PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

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PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)* see form PCT/ISA/210 (second sheet)	
Applicant's or agent's file reference see form PCT/ISA/220	**FOR FURTHER ACTION** See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP2010/050723	22.01.2010	22.01.2009

International Patent Classification (IPC) or both national classification and IPC
INV. C07K14/72 C12N9/00 C12N15/90 C12N9/90

Applicant
CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE...

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notifed the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA:	Date of completion of this opinion	Authorized Officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Fax: +49 89 2399 - 4465	see form PCT/ISA/210	Madruga, Jaime Telephone No. +49 89 2399-4739

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43bis.1(a))

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-15, 19
	No:	Claims	16-18, 20-26
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-26
Industrial applicability (IA)	Yes:	Claims	1-26
	No:	Claims	

2. Citations and explanations

see separate sheet

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item V.

1 THE APPLICATION

1.1 The present application concerns mutants of the human Estrogen Receptor alpha (ERα) ligand binding domain (LBD), with different affinity to synthetic ligands; fusion proteins comprising such mutant ERα-LBD, especially with recombinases and, in particular Cre (Cre-ER), and uses thereof are claimed.

1.2 In particular, variants are claimed having at least six amino-acid substitutions at positions L346, A350, M388, G521, Y526 (ERM-Da, known in the prior art) and in at least one of T347, V376, G400, I424, G442, Y459, L466, H524 and M528. The application discloses two specific mutants referred to as ERM-Db (L346I, A350M, M388Q, G521S, Y526D, G400V) and ERM-Dc (L346I, A350M, M388Q, G521S, Y526D, G442Y, Y459N, L466S, G521S). Said mutated ER-LBD were used to make Cre-ERM-Db and Cre-ERM-Dc, which are strictly regulated by 4,4'-dihydroxybenzyl (DHB) or 4,4'-dihydroxylbenzyl dipivalate (DHBD). The mutants of the application (ERM-Db and ERM-Dc) exhibit reduced background as compared to the mutant of the prior art (ERM-Da) and exhibit no activation in the absence of DHB/DHBD, thus, these mutants do not interfere with oestrogen signalling.

2 CITATIONS

2.1 Reference is made to the following document:

D1 US 2006/199250 A1 (ZHAO HUIMIN [US] ET AL) 7 September 2006 (2006-09-07) cited in the application

D2 CHOCKALINGAM KARUPPIAH ET AL: "Directed evolution of specific receptor-ligand pairs for use in the creation of gene switches"PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. (PNAS), NATIONAL ACADEMY OF SCIENCE, WASHINGTON, DC, US, vol. 102, no. 16, 19 April 2005 (2005-04-19) , pages 5691-5696, XP002508545ISSN: 0027-8424 cited in the application

D3 EP 1 692 936 A1 (GIE CERBM CT EUROP DE RECH EN [FR]) 23 August 2006 (2006-08-23) cited in the application

D4 FEIL ROBERT ET AL: "Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains"BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, ACADEMIC PRESS INC. ORLANDO, FL, US, vol. 237, no. 3, 1 January 1997 (1997-01-01) , pages 752-757, XP002144708ISSN: 0006-291X cited in the application

D5 WO 02/097050 A2 (NOVARTIS AG [CH]; BRACKEN KATHRYN RENE [US]; DE LOS ANGELES JOSEPH E [) 5 December 2002 (2002-12-05)

D6 EKENA K ET AL: "Identification of amino acids in the hormone binding domain of the human estrogen receptor important in estrogen binding"JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY, INC, US, vol. 33, no. 271, 16 August 1996 (1996-08-16) , pages 20053-20059, XP002075903ISSN: 0021-9258

D7 ISLAM KAZI MOHAMMED DIDARUL ET AL: "Directed evolution of estrogen receptor proteins with altered ligand-binding specificities"PROTEIN ENGINEERING DESIGN & SELECTION, vol. 22, no. 1, 1 January 2009 (2009-01-01) , pages 45-52, ISSN: 1741-0126

D8 EKENA KIRK ET AL: "Different residues of the human estrogen receptor are involved in the recognition of structurally diverse estrogens and antiestrogens"JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 8, 1997 , pages 5069-5075, ISSN: 0021-9258

D9 BUSH S M ET AL: "Use of the yeast one-hybrid system to screen for mutations in the ligand-binding domain of the estrogen receptor"STEROIDS, ELSEVIER SCIENCE PUBLISHERS, NEW YORK, NY, US, vol. 61, no. 3, 1 March 1996 (1996-03-01) , pages 102-109, XP004026431ISSN: 0039-128X

D10 ANSTEAD G M ET AL: "The estradiol pharmacophore: Ligand structure-estrogen receptor binding affinity relationships and a model for the receptor binding site"STEROIDS, ELSEVIER SCIENCE PUBLISHERS, NEW YORK, NY, US, vol. 62, no. 3, 1 March 1997 (1997-03-01) , pages 268-303, XP004057108ISSN: 0039-128X

D11 EKENA KIRK ET AL: "Determinants of ligand specificity of estrogen receptor-alpha: Estrogen versus androgen discrimination"JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 2, 9 January 1998 (1998-01-09) , pages 693-699, ISSN: 0021-9258

3 **LACK OF UNITY OF INVENTION** (Art. 17(3)(a) PCT, Rule 13.1 PCT)

3.1 Mutants of the Ligand binding domain of the human estrogen receptor alpha, even some comprising at least 6 mutations including L346I, A350M, M388Q, G521S, Y526D and having reduced affinity for estrogen and increased affinity for DHB and being controlled by DHB were known in the prior art (D1, D2, MUTANT 5-E). Fusion proteins of ER mutants of the LBD with modified ligand-binding properties were known (D1-D4), even for fusions with recombinases, including Cre (D3-D4).

3.2 The present application tries to solve the **problem** of providing further fusion proteins comprising mutants of the LBD of ERα with selective binding to synthetic ligands devoid of oestrogenic and anti-oestrogenic activities. The present application provides **many possible solutions**, namely, fusion proteins comprising a mutated ER-alpha ligand binding domain having, in addition to the mutations at positions: L346, A350, M388, G521, Y526 a mutation at, at least one additional position selected from: T347, V376, G400, 1424, G442, Y459, L466, H524, and M528.

3.3 Fusion proteins with such mutated hER-alpha LBD were known from D1-D4. The use of the mutants of D1 (or D2) for the construction of further Cre-ER variants as in D3 (or D5), was a very obvious alternative for a skilled person and cannot be considered as a common inventive concept between the different solutions.

3.4 In addition, the only common structural feature between all the mutants claimed, is the presence of the mutations at positions: L346, A350, M388, G521, Y526 , which was already known from D1 and D2 (4-S and 5-E), and these mutants showed a selective binding of the mutated ER to DHB, which is a "synthetic ligands devoid of oestrogenic and anti-oestrogenic activities".

3.5 Thus, none of the common technical features between each one of the individual mutants claimed can be regarded as a common new and inventive concept. There is no single inventive concept underlying the plurality of the

claimed inventions in the present application, in the sense of Rule 13.1 PCT. Consequently there is lack of unity and each of the mutants should be considered as a separate invention.

3.6 However, since most of the solutions are not regarded as real solutions to the problem, only two specific mutants, ERM-Db and ERM-Dc, are shown in the application to have specific properties, and the claims could be searched completely, the objection as to lack of unity of invention is not raised at present. The objection is however likely to be raised later in the procedure if the claims are not restricted in a suitable manner.

4 NOVELTY (Art. 33(2) PCT)

4.1 D1 discloses mutants of the LBD of human ER-alpha having the mutations: L346I, A350M, M388Q, G521S, Y526D ("mutant 4-S") and L346I, A350M, M388Q, G521S, Y526D, F461L, V560M ("mutant 5-E"). Said mutants exhibit high selectivity for the synthetic non-steroidal compound 4,4,dihydroxybenzil (DHB). The mutants in D1 include hER variants containing one or more of the following mutations relative to SEQ ID NO:2: Ala350Met, Leu346Ile, Met388Gln, Gly521Ser, Tyr526Asp, Phe461Leu, Val560Met, Gly442Tyr, and Leu466Ser (Example 2). D1 also discloses yeast two hybrid system fusion proteins of hER-alpha with Gal4 transactivator to test the variants (D1, [0085]). Mammalian two hybrid systems can also be used (D1, [0072]).

4.2 D2 discloses several LBD of human ER including some having the mutations: L346I, A350M, M388Q, G521S, Y526D ("mutant 4-S") and L346I, A350M, M388Q, G521S, Y526D, F461L, V560M ("mutant 5-E"). Said mutants exhibit high selectivity for the synthetic non-steroidal compound 4,4,dihydroxybenzil (DHB).

4.3 D1 and D2 do not disclose mutants having, in addition to the mutations at positions: L346, A350, M388, G521, Y526, a mutation in at least one additional position selected from: T347, V376, G400, I424, G442, Y459, L466, H524, and M528. D1 and D2 disclose fusion proteins of the mutated hERα LBD with the DNA binding domain of the yeast Gal4 transactivator; these fusion proteins, for expression in yeast, however, no fusions with a recombinase protein were disclosed in D1 or D2.

4.4 D3 discloses a Cre-ER fusion protein with a LBD having several mutations, including G521R and G400V; the triple mutant G400V/M543A/L544A called Cre-ER-T2. This allows Cre-ER to respond to synthetic antiestrogens but not

to natural (synthetic) estrogens, including tamoxifen (Tam) and 4-hydroxy-tamoxifen (OHT), and the antiestrogens ICI164,384 and ICI182,780. D3 also refers to the combined mutant G521R/G400V (called Cre-ER(VR); D3, [0033], [0036]; see also D4).

4.5 D4 discloses variants of Cre-ER, for regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. In particular, Cre-ERT2 (G400V/M543A/L544A) is 4-fold more potent than Cre-ERT in cell lines and is induced by the synthetic ER antagonists 4-hydroxy-tamoxifen (OHT) and ICI182,780 (ICI), respectively, but insensitive to 17-beta-estradiol (E2). D5 discloses other mutants, including variants at positions G521 and G400, referred to as: Cre-ER (GG), (VG), (CVR), and (GR).

4.6 D3 and D4 do not disclose a method to control the biological activity of a protein by using a fusion protein comprising a mutant ERalpha LBD as **defined in claim 1**. However, the method disclosed in D3, and D5, actually falls within the scope of claim **16,** since these documents disclose the double mutant Cre-ER(VR), comprising the substitutions G400V, G521R. Similar reasoning applies to the fusion protein of claim **20** and to dependent claims **17-18** and **21-26.**

4.7 Accordingly, the subject-matter of claims **16-18 and 20-26** lacks novelty over D3 and D5.

4.8 Thus, the present application does not satisfy the criterion set forth in Art.33(2) PCT because the subject-matter of claims **16-18 and 20-26** is not new in respect of prior art as defined in the regulations (Rule 64(1)-(3) PCT).

5 INVENTIVE STEP (Art. 33(3) PCT)

5.1 Mutants of the ligand binding domain (LBD) of the human estrogen receptor alpha (ERα), even some comprising at least 6 mutations including L346I, A350M, M388Q, G521S, Y526D and having reduced affinity for estrogen and increased affinity for DHB and being controlled by DHB were known in the prior art (D1, D2, mutant "5-E"). Fusion proteins with mutants of the LBD from hER with modified ligand-binding properties were known; even fusions with recombinases, including Cre (D3, D4).

5.2 For method **claim 1**, D3 can be regarded as the closest prior art. In view of said prior art, the present application tries to solve the **problem** of providing further fusion proteins comprising mutants of the LBD of ERα with selective binding to synthetic ligands devoid of oestrogenic and anti-oestrogenic

activities. Present claim 1 proposes several **solutions**, namely using fusion proteins comprising mutants of hER LBD, having, in addition to five mutations at positions: L346, A350, M388, G521, Y526, a mutation in at least one additional position selected from: T347, V376, G400, I424, G442, Y459, L466, H524, and M528.

5.3 The application only shows evidence that two of these mutants, namely **ERM-Db** (having the additional mutation G400V) and **ERM-Dc** (having the additional mutations: G442Y, Y459N and L466S) show the desired effect.

5.4 A skilled person starting from D3 would be aware of the need to produce variants of Cre-ER with improved selectivity for synthetic ligands and having a lower background due to endogenous oestrogens (D3, [0007]-[0012]). The skilled person would be aware of D1 and D2, being in the technical field of hER variants with altered ligand specificity. Thus, the skilled person would combine the teachings of D3 and D1 (or D2) and arrive to the solution of claim 1.

5.5 The skilled person would use the mutants of D1, including the mutants 4-S and 5-E from table 3 in order to construct further Cre-ER variants without the use of any inventive activity. In addition, since in D3, the mutations at G400 and G521 were already combined, the skilled person would have a clear incentive to combine the well known G400V mutation with the mutations in the 4-S and 5-E mutants of D1, especially in view of the fact that said G400V mutation was well known to reduce sensitivity to natural ligand estradiol (D3, [0038]). Furthermore, the skilled person would also be motivated to combine the mutations L346I, A350M, M388Q, G521S, Y526D (from 4-S or 5-E) with other interesting mutations described in D1 (and D2) which have similar interesting properties concerning their selectivity for synthetic ligands and/or reduced affinity for natural ligands (estrogens), including mutations: Gly442Tyr, Leu466Ser, Thr347Cys, Met528Asp, Ile424Val (D1, [0099], [0100]-[0104], examples 2-3, Table 7).

5.6 The same reasoning applies for independent claims 16, 19, 20, 22-26; the subject-matter of said claims and of the dependent claims thereof, does not seem to add any technical features which could render the subject-matter of any of the independent claims inventive.

5.7 Thus, at present the subject-matter of claims **1-26** cannot be regarded as involving an inventive step.

5.8 In summary, fusion proteins comprising Cre or other recombinases and variants of the hER LBD having, in addition to the mutations at positions: L346, A350, M388, G521, Y526, at least one additional mutation at a position selected from: T347, V376, G400, 1424, G442, Y459, L466, H524, and M528, even wherein the mutations are the specific substitutions: L3461, T347C, A350M, V376A, M388Q, M388F, 1424V, G442Y, Y459N, L466S, G521 S, G521 R, H524Y, Y526D, and M528E, and even the specific ERM-Db mutant (L346I, A350M, M388Q, G521S, Y526D + G400V) lack an inventive step in view of a combination of D3 with D1.

5.9 Other prior art documents (D5-D11) disclose several mutants of the LBD of the human ER-alpha having an impact in ligand binding, including several of the specific substitutions referred to in the claims and description, even combinations thereof. Said documents might become relevant later in the procedure.

5.10 Thus, the present application does therefore not satisfy the criterion set forth in Art. 33(3) PCT and the subject-matter of claims **1-26** does not involve an inventive step (Rule 65(1)(2) PCT).

5.11 However, it appears that the specific mutant ERM-Dc, would not appear to be obvious from a combination of prior art documents D3 (or D5) and D1 (or D2).

Re Item VIII

6 CLARITY, SUPPORT AND SUFFICIENCY OF DISCLOSURE

6.1 The application does not meet the requirements of Article 6 PCT because claims are not clear for the following reasons:

6.2 It appears from the description as a whole and in particular from the examples, that **all the mutations** in the ERM-Db and ERM-Dc mutants are essential technical features of the present invention. This essential technical feature is however not present in any of the independent or even the dependent claims. For these reasons the claim lack clarity according to Art. 6 PCT taken in combination with Rule 6.3 (b) PCT (see also PCT Preliminary Examination Guidelines III.4.3).

6.3 Several terms used in the claims, in particular independent claims **1, 16, 19 and 20** lack clarity in the sense of Art. 6 PCT since it is not known what are the technical features involved by said terms, especially since they are terms

open to interpretation. The terms and expressions concerned are: "selective binding", "a synthetic ligand devoid of oestrogenic and anti-oestrogenic activities", "at least six amino-acid substitutions".

6.4 Moreover, even if the definition of the "ERM polypeptide selective binding a synthetic ligand devoid of oestrogenic and anti-oestrogenic activities" could be rendered clear, this is regarded as a result to be achieved and thus, said expression cannot be used as a functional feature, but rather as an attempt to define the subject-matter in terms of the result to be achieved which merely amounts to a statement of the underlying problem (Art. 6 PCT).