of all of FANCM, FAAP24, MHF1 and MHF2 had an additive effect, i.e., there was a moderate increase in foci at the telomeres.

[0037] Tests on other FA proteins (i.e., FANCB, FANCG, and FANCI), comparable to those discussed *supra* for FANCM, showed the depletion of FANCI had no impact on foci formation, and deletion of FANCB and FANCG had a moderate, but much reduced role in foci formation. There was a moderate increase in ChK1-pS345 foci formation, not as large as the increase resulting from deletion of FANCM; however, the increase in BLM foci was equivalent

to FANCM, suggesting that the FA core complex, could have an active role in inhibiting BLM recruitment.

EXAMPLE 4

[0038] These experiments examine the role of FANCM in telomere replication. SMARD assays were carried out, following Drosopoulos, et al., J. Cell Biol., 197(2):253-266 (2012), incorporated by reference, in the subtelomeric regions of FANCM depleted cells. (“SMARD” is an acronym for “single molecule analysis of replicated DNA”). To elaborate, cells were transfected with siRNA, as discussed *supra*, to deplete FANCM. Then, the cells were pulse labeled with IdU and CldU for 4 hours each, in sequence. After the labeling, the genomic DNA of the cells was digested, subjected to pulsed field gel electrophoresis to separate it, and any genomic DNA enriched in subtelomeres and telomeres was identified using a telomeric specific PNA probe and classic Southern blotting.

[0039] Next, the thus identified DNA was stretched and stained with anti-IdU and anti-CldU antibodies, labeled red and green, and classic FISH methodologies. The images were visualized and fibers containing the halogenated nucleotides counted, using standard methods.

[0040] The results showed a three-fold decrease in replication efficiency in FANCM depleted cells. The decrease is not believed correlatable to decreases in overall DNA replication because comparison of the cell cycle profile of the FANCM depleted cells was comparable to controls.

[0041] These results do, however, support the conclusion that when FANCM is absent or reduced, replication forks tend to pause and/or stall at ALT telomeres, resulting in increased replication stress.

EXAMPLE 5

[0042] “BLM” is known to be involved in a number of DNA metabolic pathways including replication, replication stress response, and telomere biology. See, Bizard, et al., Cold

Spring Harb. Perspect. Biol., 6(7):a016477 (2014); and Crouteau, et al., Annu. Rev. Biochem., 83:519-552 (2014). It is known to play an important role in DNA end resection during homologous recombination, at telomeres. See, e.g., Gravel, et al., Genes Dev., 22(20:2767-2722 92008); and Kibe, et al., Mol. Cell, 61(2):236-246 (2016).

[0043] To study BLM's function, cells were co-depleted of FANCM and BLM, using the siRNA methodology discussed *supra*, and DNA end resection was monitored via immunoblotting, using antibodies which recognize RPA32-pS4pS8 and RPA32-pS33.

[0044] The depletion of BLM dramatically attenuated DNA end resection in cells also depleted of FANCM, proving that BLM is required for DNA end resection during replication response at ALT telomeres. It should be pointed out that, e.g., Cesare, et al., Nat. Rev. Genet., 11(5):319-330 (2010) show that ALT cells rely on homologous recombination to maintain telomere length and to repair stalled replication forks. Reduced DNA end resection compromises the homologous recombination, and hinders DNA repair processes. The results of this experiment are consistent with an observed, dramatic increase of cells with micronuclei when the two subject molecules were depleted. Further, standard crystal violet assays showed a strong lethal interaction – resulting from the depletion of the two molecules.

EXAMPLE 6

[0045] These experiments were designed to determine if BRCA1, another “player” in DNA metabolism, is involved in the ALT telomere replication stress system. This molecule is well known as being very involved in several facets of DNA metabolism, including checkpoint activation, and homologous recombination. Acharya, et al., Phos One, 9(8):103819 (2014), showed that it was found at ALT telomeres, and that it stimulated helicase activity of BLM.

[0046] To study the role of BRCA1, FANCM depleted cells, as described *supra*, were co-stained with a BRCA1 specific antibody, and telomere specific probes also as described *supra*. Both U2-OS and SaOS-2 cells were tested using standard methods.

[0047] The results showed that a small percentage of control cells showed BRCA1 foci, but these rarely colocalize with telomeres.

[0048] On the other hand, over 90% of BRCA1 specific foci in the FANCM depleted cells, colocalize with telomeres.

[0049] Of particular interest is that recruitment of BLM and BRCA1 to stressed ALT telomeres, is mutually dependent.

EXAMPLE 7

[0050] Further experiments were carried out to elucidate the function of BRCA1 in the repair processes under consideration. It is known that, during repair of double strand breaks (“DSBs”), BRCA1’s most important function is to counter 53BP1, and to stimulate DNA end resection so that DSBs can be repaired via homologous recombination, which is recognized as the “high fidelity” repair pathway. It’s also been shown that when cells are subjected to the replication stress inducer ultraviolet light, BRCA1 promotes DNA end resection.

[0051] The experiments which follow showed that BRCA1 has a role in DNA end resection at telomeres, in FANCM deficient ALT cells.

[0052] As with the other examples set forth herein, US-OS cells were transfected with siRNA targeted to luciferase (control), BRCA1, FANCM, or to both BRCA1 and FANCM. Cells were lysed and lysates were blotted with antibodies in accordance with standard methods.

[0053] The results indicate that codepletion of BRCA1 and FANCM severely impedes phosphorylation of RPA32 at Ser4/8 and 33, showing that BRCA1 is required for DNA end resection at ALT telomeres.

[0054] RPA is known to be involved in repair of damaged DNA. Once the RPA is phosphorylated at Ser4 and Ser8, the thus phosphorylated RPA molecule is recruited to the site of damage, and binds the single stranded DNA.

[0055] Thus, measurement of phosphorylation of RPA is an indication of RPA activation, and of DNA end resection – which in turn is an indication of how much single strand DNA has been produced. One therefore determined the amount of repair that is going on in FANCM depleted, ALT cells. Expressed more simply, a decrease in phosphorylated RPA levels suggests reduced DNA end resection and reduced DNA repair.

[0056] Further, as was the case with BLM, there was a very dramatic increase in micronuclei in codepleted cells, and strong synthetic lethality in the ALT cells tested, phenomena not seen in telomerase positive cells HeLa and MG63, which do not show ALT features. Nor was synthetic lethality seen when BRCA1 and BLM1 were codepleted, while FANCM was not.

[0057] The foregoing disclosure sets forth features of the invention, which involves methods for eliminating dysfunctional ALT cells, by inhibiting FANCM, as well as one or both of BRCA1 and BLM. These three molecules (FANCM, BRCA1, and BLM), are shown to be involved in alleviating replication stress in ALT cells. By inhibiting them, the viability of these dysfunctional cells is dramatically reduced.

[0058] The inhibition can be at the nucleotide level using, e.g., siRNA or other inhibitors of protein expression, but also can take place at the protein level, by using protein inhibitors, such as small molecules, i.e., non-peptide based, heterocyclic molecules, inhibiting antibodies, antibody fragments which are inhibitory, and so forth.

[0059] The methodology may be used in combination with other methodologies for inhibiting and/or eliminating dysfunctional cells. For example, several types of cancer cells are characterized by ALT. By inhibiting the mechanism which alleviates the ALT, standard drug

therapies can be modified to, e.g., reduce the strain on normal cells caused by severe therapies. Such severe forms of therapy are no longer needed when the repair mechanism is inhibited.

[0060] Other aspects of the invention will be clear to the skilled artisan and are not set forth here.

[0061] The terms and expression which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expression of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

CLAIMS

1. A method for inhibiting replication of ALT cells comprising contacting said ALT cells with (i) an inhibitor of FANCM and (ii) an inhibitor of one or both of BRCA1 or BLM, each of (i) and (ii) being administered in an amount sufficient to inhibit FANCM, BRCA1, and BLM.
2. The method of claim 1, wherein said ALT cells are cancer cells.
3. The method of claim 1, wherein each of said inhibitors inhibits DNA expression.
4. The method of claim 1, wherein each of said inhibitors are siRNA molecules.
5. The method of claim 1, wherein each of said inhibitors is a binding protein which inhibits said FANCM, BRCA1, or BLM.
6. The method of claim 5, wherein said binding protein is an antibody or a binding fragment of an antibody.
7. The method of claim 4, wherein said siRNA molecules consist of 19-27 nucleotides.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/17863

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 15/00; C12Q 1/68; G01N 33/53; G01N 33/53; C07H 21/04 (2017.01)

CPC - C12N 15/87; C12Q 1/68; C12Q 1/6883; C12Q 1/6886; G01N 33/53; C12N 15/113; C12Q 1/6876

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	Murshedi, ROLE OF FANCM IN TELOMERE MAINTENANCE IN ALTERNATIVE LENGTHENING OF TELOMERES (ALT) HUMAN CELLS. Science Graduate Institute of Medical Science, University of Toronto. A thesis for Degree of Master. 2010. PDF File: pg i-x and 1-79. [online]. [Retrieved on 2017.03.21]. Retrieved from the Internet: <URL: https://tspace.library.utoronto.ca/bitstream/1807/24237/1/AlMurshedi_Fathiya_M_20106_MSc_thesis.pdf > Abstract; pg 2, para 1; pg 5, para 1; pg 15, Fig 1-4; pg 16, Fig 1-5, pg 19, Table 1-2; pg 20, middle para; pg 22, Fig 1-6; pg 25, para 1; pg 32, lower para; pg 33, up para; pg 40 middle para; pg 48, para 1; pg 52, para 1; pg 54, para 1; pg 63, para 1; and pg 65, top para	1-4, 7
Y	ACHARYA et al., Association of BLM and BRCA1 during Telomere Maintenance in ALT Cells. PLoS One. 2014, Vol. 9(8):e103819. PDF File: pg 1-13. Abstract; pg 1, col 1; pg 2, col 1, para 1, and col 2, top para, and last para; pg 4, col 1, lower para; pg 7, col 2; and pg 8, col 1, up para, and col 2, para 2	1-4, 7
A	SCHWAB et al., ATR activation and replication fork restart are defective in FANCM-deficient cells. EMBO J. 2010, Vol. 29(4), p. 806-18. Entire documentation, especially Abstract; pg 806, col 1, and col 2, top para, para 1, and last para; pg 807, col 1, para 2, and col 2, para 2; and pg 810, col 1, para 1, and col 2, last para	1-4, 7
A	STAVROPOULOS et al., The Bloom syndrome helicase BLM interacts with TRF2 in ALT cells and promotes telomeric DNA synthesis. Hum Mol Genet. 2002, Vol. 11(25), p. 3135-44. Entire documentation, especially Abstract; pg 3136, col 2, and Fig 1	1-4, 7
A	US 2012/0329047 A1 (REDDEL et al.) 27 December 2012 (27.12.2012), Abstract, para [0001], [0003], [0055], and [0068]	1-4, 7



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	"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
"A" document defining the general state of the art which is not considered to be of particular relevance	"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
"E" earlier application or patent but published on or after the international filing date	"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
"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)	"&" document member of the same patent family
"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	

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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	US 2013/0090370 A1 (GENECARE RESEARCH INSTITUTE CO., LTD.) 11 April 2013 (11.04.2013), para [0014], [0109], [0112], and [0125]	1-4, 7
A	US 2013/0331365 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 12 December 2013 (12.12.2013), para [0027], [0031], [0041], and [0071]	1-4, 7
P,A	VERMA et al., Noncanonical views of homology-directed DNA repair. Genes Dev. 2016 May, Vol. 30(10), p. 1138-54. Entire documentation, especially Abstract; pg 1138, col 1, para 1; pg 1139, Table 1; pg 1140, col 1, lower para; pg 1143, col 1, middle para; pg 1146, col 1, up para, and col 2, middle para, and pg 1148, para 1	1-4, 7

INTERNATIONAL SEARCH REPORT

International application No.

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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 examined, the appropriate additional examination fees must be paid.

Groups I+, Claims 1-7, directed to a method for inhibiting replication of alternative lengthening of telomeres (ALT) cells comprising contacting said ALT cells with an inhibitor. The inhibitors will be searched to the extent that the inhibitors encompass wherein each of said inhibitors are siRNA molecules. It is believed that claims 1-4 and 7 encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass wherein each of said inhibitors are siRNA molecules. Additional inhibitors will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected inhibitors. 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 wherein each of said inhibitors is a binding protein [claims (1-2), 5-6].

*****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.:
1-4 and 7

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 17/17863

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

The inventions listed as Groups I+ 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:

Special Technical Feature

Among Groups I+, an inhibitor that is a siRNA molecule is structurally and functionally different from an inhibitor that is a binding protein, and vice versa.

Common Technical Features

The inventions of Groups I+ share the technical feature of a method for inhibiting replication of alternative lengthening of telomeres (ALT) cells comprising contacting said ALT cells with (i) an inhibitor of Fanconi anemia complementation group M (FANCM) and (ii) an inhibitor of one or both of breast cancer 1 (BRCA1) or Bloom syndrome protein (BLM), each of (i) and (ii) being administered in an amount sufficient to inhibit FANCM, BRCA1, or BLM (claim 1).

However, these shared technical features do not represent a contribution over prior art as being obvious over a thesis for the degree of Master of Science entitled 'The ROLE OF FANCM IN TELOMERE MAINTENANCE IN ALTERNATIVE LENGTHENING OF TELOMERES (ALT) HUMAN CELLS' by Murshedi (Graduate Institute of Medical Science, University of Toronto, 2010. p. i-x, 1-79.

Retrieved from the Internet: <URL:

https://tspace.library.utoronto.ca/bitstream/1807/24237/1/AIMurshedi_Fathiya_M_20106_Msc_thesis.pdf>, in view of an article entitled 'Association of BLM and BRCA1 during Telomere Maintenance in ALT Cells' by Acharya et al. (hereinafter 'Acharya'; PLoS One. 2014, Vol. 9(8):e103819. PDF File: pg 1-13) as follows:

Murshedi discloses a method for inhibiting replication of alternative lengthening of telomeres (ALT) cells (pg 52, para 1 - 'siRNA knockdown of FANCM was conducted in WI38-VA13/2RA and GM00847 ALT ...cell lines... after the first siRNA transfection,...all cell lines received the FANCM siRNA had decreased growth ...Reduced growth was more pronounced in the ALT cell lines', wherein 'siRNA knockdown of FANCM... Reduced growth was more pronounced in the ALT cell lines' is 'a method for inhibiting replication of alternative lengthening of telomeres (ALT) cells'; pg 65, top para - 'FANCM is required for normal telomere maintenance in ALT cells by the involvement of FANCM in regulation of HR-mediated replication/amplification of telomeric repeat DNA'; Abstract - 'alternative lengthening of telomeres (ALT)'; pg 25, middle para - 'Fanconi Anemia Complementation group M (FANCM)'; pg 63, para 1 - 'FANCM siRNA knockdown affected cell growth and cell death in both ALT and telomerase positive cells...Absence or depletion of FANCM can be speculated to affect cell growth and survival through failure to resolve stalled replication intermediates, achieve or regulate appropriate homologous recombination (HR)')
--comprising contacting said ALT cells with (i) an inhibitor of Fanconi anemia complementation group M (FANCM) (pg 52, para 1 - 'siRNA knockdown of FANCM was conducted in WI38-VA13/2RA and GM00847 ALT ...cell lines...after the first siRNA transfection, ... all cell lines received the FANCM siRNA had decreased growth ...Reduced growth was more pronounced in the ALT cell lines'; pg 25, middle para - 'Fanconi Anemia Complementation group M (FANCM)'),

-- being administered in an amount sufficient to inhibit FANCM (pg 52, para 1 - 'siRNA knockdown of FANCM was conducted in WI38-VA13/2RA and GM00847 ALT ...cell lines...after the first siRNA transfection, ... all cell lines received the FANCM siRNA had decreased growth ...Reduced growth was more pronounced in the ALT cell lines').

Murshedi further discloses that both breast cancer 1 (BRCA1) and Bloom syndrome protein (BLM) are associated with ALT (pg 19, Table 1-2: DNA damage response ...BRCA1; HR...BLM; pg 20, para 1 - 'the role of DNA repair proteins in ALT maintenance is Bloom syndrome protein (BLM)'; pg 5, para 1 - 'breast cancer susceptibility gene 1 (BRCA1)'; pg 63, para 1 - 'FANCM ... homologous recombination (HR)'; pg 22, Fig 1-6), in gene replication (pg 19, Table 1-2; pg 15, Fig 1-4, Legend - 'Homologous recombination as the telomere maintenance mechanism in ALT'; Fig 1-4; pg 15, lower para - 'the total gain of telomeric sequence observed in ALT, a rolling-circle amplification of telomeres model ...continuous replication of the rolling circle and results in extensive lengthening of the telomeres'; pg 16, Fig 1-5, Legend - 'A model for rolling-circle amplification of telomeres. In this model, BLM'; Fig 1-5).

Murshedi does not specifically teach wherein the method further comprising contacting said ALT cells with (ii) an inhibitor of one or both of breast cancer 1 (BRCA1) and Bloom syndrome protein (BLM), each of (ii) being administered in an amount sufficient to inhibit BRCA1 and/or BLM. Acharya discloses tumors utilize recombination-based alternative lengthening of telomeres (ALT) to maintain telomere which is associated with BLM and BRCA1 co-localization at telomeres (Abstract - 'tumors utilize recombination-based alternative lengthening of telomeres (ALT) to maintain telomere... BLM and BRCA1 co-localize with RAD50 at telomeres during S- and G2-phases... in immortalized human cells using ALT'; pg 2, col 2, last para - BLM and BRCA1 physically interact and the BLM-BRCA1-telomere co-localized foci are specific to ALT cells'), wherein BRCA1 stimulates BLM activity in replication (pg 4, col 1, lower para - 'BRCA1 stimulates BLM unwinding activity on a telomeric fork'; pg 7, col 2 to pg 8, col 1, up para - 'the temporal requirements of the BLM-BRCA1 interaction...their enhanced association at G2 suggests a function during late replication or during recombination-associated events at the telomere'), as well as a method for inhibiting BRCA1 by administering a BRCA1 inhibitor, a siRNA, in an amount sufficient to inhibit (knockdown) BRCA1 (pg 2, col 2, top para - 'Knockdown of BRCA1 by siRNAs eliminates co-localization'; Abstract - 'BLM and BRCA1 co-localize... in immortalized human cells using ALT'), for inhibiting recombination-based ALT (pg 8, col 1, up para - 'Given the importance of recombination in ALT, an inhibitory activity of BRCA1 might be essential to limit recombination at telomeric regions resulting from a strand break due to damage or replicative stress'; Abstract - 'tumors utilize recombination-based alternative lengthening of telomeres (ALT) to maintain telomere').

It would have been obvious to one of ordinary skill in the art at the time the invention was made to combine the teachings of Murshedi and Acharya, to obtain a method for inhibiting replication of alternative lengthening of telomeres (ALT) cells comprising contacting said ALT cells with (i) an inhibitor of Fanconi anemia complementation group M (FANCM), being administered in an amount sufficient to inhibit FANCM, as taught by Murshedi, and further wherein the method further comprising contacting said ALT cells with (ii) an inhibitor of one or both of breast cancer 1 (BRCA1) and Bloom syndrome protein (BLM), each of (i) and (ii) being administered in an amount sufficient to inhibit FANCM, BRCA1, and/or BLM, based on the combination of Acharya and Murshedi, in order to combine the methods and inhibitors available in the art for facilitating inhibiting replication of ALT cells with a desired effect and without undue experimentation.

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

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/17863

Continuation of:

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

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

Groups I+ therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Note:

I) Claim 1 is objected as lacking a definition for the first appeared abbreviation "ALT", "FANCM", "BRCA1", and "BLM" in claims. For the purposes of this ISR, the first "ALT", "FANCM", "BRCA1", and "BLM" in claim 1 is rewritten as "alternative lengthening of telomers (ALT)", "Fanconi anemia complementation group M (FANCM)", "breast cancer 1 (BRCA1)", and "Bloom syndrome protein (BLM)", respectively, base on the Specification and Wikipedia (Specification: para [001] - 'alternative lengthening of telomers, or "ALT"; Wikipedia FANCM - 'Fanconi anemia, complementation group M, also known as FANCM'; Wikipedia BRCA1 - 'BRCA1... the official name (breast cancer 1)'; Wikipedia Bloom syndrome protein - 'Bloom syndrome protein is a protein that in humans is encoded by the BLM gene'; All Wikipedia references are not provided).

II) Claim 1 is further objected as using an improper alternative format for the limitation "one or both of BRCA1 or BLM" as well as the list of "FANCM, BRCA1, and BLM", which is inconsistent with the limitation of "each of (i) and (ii) being administered". For the purposes of this ISR, the "or" in the limitation "BRCA1 or BLM" is replaced by "and" to have the limitation "one or both of BRCA1 and BLM"; and the "and" in the list of "FANCM, BRCA1, and BLM" is replaced by "and/or"; and claim 1 is construed as follows:

1. A method for inhibiting replication of alternative lengthening of telomers (ALT) cells comprising contacting said ALT cells with (i) an inhibitor of Fanconi anemia complementation group M (FANCM) and (ii) an inhibitor of one or both of breast cancer 1 (BRCA1) and Bloom syndrome protein (BLM), each of (i) and (ii) being administered in an amount sufficient to inhibit FANCM, BRCA1, and/or BLM.