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D E C I S I O N
of 30 January 2006

Case Number: W 0016/05 - 3.3.08

Application Number: PCT/US 2004/014494

Publication Number: WO 2004/101788

IPC: C12N 15/11

Language of the proceedings: EN

Title of invention:

Small interfering RNA libraries and methods of synthesis and use

Applicant:

University of Pittsburgh of the Commonwealth System of Higher Education

Headword:

Random siRNA library/UNIVERSITY OF PITTSBURGH

Relevant legal provisions:

PCT Art. 17(3)(a)
PCT R. 13, 40

Keyword:

"Lack of unity between inventions 1 and 9 (no)"
"Lack of unity among inventions 1, 5 and 6 (yes)"

Decisions cited:

-

Catchword:

-



Case Number: W 0016/05 - 3.3.08

International Application No. PCT/US 2004/014494

D E C I S I O N
of the Technical Board of Appeal 3.3.08
of 30 January 2006

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Decision under appeal: Protest according to Rule 68.3(c) of the Patent
Cooperation Treaty made by the applicants
against the invitation of the European Patent
Office (International Preliminary Examining
Authority) to restrict the claims or pay
additional fees dated 12 November 2004.

Composition of the Board:

Chairman: L. Galligani
Members: B. Guenzel
P. Julià

Summary of Facts and Submissions

- I. International patent application PCT/US04/14494 with the title "Small interfering RNA libraries and methods of synthesis and use" was filed on 10 May 2004 with 74 claims and published as WO 2004/101788.

Independent claims 1, 8, and 13 were directed to methods for preparing small interfering RNA (siRNA) libraries. Independent claim 18 related to a method of preparing a double stranded RNA library. Claims 22 and 23 concerned, respectively, a library as prepared by the method of any of claims 1 to 21 and a random or semi-random siRNA library. Whereas independent claim 31 was directed to a method of using a siRNA library, independent claims 53 and 55 related to a subpopulation (of cells) obtained by said method and to a siRNA isolated from said population, respectively. Independent claims 57, 60 and 61 were concerned, respectively, with a nucleic acid molecule, a DNA expression cassette encoding or comprising said nucleic acid molecule and a method of attenuating the expression of estrogen receptor alpha using said nucleic acid molecule. Independent claims 63 and 67 referred, respectively, to a method of generating DNA hairpins and to a plasmid containing two recombinase recognition sites oriented towards each other. All other claims were dependent on these independent claims and covered further embodiments of the invention.

- II. On 12 November 2004 the European Patent Office, acting as International Searching Authority (ISA), issued an "Invitation to pay additional fees" (PCT Article 17(3)(a) PCT and Rule 40.1 PCT). The ISA

considered that the international application did not comply with the requirements of unity of invention (Rules 13.1, 13.2 and 13.3 PCT) and invited the applicant to pay eight additional fees. The applicant was inter alia informed that, according to Rule 40.2(c) PCT, the payment of any additional fee could be made under protest.

III. In the above mentioned invitation, the ISA stated that the application lacked unity as required by Article 17(3)(a) PCT and Rule 13 PCT and that it related to nine groups of inventions identified as follows:

"1. claims: 1-7 (completely) and 17, 22-56 (partially)

A method for preparing a small interfering RNA (siRNA) library comprising: (1) generating a population of oligoDNAs, each of which comprises a random or semi-random sequence flanked by a 3' restriction site and a 5' restriction site, wherein the 3' restriction site is different from the 5' restriction site; (2) cloning the oligoDNAs into plasmids having two recombinase sites oriented toward each other so as to orient the random sequence flanked by the 3' restriction site and the 5' restriction site between the dual recombinase sites; (3) replicating the plasmids within a population of host cells that produce the appropriate recombinase enzyme for the particular recombinase recognition sites present in the plasmids; (4) exposing the plasmids to the recombinase enzyme within the host cells; (5) isolating plasmid DNA from the population host cells and digesting the isolated plasmids with a restriction enzyme specific for either the 3' restriction site or the 5' restriction site; (6) autoligating the digested

fragment containing a dual cassette wherein said dual cassette comprise a sense random sequence and a complementary antisense sequence separated by a spacer sequence which comprises either said 3' or said 5' restriction site; (7) digesting the dual cassettes with the restriction enzyme specifically flanking the inverted repeats and cloning the population of dual cassettes into a RNA expression vector system, which includes a RNA expression vector having a RNA polymerase promoter and a RNA polymerase termination sequence, such that the dual cassettes are inserted between the RNA promoter and the termination sequence; (8) pooling the vectors to form a siRNA library. Libraries prepared by said method."

"2. claims: 8-12 (completely) and 17, 22-56 (partially)

A method for preparing a siRNA library comprising: (1) generating a population of oligoDNAs, each of which comprises a random or semi-random sequence bounded by restriction sites optionally followed by a priming sequence; (2) extending the complementary strand to make double-stranded oligoDNAs; (3) cloning the oligoDNAs into a RNA expression vector system having vectors comprising a first RNA promoter and a second RNA promoter, the first and second promoters oriented inward to direct RNA transcription of respective sense and antisense complementary sequences positioned between the first and second RNA promoters, wherein the oligoDNAs sequences are cloned into said vectors between said first and said second promoters; and (4) pooling the vectors containing the inserts from step (3) to form a siRNA-encoding library. Libraries prepared by said method."

"3. claims: 13-16 (completely) and 17, 22-56 (partially)

A method for preparing a siRNA library comprising: (1) generating a population of oligoDNAs, each of which comprises (5' to 3') a preselected restriction site, a random or semi-random sequence, a first sequence contributing to a priming loop, a spacer sequence, and a second sequence contributing to a priming loop, whereby the first sequence contributing to a priming loop and the second sequence contributing to a priming loop anneal to form a loop; (2) exposing the population of oligoDNAs to a DNA polymerase under suitable conditions to extend the complementary strand from the end of the second sequence contributing to a priming loop through the template strand preselected restriction site; (3) denaturing the double stranded extended oligoDNAs for form single stranded extended oligoDNAs; (4) synthesizing the complementary strand to the single stranded extended oligoDNAs for form siRNA-encoding DNAs under conditions that minimize self-annealing of the template; (5) pooling the siRNA-encoding DNAs to form a siRNA library. Libraries prepared by said method."

"4. claims: 18-21 (completely) and 22-56 (partially)

A method of preparing a double stranded RNA library comprising: (1) obtaining a cDNA library; (2) cloning the cDNAs into plasmids comprising a cloning site flanked by two RNA polymerase promoters wherein a first RNA promoter transcribes the sense strand and a second RNA polymerase promoter transcribes the antisense strand, and wherein the digested cDNA is introduced

into the plasmid between the first and the second RNA polymerase promoters, resulting in unique expression cassettes; (3) combining the resultant plasmids to form a double stranded RNA-encoding library. Libraries obtained by said method."

"5. claims 23(completely) and 24-30 (partially)

A random or semi-random siRNA library (obtained by any method) or subpopulation thereof."

"6. claims 30-56 (completely)

Methods of using a siRNA library (random or not) comprising introducing the siRNA library into a population of cells and subjecting the population of cells to a selection process to select a subpopulation of cells that exhibit a different behavioural, biochemical, chemical, functional, molecular, morphological, phenotypic or physical property than the remainder of the population. Subpopulations isolated in accordance with the methods and siRNA isolated from said subpopulation."

"7. claims: 57-62 (completely)

Nucleic acid molecules comprising at least ten consecutive nucleic acids selected from the sequences shown in SEQ ID NO: 45, 46, 49 and 50 or their complementary sequences and their use in a method for attenuating the expression of estrogen receptor alpha."

"8. claims: 63-66 (completely)

A method of generating DNA hairpins comprising: (1) generating a population of oligoDNAs, each of which is flanked by a 3' restriction site and a 5' restriction site, wherein the 3' restriction site is different than the 5' restriction site; (2) cloning the oligoDNAs into plasmids having two recombinase sites oriented toward each other so as to orient the oligoDNAs flanked by 3' restriction site and the 5' restriction site between the dual recombinase sites; (3) replicating the plasmids within a population of host cells that produce the appropriate recombinase enzyme for the particular recombinase recognition sites present in the plasmids; (4) exposing the plasmids to the recombinase enzyme within the host cells; (5) isolating plasmid DNA from the population host cells and digesting the isolated plasmids with a restriction enzyme specific for either the 3' restriction site or the 5' restriction site; (6) autoligating the digested fragment containing a dual cassette wherein said dual cassettes comprise a sense sequence and a complementary sequence corresponding to said oligoDNAs separated by a spacer sequence which comprises either said 3' or said 5' restriction site."

"9. claims: 67-74 (completely)

A plasmid containing two recombinase recognition sites oriented towards each other and having a sequence of DNA between the two recombinase recognition sites, wherein said sequence comprises at least one restriction endonuclease recognition sequence."

In the light thereof, the ISA further identified the following groups of inventions:

Group A: inventions 1 to 6 referring to the generation and use of siRNA libraries.

Group B: invention 7 referring to specific nucleic acid molecules and their use for attenuating expression of estrogen receptor alpha.

Group C: invention 8 referring to a method for generating hairpins.

Group D: invention 9 referring to plasmids containing two recombinase recognition sites oriented toward each other and comprising between the two recombinase recognition sites a DNA having at least one restriction endonuclease recognition sequence.

IV. The ISA failed to see which was the inventive common concept linking the groups of inventions A, B, C and D. Therefore, each of these groups was considered as an independent invention in the sense of Rule 13.1 PCT. Moreover, the common concept underlying inventions 1 to 6 was identified as being a siRNA library. However, the generation of random siRNA libraries using two U6 promoters placed in opposed orientation and the use of said random siRNA library for reverse genetic screens were already disclosed in the prior art (cf. Miyagishi et al., Nature Biotechnology, 2002, Vol. 19(5), 497-500) (D1). The generation of random siRNA libraries for genetic studies was also disclosed in WO 03/020931 (D2). Therefore, the common concept linking inventions 1 to 6 was not regarded as inventive. Thus, group A was

considered to lack unity in the sense of Rule 13.1 PCT and each of the inventions 1 to 6 was an independent invention.

V. With letter dated 27 December 2004, the applicant paid four additional search fees for the identified inventions (subgroups) 5, 6, 8 and 9. The additional fees were paid under protest since the applicant argued that the claims of groups A, C and D (inventions or subgroups 1-6, 8 and 9) could be searched and examined together for the following reasons:

1) subgroup 1 should be searched and examined with subgroup 8.

A comparison of claim 63 (subgroup 8) and claim 1 (subgroup 1) revealed that the process steps were virtually identical. Steps 1 to 6 of claim 63 were the same as steps 1 to 6 of claim 1, with the exception that step 1 of claim 1 specified that each oligoDNAs comprised a random or semi-random sequence. Claim 65, also within subgroup 8, specified that the oligoDNAs were random or semi-random. Thus, the subject-matter of subgroups 1 and 8 overlapped to such an extent that they should be searched and examined together.

2) subgroup 9 should be searched and examined with subgroup 1 and/or 8.

The claims of subgroup 9 concerned a plasmid having two recombinase sites oriented toward each other. This was a component of the method recited in claims 1 (subgroup 1) and 63 (subgroup 8). Accordingly, the claims of

subgroup 9 should be searched in conjunction with a search of either (and both) of subgroups 1 and 8.

3) group A should not be split into subgroups 1 to 6 but should be searched together.

None of the documents cited by the ISA, namely Miyagishi et al. and WO 03/02931 (cf. point IV *supra*), was sufficient to place truly random siRNA libraries within the state of the art.

Miyagishi et al. (*supra*) disclosed the generation of a separate sense and antisense RNA for effective RNAi after hybridization, using two separate, tandem Pol III (U6) promoters. These were specifically targeted to products of the GFP or other commonly used reporter genes and required known, and therefore not random sequences. The authors reported only that two U6 promoters had been oriented toward each other, but they did not state or imply that any 20 nt siRNA was cloned between them and that it had any resulting siRNA activity. The discussion regarding random libraries was entirely speculative and hypothetical in nature. Moreover, there was no enabling information as to how a random library could be made or used (e.g., how genetic screens were to be designed and performed) or even what concerns needed to be addressed. Nor did this document provide any expectation that a random library could be functional. Reference was also made to technical hurdles that needed to be addressed in constructing random siRNA libraries. In particular, the possible generation of inverted repeats (due to the presence of two identical opposing Pol III promoters) that could

cause plasmid instability (increased potential bacterial recombination) in *E. coli*.

WO 03/020931 disclosed the generation of siRNA libraries directed against existing RNA sequences. Examples 10-11 speculated that a library with random sequences could be possible. However, it failed to address the technical hurdles that impeded the generation of these random siRNA libraries. In particular, the hairpin structure had great homology to itself and would tend to self-anneal (more particularly for regions rich in GC content). Thus, it was necessary to use an amount of primer in great excess of the template to facilitate complementary strand priming and to use polymerization conditions that maintained the denaturation of the hairpin structure (so as to prevent non-random GC biased self-annealing). However, this was not taught in that document and thus, as such, the document did not place a random siRNA library within the state of the art.

VI. On 12 May 2005 a notification regarding review of justification for invitation to pay additional search fees was issued by the ISA (Rule 40.2(e) PCT). The review panel decided that invention 8 could be searched with invention 1 and thus refunded one extra search fee. However, the group of inventions 5, 6 and 9 were regarded as independent inventions in the sense of Rule 13(1) PCT. The following reasons were given in an Annex I:

Document Miyagishi et al. (*supra*) did not disclose a random siRNA library but suggested the use of two opposed U6 promoters on each side of an about 20-nt

fragment for the generation of siRNAs and the use of this opposed U6 promoters system for the production of a randomized siRNA library. The possible problem associated with the use of two identical opposed promoters (increased bacterial recombination) was not addressed in that document. However, the application was not restricted to the use of two different opposed promoters. On the contrary, figure 4A of the application, which illustrated an embodiment of the invention, showed a construct corresponding to the constructs suggested in this document, i.e. comprising two opposed identical U6 promoters.

With regard to document WO 03/020931, it was considered that it was no problem for the skilled person to adjust the amount of primer in the method disclosed in examples 10 and 11 and figure 6 of that document so as to avoid any self-annealing or to adjust the polymerization conditions so as to avoid the production of a siRNA library biased against GC-rich sequences.

The vectors comprised in the identified invention 9 were not restricted to vectors for use in the methods of the application but encompassed every vector having any sequence with a restriction site located between two inverted recombinase sites.

Thus, the review panel invited the applicant to pay a protest fee.

VII. The protest fee was paid on 10 June 2005.

Reasons for the Decision

1. Pursuant to Article 154(3) EPC the Board of Appeal is responsible for deciding on a protest made by an applicant against the payment of an additional fee charged by the EPO under the provisions of Article 17(3)(a) PCT.
2. The protest complies with the requirements of Rule 40.2 (c) and (e) PCT and it is therefore admissible.
3. According to Rule 13.1 PCT, the international patent application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept. This requirement is fulfilled only when there is a technical relationship among those inventions involving one or more of the same or corresponding "special technical features", i.e. those technical features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art (Rule 13.2 PCT).
4. In the present case, the ISA found lack of unity at two levels, namely in respect of the groups of inventions designated as A, B, C and D and in respect of the groups of inventions 1 to 9, wherein 1 to 6 were subgroups of A, and 7, 8 and 9 corresponded to B, C and D, respectively. In response to the objection, the applicant decided to pay under protest only four additional search fees, namely for the inventions 5, 6, 8 and 9. One of the extra fees, namely that in respect of invention 8, was reimbursed after review by the review panel. Thus, the present protest is in respect of the finding of lack of unity between inventions 1

(the first invention searched) and inventions 5, 6 and 9.

Inventions 1 and 9

5. The board agrees with the appellant that there is a technical relationship between inventions 1 and 9 for the following reasons:

The first technical feature characterizing the plasmids of invention 9, namely the presence of *"two recombinase recognition sites oriented towards each other"*, is shared by the plasmids used for cloning a population of random oligoDNAs in step (2) of the method of claim 1 (invention 1). Moreover, in order *"to orient the random sequence flanked by the 3' restriction site and the 5' restriction site between the dual recombinase sites"* as required in said step (2) of claim 1, the plasmids must necessarily have *"a sequence of DNA between the two recombinase recognition sites, wherein said sequence comprises at least one restriction endonuclease recognition sequence"* as shown in the description of the published application (cf. page 27, lines 9 to 12, page 28, lines 35 to 37, page 29, lines 26 to 28 and Method 1A, Figure 1A). This feature corresponds in fact to the second technical feature characterizing the plasmids of the invention 9.

Although the plasmids of the invention 9 might be used for a purpose other than the preparation of a random siRNA library, i.e. they are not restricted to plasmids for use in the method of claim 1 (invention 1), these plasmids are nevertheless always suitable for use in such a method - as appropriate intermediate products -

due to their essential technical features. Moreover, plasmids having these essential characterizing features must - always and necessarily - be used in the method of claim 1 (invention 1).

6. Therefore, the board considers that there is a technical relationship between inventions 1 and 9 involving the same special technical features in the sense of Rule 13.2 PCT, namely the features defining the plasmids of invention 9 which must necessarily be used (as intermediate products) in the method of invention 1. Thus, an extra search fee is to be reimbursed to the applicant.

Invention 1 and inventions 5 and 6

7. It remains now to be assessed whether such a technical relationship is also found among the inventions 1, 5 and 6. In other words, whether there is a single general inventive concept underlying all these inventions.
8. The ISA identified "a random siRNA library" as the common concept linking the inventions 1 to 6 (cf. point IV *supra*). This has not been denied by the applicant and, although extra search fees were paid for inventions 5 and 6 only, no other (alternative) common concept underlying the inventions 1, 5 and 6 has been identified by the applicant. Nor is the board able to identify any other. Thus, the concept of a random siRNA library should serve as a unitary link among the methods for preparing a siRNA library (invention 1), the library itself (invention 5) and the methods of using it (invention 6) (cf. point III *supra*).

9. The ISA considered, however, that the concept of "a random siRNA library" was already disclosed by documents D1 and D2. This was also confirmed by the review panel (cf. points IV and VI *supra*). Thus, no single general inventive concept was seen among the groups of inventions in question.
10. Document D1 discloses a strategy for generating siRNA using an expression vector in which 19-nt sense and antisense sequences against a target gene are placed under the control of U6 promoters (cf. page 497, figure 1). Cotransfection experiments with analysis of the resulting repression in the expression of several reporter genes demonstrate that U6 promoter-driven siRNA is stably produced in cells and that it disrupts effectively and specifically the expression of a gene of interest. Document D1 concludes stating that "*it could be possible to generate siRNA using opposing U6 promoters, similar to the opposing T7 promoters¹⁷, with two different U6 promoters placed in opposing orientations with ~20 nt between them. In our preliminary experiment, we have been able to develop opposing U6 promoters. This opposing promoter system may allow the production of **randomized siRNA libraries** and may eventually allow reverse genetic screens using RNAi, utilizing an approach similar to that reported earlier using hybrid ribozyme libraries^{18,19}.*" (bold-type introduced by the Board) (cf. page 499, left-hand column, last paragraph).

Thus, as such the concept of a random siRNA library is already anticipated in document D1 and consequently, it

cannot be seen as the inventive common concept linking the inventions 1, 5 and 6.

11. The applicant has argued that, although document D1 refers to a random siRNA library, this reference is merely speculative. In its view, document D1 does not actually make a random siRNA library available to the skilled person since the technical difficulties and hurdles that have to be overcome in order to obtain such a random siRNA library are not addressed in this document (cf. point V *supra*). However, the board cannot follow this argumentation for the following reasons:

The construction of opposing U6 promoter systems does not require any particular skill. Nor is it required for using these systems in the production of a random siRNA library or for performing the (reverse) genetic screens referred to in document D1, as clearly shown by the references to the known prior art. Thus, document D1 gives clear instructions to be followed and provides the appropriate technical means to achieve a random siRNA library.

Moreover, in the light of the application itself, the technical difficulties referred to by the applicant, in particular an alleged plasmid instability caused by an increase of (bacterial) recombination arising from the presence of inverted repeats comprised between the two identical opposed promoters (cf. point V *supra*), do not appear to be relevant. In fact, the application refers to the use of expression vectors for directly cloning random oligoDNAs between two identical opposed promoters as a preferred embodiment (cf. page 13, lines 12 to 27 of the published application) that is further

illustrated in examples 5, 7 and 8 (cf. page 30, line 32 to page 31, line 6 and figure 4A, pages 31 and 32 and figures 5 and 7A-7B of the published application) and explicitly claimed as well (cf. claims 9 and 12 of the application as published). Thus, although the use of expression vectors with two different opposed promoters might be more advantageous (higher stability, increased yield, etc.), the use of two identical opposed promoters - as proposed in document D1 - appears to be workable and appropriate.

12. It follows from the foregoing that document D1 discloses - in an enabling manner - the common concept linking inventions 1, 5 and 6, i.e. a random siRNA library. Therefore, as correctly stated by the ISA and also confirmed by the review panel, a lack of unity arises for these inventions as the only possible inventive link is then missing. The request of extra search fees made by the ISA for the inventions 5 and 6 was thus justified.

13. In view of this finding, the Board considers that it is not necessary to further analyse in detail the teaching of document D2.

Order

For these reasons it is decided that:

1. The protest is partially justified.
2. The reimbursement of one additional search fee paid by the applicant is ordered.

The Registrar:

The Chairman:

G. Rauh

L. Galligani