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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 August 2008 (28.08.2008)

PCT

(10) International Publication Number
WO 2008/103060 A1

(51) International Patent Classification:
C12N 15/11 (2006.01) *A61K 31/713* (2006.01)

(21) International Application Number:
PCT/PL2008/000017

(22) International Filing Date:
15 February 2008 (15.02.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
P 381824 22 February 2007 (22.02.2007) PL

(71) Applicant *(for all designated States except US)*: **INSTYTUT CHEMII BIOORGANICZNEJ PAN** [PL/PL]; ul. Noskowskiego 12/14, PL-61-704 Poznan (PL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **KRZYZOSIAK, Wlodzimierz** [PL/PL]; os. B. Smialego 2/128, PL-60-682 Poznan (PL). **KROL, Jacek** [PL/PL]; Wierzchoslawice 17a/27, PL-81-141 Wierzchoslawice (PL). **FISZER, Agnieszka** [PL/PL]; ul. Iwaszkiewicza 2/49, PL-96-100 Skierniewice (PL).

(74) Agent: **TWARDOWSKA, Aleksandra**; Jan Wierzchon & Partners, ul. Zurawia 47/49, PL-00-680 Warszawa (PL).

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

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

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

WO 2008/103060 A1

(54) **Title:** A SIRNA SEQUENCE, VECTOR, A MOLECULAR TARGET FOR SIRNA REAGENTS AND VECTORS INTRODUCED INTO CELLS AND TISSUES, A METHOD OF EVALUATING THE SPECIFICITY OF THE SILENCING OF THE MUTATED TRANSCRIPT, A METHOD OF EXAMINING THE INTERACTIONS OF ENZYMES ON THE RNAI PATHWAY WITH TRANSCRIPTS AS WELL AS THE APPLICATION OF THE SIRNA SEQU

(57) **Abstract:** The subject of the present invention are a siRNA sequence, a vector, a molecular target for siRNA reagents and vectors introduced into cells and tissues, a method of evaluating the specificity of the silencing of the mutated transcript, a method of examining the interactions of the RNA interference pathway with transcripts containing repeating sequences as well as the application of a siRNA sequence and the vector in the therapy of trinucleotide repeat expansion diseases (TREDs). The solution relates to a new concept in the treatment of inherited neurological diseases caused by the expansion of tri-nucleotide elements using RNA interference and new tools for the embodiment of said concept.

A siRNA sequence, vector, a molecular target for siRNA reagents and vectors introduced into cells and tissues, a method of evaluating the specificity of the silencing of the mutated transcript, a method of examining the interactions of enzymes on the RNAi pathway with transcripts as well as the application of the siRNA sequence and vector

The subject of the present invention are a siRNA sequence, a vector, a molecular target for siRNA reagents and vectors introduced into cells and tissues, a method of evaluating the specificity of the silencing of the mutated transcript, a method of examining the interactions of the RNA interference pathway with transcripts containing repeating sequences as well as the application of a siRNA sequence and the vector in the therapy of trinucleotide repeat expansion diseases (TREDs). The solution relates to a new concept in the treatment of inherited neurological diseases caused by the expansion of tri-nucleotide elements using RNA interference and new tools for the embodiment of said concept.

The expansion of unstable regions repeats in individual genes is the cause of over 20 different inheritable diseases collectively dubbed *Triplet Repeat Expansion Diseases* (TREDs). These include fragile X-chromosome syndrome (FXS), myotonic dystrophy type 1 (DM1), Huntington's disease (HD) and a series of spinocerebellar ataxias (SCAs). Repeat sequences which undergo pathological expansion occur in all genetic regions, both translated and untranslated.

Most TRED-related genes contain CAG repeats in the coding region, and the number of repeats capable of causing the diseases is usually between 40 and 100. Repeat expansions in non-coding regions are larger and more diverse. It is commonly believed that repeats occurring in translated regions cause disease through altering the functioning of proteins, which in most cases contains expanded glutamine repeats. But,

their toxic activity may not be the only mechanism responsible for the disruption of neuronal function. Transcripts with extended repeats in non-coding regions do not cause any qualitative changes in the protein, but may be inhibited at the transcription level, or their translation may be repressed. They can also activate other proteogenic mechanisms at the RNA level including the sequestration of specific proteins by mutant transcripts. A wider role of pathogenic mechanisms at the RNA level in TREDs is indicated by the neurodegeneration occurring in a portion of carriers of the pre-mutation alleles of the *FMR1* gene (Fragile X associated Tremor Ataxia Syndrome, FXTAS). Research shows that the clinical phenotype of FXTAS is a result of not only an extended region of CGG repeats in the FMR1 transcript, but also an increased level of said transcript in the carriers.

The stem of the hairpin formed by CNG (N = U, A, C, G) repeats consists of periodically occurring C-G and G-C pairs, as well as unpaired N-N. If no interacting flanking sequences occur in the transcript, then the (CNG)n sequences exhibit a tendency to form several conformational variants. A characteristic of the hairpin formed by identical CNG repeats is an increase in rigidity directly proportional to its length. Structures formed by CUG and CAG repeats assume a geometry typical of A-RNA and may induce cellular responses typical of long, double-stranded RNA. One such response is RNAi, in which short RNA duplexes generated by the Dicer nuclease are used by the RISC complex in the specific silencing of genes containing complementary sequences.

Most diseases caused by the expansion of trinucleotide repeats are autosomal dominant, meaning that they are caused by a mutation in one of two gene variants. The product of a mutated gene, a transcript or protein or both, are cytotoxic. Thus, the removal of a mutated transcript should counteract the disease. TRED therapy is a sizeable challenge due to the sites of occurrence of the main symptoms of the disease meaning the CNS, as well as the need to eliminate the mutated gene products of a specific allele of a specific gene.

Patent descriptions CA2565098 (published 2005-12-08), US2004220132 (published 2004-11-04) and WO2004047872 (published 2004-06-10) describe a method of treating neurodegenerative diseases through the intra-cranial administration of siRNA. Those solutions present a method, short interfering RNAs as well as a procedure of treating neurodegenerative diseases which encompass the surgical implantation of a catheter, such that its reagent release site is adjacent to a specific

region of cerebral infusion, and releases, through the catheter egress, at least one substance capable of halting the biosynthesis of proteins causing neurodegeneration. That invention also describes vectors encoding short interfering RNAs as well as methods of treating neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's, type 1, 2 or 3 spinocerebellar ataxias and/or DRPLA.

Patent descriptions US2006270623 (published 2006-11-30), US2005277133 (published 2005-12-15), GB2415961 (published 2006-01-11) and WO2005105995 (published 2005-11-10) describe a RNAi-based method of treating diseases caused by polyQ (polyglutamine) repeats using a short interfering nucleic acid (siNA). That solution relates to compounds, their composition, research and diagnostic methods and treatment methods of diseases, as well as conditions related to allelic variants of polyglutamine repeats responsible for modulating gene expression and/or activity. Said solution also relates to compounds, their composition, and methods relating to diseases and conditions connected with allelic variants of polyglutamine repeats responsible for the modulation of the expression and/or activity of genes involved in pathways related to genes containing polyQ or with other cellular processes which may influence the course and/or development of diseases related to poly-Q and their symptoms, such as Huntington's disease and its symptoms, such as advancing chorea, stiffness, dementia and seizures, spinocerebellar ataxia, spinal-bulbular muscular atrophy (SBMA), Dentatorubral and Pallidoluysian Atrophy (DRPLA) as well as any other disease or symptom related to the level of proteins containing repeat expansions, in a cell or tissue, individually or in connection with other therapies.

Patent description CA2526893 (published 2004-11-25) describes a method of inhibiting the expression of a gene related to Huntington's disease through the application of double-stranded RNA (dsRNA). The authors described the application of an inhibitor of the expression of a Huntington's disease gene as well as a drug counteracting and/or curing Huntington's disease. The specific target mRNA sequence is located upstream of the CAG repeats in the Huntington's gene. The expression of this gene is inhibited through the application of dsRNA, of a sequence homologous with the mRNA sequence. Using this method, one may effectively obtain siRNAs containing from 21 to 23 bp. of dsRNA homologous with the mRNA sequence. Huntington's disease can be counteracted and/or cure through the administration or transfer of the above dsRNA as an inhibitor of the expression of the Huntington's gene, or prophylactically and/or therapeutically into a live mammal or live cells.

Patent description US2005255086 (published 2005-11-17) describes an application of nucleic acid molecules for the silencing of the Huntington's disease gene. Said solution relates to siRNAs directed against the Huntington's disease gene, methods of their application as well as vector system for said siRNA molecules. This solution described allele-specific siRNA reagents which effectively silence the mutated transcript, both in experiments on cultured cells as well as studies using the mouse model.

Despite the many extant solutions using RNAi regents, applicable in treatment of diseases caused by trinucleotide repeat expansions, which are related to the expansion of unstable repeat regions in individual genes, there still exists a real need to obtain an effective solution facilitating the allele-specific elimination of mutated gene products, taking into account the specific site where the characteristic symptoms of the disease develop, meaning the central nervous system.

The goal of the present invention is to produce effective, allele-specific RNAi reagents (i.e. siRNA), which may be applicable in the therapy of TREDs.

The embodiment of such a stated goal and the solution of problems related to the fact that RNAi reagents RNAi (i.e. siRNA) should be designed in a way that their sequence and length corresponds to or are not shorter than the products generated by Dicer ribonuclease from transcripts of the mutated genes containing trinucleotide repeats; have been achieved in the present invention.

The subject of the present invention is a siRNA sequence, characterised in that it is composed of the repeats of one type of trinucleotide motif, either CUG, CCG, CGG or CAG, occurring in the form of a single strand, a hairpin structure or an unstable duplex with periodically occurring unpaired bases, wherein the siRNA is directed against a complementary sequence occurring in the transcripts of mutated genes which cause inheritable neurological diseases of the TRED group.

Preferentially, its length is equal to or longer than the length of repeated sequence fragments generated by the Dicer ribonuclease from mutated transcripts.

Preferentially, it contains chemical modifications in the form of: 2'-O-methyl, 2'-deoxy-2'-fluoro, oxyethane or LNA.

The next subject of invention is a vector, characterised in that it releases a siRNA sequence into a cell which is composed of the repeats of one type of trinucleotide motif, either CUG, CCG, CGG or CAG, occurring in the form of a single strand, a hairpin structure or an unstable duplex with periodically occurring unpaired bases, wherein the

siRNA is directed against a complementary repeat sequence occurring in the transcripts of mutated genes which cause inheritable neurological diseases of the TRED group.

Preferentially, the length of the siRNA fragments it releases into a cell is equal to or longer than the length of repeated sequence fragments generated by the Dicer ribonuclease from mutated transcripts.

Preferentially, it is a viral or plasmid vector.

The next subject of invention is a molecular target for siRNA reagents and vectors introduced into cells and tissues, characterised in that it is a characteristic hairpin structure formed by a region of trinucleotide repeats, one of CUG, CCG, CGG or CAG, in the transcripts of genes undergoing mutations leading to inheritable neurological diseases of the TRED group.

The next subject of invention is a method of evaluating the specificity of the silencing of a mutated transcript, characterised in that in addition to the level of the silenced gene the analysis encompasses the level of the healthy allele as well as the levels of a series of other transcripts containing regions of identical trinucleotide motif repeats, either CUG, CCG, CGG or CAG, by amplifying repeat sequence regions from cDNA using PCR and allelospecific PCR in real time.

The next subject of invention is a method of evaluating the interactions of enzymes of the RNA interference pathway, characterised in that the interactions of Dicer and Ago2, enzymes of the RNA interference pathway, with transcripts containing repeat sequences composed of the repeats of one type of trinucleotide motif, either CUG, CCG, CGG or CAG, using trancripts generated *in vitro* as well as recombinant nucleases, in cell extracts and in the cell, are examined, as well as methods of identifying and characterising the products of said interactions.

The next subject of invention is use of a siRNA, composed of the repeats of one type of trinucleotide motif, either CUG, CCG, CGG or CAG, occurring in the form of a single strand, a hairpin structure or an unstable duplex with periodically occurring unpaired bases, wherein the siRNA is directed against a complementary repeat sequence occurring in the transcripts of mutated genes which cause inheritable neurological diseases of the TRED group, in the treatment of diseases caused by trinucleotide repeat expansion (TREDs).

Preferentially, the length of the sequence used is equal to or longer than the length of repeated sequence repeat fragments generated by the Dicer ribonuclease from mutated transcripts associated with TREDs.

Preferentially, the sequence used contains chemical modifications in the form of: 2'-O-methyl, 2'-deoxy-2'-fluoro, oxyethane or LNA.

The next subject of invention is use of a vector, which releases siRNA into cells which is composed of the repeats of one type of trinucleotide motif, either CUG, CCG, CGG or CAG, occurring in the form of a single strand, a hairpin structure or an unstable duplex with periodically occurring unpaired bases, wherein the siRNA is directed against a complementary duplicated sequence occurring in the transcripts of mutated genes which cause inheritable neurological diseases of the TRED group, in the treatment of diseases caused by trinucleotide repeat expansion (TREDs).

Preferentially, the length of the sequence released is equal to or longer than the length of repeated sequence fragments generated by the Dicer ribonuclease from mutated transcripts associated with TREDs.

Preferentially, the vector is a viral or plasmid vector.

The attached Figures facilitate a better understanding and present the nature of the present invention.

Figure 1 represents transcripts containing long CNG repeat sequences as cleaved by the Dicer ribonuclease.

Reactions of the 5' –end-labeled pre-miR-24 (A), CUG17_cl (B), and CUG35_cl (C) transcripts with Dicer. Lanes represent: Ci – incubation control without Dicer; F – formamide generated marker; T – guanine-specific ladder. The positions of selected G nucleotides are indicated. It should be underlined that Dicer cleavage products containing a 3'OH group migrate slower in the gel than T1 and formamide products of the same length, which possess a 2'3'-cyclic phosphate, and that this difference is about 1 nucleotide in the range of 20-40 nt under the conditions used. Primary cleavage sites (black rectangles and arrows) as well as secondary cleavage sites (white arrows) are shown on autoradiograms as well as on models of secondary structure. (D) The half-life (in minutes) of substrates: ^{32}P-labeled transcripts CNG25_cl (white bars) as well as CNG35_cl (grey bars). The presented results are an average of two independent experiments. Dicer cleavage patterns obtained for 5' end-labeled transcripts of

CUG_25_cl (E), CUG25 (F), as well as CAG25_cl, CCG25_cl, and CGG25_cl (G). Cleavage products were analyzed as described for (A)-(C). X3 and X8 mark three and eight repeats of the CNG triplet in the central region of the structure of CNG25 and CNG35, respectively.

Figure 2 represents changes in the levels of mutated cellular transcripts containing CAG and CUG repeats after Dicer knockdown.

(A) The cellular level of Dicer mRNA was determined using RT-PCR in real time using a cDNA template obtained from the cells treated with control siRNA (black bars) and Dicer-specific siRNA (grey bars). The results presented in the Figure are an average of three independent experiments. The right panel shows an analysis of Dicer and GAPDH protein levels using Western blot, following the silencing of Dicer.

(B) A Northern blot of a mutated (1000 CUG) and normal (15 CUG) transcripts of DMPK following the treatment of fibroblasts with siRNAs specific for Dicer (lane -Dicer) or control siRNA (lane +Dicer). The signal intensity representing levels of normal (black bars) and mutated (grey bars) transcripts have been measured densitometrically (ImageQuant 5.1, Molecular Dynamics) and is proportional to the height of the bars in the graph. The signal intensity has been normalized to GAPDH mRNA and compared using a t-test for statistical significance (Prism v 4, GraphPad Software, San Diego, CA).

(C) The cellular level of DMPK mRNA was analyzed using RT-PCR in real time using a cDNA template obtained from the cells treated with control siRNA (DM1) or Dicer-specific siRNA (DM1-Dicer), and TaqMan probes for the DMPK and GAPDH cDNA sequences. The DMPK transcript levels have been normalized to GAPDH mRNA levels and compared using a t-test for statistical significance. The results shown represent averages of four independent experiments.

(D) The left panel shows an STR region analysis performed in order to determine transcript levels using a semiquantitative RT-PCR protocol, following the treatment of HD and SCA1 fibroblasts with Dicer-specific siRNA (lane cDNA-Dicer). PCR products from DNA and cDNA templates obtained from HD and SCA1 cells following treatment with control siRNA were used as controls. The lengths of the STR regions for HD and SCA1 cells are indicated. The right panel shows an analysis of STR regions to determine transcript levels of HD and SCA1 after the silencing of Dicer (lane cDNA-Dicer). The signal intensity is an average of five independent experiments and is

proportional to the height of the control (black bars) and mutated (grey bars) of the transcript measured densitometrically.

(E) A schematic representation of STR-SNP haplotypes in transcripts of mutated and normal HD and SCA1 alleles.

(F) Analysis of normal and mutated HD and SCA1 transcripts using allele-specific RT-PCR in real time. The experiments were performed at least three times. The p value was calculated using a t-test for significance.

Figure 3 represents analysis of short products consisting of CNG repeats, produced by Dicer in cells derived from TREDs patients.

Northern hybridization of total RNA from control, HD, SCA1 and DM1 fibroblastswith normal and decreased levels of Dicer (lanes marked –Dicer), using complementary oligonucleotide probes against (CAG)7 or (CUG)7. The levels of expression of let-7, miR-21 and miR-16 were also determined, wherein the hybridization of the U6 probe served as a control for total RNA amounts.

Figure 4 represents the silencing of Dicer which demonstrates the downstream effects caused by the siCUGs generated in cells.

(A) SCA1 and HD transcript analysis using semi-quantitative RT-PCR following the treatment of DM1 cells with control siRNA (lane DM1) as well as Dicer-specific siRNAs (lane DM1-Dicer). Another control used were cells from a healthy individual (WT). The genotypes of STR regions for SCA1 and HD in the cells used are shown.

(B) Bar graphs represent the cellular levels of the analyzed transcripts, determined using RT-PCR of STRs or Northern blot (for DMPK), measured densitometrically. Signal intensity has been normalized to the GAPDH mRNA level and compared using a t-test.

(C) The left panel represents the levels of Ago2 and GAPDH proteins using a Western blot, following the treatment of healthy and DM1 cells with control or Ago2 specific siRNA. The DMPK transcript level was determined using RT-PCR in real time, whereas the analysis of HD and SCA1 transcripts was performed using semiquantitative RT-PCR. The graphs represent average results from three independent experiments. The p value was calculated using a t-test for statistical significance.

Figure 5 represents the cellular effects elicited by exogenous siRNA composed of repeats.

(A) A 21 nt siRNA used in the experiment. The structure of these reagents in the cell is difficult to determine, and as an alternative to the presented unstable duplex may be single strands or unstable hairpin structures.

(B) An analysis of the transcript levels containing trinucleotide repeats using RT-PCR and STR amplification, or Northern blotting (for DMPK) after the treatment of DM1, HD, SCA1 cells with (CAG)7 or (CUG)7 siRNAs.

(C) Bar graphs show the cellular level of the analyzed transcripts determined using RT-PCR or Northern blot protocols, measured densitometrically. The experiments were performed at least thrice, and the signal intensity was compared to GAPDH mRNA levels using a t-test for statistical significance.

(D) A Northern blot of the level of normal and mutated DMPK transcripts following treatment of cells with (CAG)7 or (CUG)7 siRNAs.

Figure 6 represents an analysis of the effectiveness and specificity of siRNA activity of various sequence lengths: the effectiveness of silencing of complementary transcripts increases with the increase of siCUG length.

(A), (B) Analysis of HD transcript levels in cell lines containing mutations in this gene, containing various (indicated) length variants, following the application of siCUGs of varying lengths, from 6 to 9 CUG repeats. O, L and C represent respective controls: untreated cells, cells treated with Lipofectamine alone and cells transfected with control siRNA.

(C) HD transcript levels which show the dose-dependence of the effectiveness and specificity of siRNA activity composed of 9 repeats of CUG.

Figure 7 represents a schematic showing the Dicer-dependent decrease of mutated transcripts and their silencing by endogenous and exogenous siRNA composed of repeats, along the RNAi pathway. Transcripts containing long CUG and CAG repeat regions which form hairpins are both substrates for Dicer and comprise a target sequence for the RNAi mechanisms, induced by short sequences consisting of complementary repeats.

Below are example embodiments of the present invention described above.

Example 1

All hairpin structures formed by CNG repeats of sufficient length are Dicer substrates.

The relationship between the type and length of trinucleotide repeats and the effectiveness of their binding and cleavage by Dicer were characterized. Fig.1A represents analysis of the Dicer cleavage of its natural substrate, the miRNA precursor pre-miR-24. Two cuts are made in the stem of the precursor, a 23 nucleotide product is generated from the 5'-side of the transcript, which corresponds to the mature miRNA. Transcripts composed of 17 CUG trinucleotide repeats form a structure with an unpaired U-U, which results in lower stem stability and resistance to Dicer cleavage. Transcripts composed of 17 repeats of CAG, CCG or CGG, which form similar structures were also not cleaved by Dicer (Fig.1B for CUG17_cl). In addition to the repeat sequence, the transcripts contain 5-nucleotide, pairing sequences at either ends, mimicing the base-paired sequences which commonly occur in many natural transcripts. Hairpin structures composed of long sequences of CUG repeats are more stable and susceptible to cleavage by Dicer, as shown for CUG35_cl (Fig.1C) and others (Fig. 1D). Transcripts CNG25_cl and CUG25 are also good substrates for Dicer (Fig.1E-G), wherein those composed of CUG and CAG repeats are more susceptible than those composed of CCG and CGG. The exact sites of cleavage by Dicer were then analyzed. For the CUG25_cl transcript, the primary cut sites generate fragments of 21, 22 and 24 nucleotides long from the 5' end as well as 20 and 22 nt. from the 3' end, and often occur at unpaired nucleotides (Fig. 1E). Secondary cut sites shorten the primary cut products to 21 nt fragments. The CUG25 transcript resulted in fragments of 21, 22 and 24 nucleotides from the 5'-end as well as 20 and 22 nucleotides from the 3'-end (Fig. 1F).

Example 2

The level of mutated transcripts related to TREDs is lowered in cells by the Dicer ribonuclease.

It was shown in vitro that Dicer is engaged in the regulation of transcripts containing elongated hairpin structures. The levels of control transcripts and ones containing trinucleotide repeats were analyzed in cells from both healthy individuals and those suffering from disorders of HD, SCA1 and DM1, both prior to and following the silencing of Dicer. The effectiveness of Dicer silencing at the mRNA and protein level is shown in Fig. 2A. In DM1 cells using Northern blotting, it was observed that the level of the mutated DMPK allele which was around 40 % higher in cells with silenced Dicer than that in control cells (Fig. 2B), confirmed using RT-PCR analysis in real time (Fig.

2C). For HD and SCA1 cells, transcript levels were analyzed using RT-PCR and it was determined that the mutated allele level was about 50 % higher in cells with silenced Dicer in relation to control cells (Fig. 2D). The levels of normal allele transcripts remained unchanged or was only slightly higher.

Example 3

Short fragments composed of CAG and CUG repeats are detectable in cells.

Northern analysis showed that in HD, SCA1 and DM1 cells, there occur short, ca. 21 nucleotide RNAs composed of CAG (in HD and SCA1) as well as CUG in DM1 (Fig. 3). Following the treatment of cells with Dicer-specific siRNA a drop in the level of analyzed miRNAs is observed.

Example 4

Short fragments composed of CUG repeats induce secondary effects in cells.

It was shown that short siCUG fragments generated in DM1 cells enter the RNAi pathway and act as siRNA, silencing transcripts containing long complementary repeats. The levels of transcripts containing CAG or CUG repeats, which were potential siRNA targets, were analyzed. In DM1 cells (15/1000 CUG repeats), it was shown that along with a decreased level of Dicer, the level of SCA1 increases (about 30%) as does HD (about 35%) in comparison to control cells (Fig. 4A, B). Changes in the expression of Dicer did not influence the levels of the other transcripts examined. It was shown that the siCUG generated in DM1 cells are active on the RNAi pathway through analogous experiments performed using Ago2 silencing. The silencing of the Ago2 transcript in DM1 cells resulted in the increase of HD and SCA1 mRNA by about 45% and 40% respectively, whereas the DMPK level remained unchanged (Fig. 4C).

Example 5

Mutated transcripts are silenced by exogenous siRNAs complementary to the repeats contained therein.

We achieved effective and specific silencing of mutated transcripts of HD, SCA1 and DMPK following the treatment of cells with 5 nM siRNA composed of complementary trinucleotide repeats. (CUG)7 and (CAG)7 RNA reagents transfected using Lipofectamine 2000 were designed based on the analysis of CUG35_cl transcript cleavage products generated by Dicer. The level of the selected transcripts following siRNA treatment were analyzed using RT-PCR and Northern blot. DM1 cells exhibited considerable silencing of the mutated DMPK allele using (CAG)7 as well as a slight

decrease of the levels of normal long alleles of HD and SCA1. Using (CUG)7, the earlier described endogenous effect was slightly enhanced (Fig. 5). Likewise for the HD and SCA1 cells, we showed the specific silencing of the mRNA of HD and SCA1 mutated alleles respectively, following transfection with (CUG)7.

Example 6

Longer siCUGs are capable of more effective silencing of mutated alleles.

It was shown that in comparison to siCUGs composed of 7 repeats described earlier, RNA consisting of 8 and 9 repeated motifs are capable of greatly enhanced silencing of mutated alleles, which was shown using the HD cell line (Fig. 6A, B). At the same time it was shown that the most effective RNA, (CUG)9, can specifically silence mutated alleles at a concentration as low as 2 nM (Fig. 6C).

Methods

Preparation of RNA substrates

DNA templates for transcripts of CNG17_cl, 25_cl, 35_cl; CUG25; as well as pre-miR-24 were obtained using chemical synthesis and purification in a polyacrylamide gel. Each oligomer contained a promoter sequence for T7 RNA polymerase (or SP6 for pre-miR-24) on the 3' end. DNA templates for *in vitro* transcription were obtained through the amplification of gel-purified fragments of SCA1, HD and DMPK, containing different numbers of CNG repeats. The following primer sequences were used: for SCA1 (long flanking sequence) (R 5'CCGAGGGACAAAGTGGCTGC; F 5'TAATACGACTCACTATAGGAACCTATGCCA GCTTCATCC); HD (R 5'TGCAGCGGCTCCTCAGCC; F 5'TAATACGACTCACTATAG GACGGCCGCTCAGGTTC); DMPK (R 5'TGGGGCTCCGAGAGCAGCGCAA; F 5'TAATACGACTCACTATAGGCACAGAAGCCGCGCCCAC); (short flanking sequence) SCA1 (R 5'GAACTGGAAATGTGGACGTA; F 5'TAATACGACTCACTATACAACATG GGCAGTCTGAGC). The transcripts were 5' end labeled using 10U of T4 polynucleotide kinase (Epicentre) and 50 μCi [γ^{32}P]ATP (4500 Ci/mmol; ICN) at 37°C for 10 min. Internally labeled ^{32}P RNA were obtained during the transcription reaction *in vitro* using [α-^{32}P]CTP. The labeled RNA were purified in a gel and stored at -80°C prior to use.

RNA cleavage assay using recombinant Dicer

To cleave RNA, we used commercially available Dicer from Stratagene as well as from Ambion, respectively containing ~20% and ~60% of full length protein (which was evaluated using SDS-PAGE) as well as a highly purified enzyme, for which no degradation products were observed. No differences were observed in the cleavage pattern for the enzymes used. Prior to the reaction with Dicer, the RNA was subjected to denaturation and renaturation by heating to 80°C for 1 min and the slowly cooling to 37°C for an appropriate time, as shown in the figures. The reactions were stopped through the addition of an equal volume of loading buffer (8 M urea with 20 mM EDTA and dyes). The samples were analysed in a 15% polyacrylamide gel with 7.5 M urea in 1X TBE with products of alkaline hydrolysis as well as limited cleavage using T1 ribonuclease of the same RNA molecule. The alkaline lysis marker was obtained by the incubation of the labeled RNA in a formamide buffer containing 0.5 mM $MgCl_2$ at 100°C for 10 min. Partial T1 cleavage of the RNA was performed in semi-denaturing conditions (10 mM sodium citrate pH 4.5, 0.5 mM EDTA, as well as 3.5 M urea) for 10 min at 55°C with 0.2 U/µl of enzyme. The products were detected using autoradiography. Quantitative analysis of cleavages was performed using a Typhoon PhosphorImager (Molecular Dynamics). The primary cuts were distinguished from secondary ones through a comparison of hydrolysis patterns of transcripts labelled at either 5' or 3' ends as well as through an analysis of the intensity of signals originating from the cleavage of 5' end-labelled transcripts at different time points of the reactions.

RNA isolation, RT-PCR and real-time PCR

Total RNA was isolated from human fibroblasts (from Coriell Cell Repositories; GM00321 – healthy, GM04033 – *DMPK* mutant (1000 CUG), GM03991 – *DMPK* mutant (80 CUG), GM04208 – *HD* mutant, GM06927 – *SCA1* mutant) using TRIzol (Invitrogen) according to the manufacturer's instructions. In order to determine the effectiveness of silencing using siRNA, RNA (1 µg) was used as a template for cDNA synthesis with random hexamer or oligo-dT primers (Promega) using the SuperScript II kit (Invitrogen). To amplify the GAPDH and β-actin fragments, we used the following primers and RT-PCR conditions: GAPDH (F 5'GAAGGTGAAGGTCGGAGTC; R 5'GAAGATGGTGATGGGATTTC; 30 cycles of 94°C for 15s, 60°C for 15s and 72°C for 20s), β-actin (F 5'TTCGCGGGCGACGATGC; R 5'CGTACATGGCTGGGG TGTTG; 30 cycles of 95°C for 30 s, 60°C for 30s and 72°C for 45 s). To amplify the

STR region we used the following primers and RT-PCR conditions: HD (F 5'ATGGCGACCCTGGAAAAGCTGAT; R 5'TGAGGCAGCAGCGGCTG T; 25 cycles of 95°C for 30 s, 60°C for 20s and 72°C for 45 s), SCA1 (F 5'CAACATGGGCAGTCTGAGC; R 5'GAACTGGAAATGTGGACGTA; 25 cycles of 95°C for 20 s, 60°C for 20s and 72°C for 45 s), SCA3 (F 5'GGAAGAGACGAGAAGCCTAC; R 5'TCACCTAGATCACTCCCAAGT; 25 cycles of 95°C for 20 s, 55°C for 20s and 72°C for 45 s), AR (F 5'TGCGCGAAGTGATCCAGAAC; R 5'CTTGGGGAGAACCATCCTCA; 30 cycles of 95°C for 15 s, 57°C for 15s and 72°C for 20 s), MAB21L1 (F 5'GGCTGG GCTCAAAGATAAAA; R 5'TAGAGCTGGAGCCAACTTCG; 25 cycles of 95°C for 20 s, 55°C for 20s and 72°C for 45 s), DMPK (F 5'AGGCTTAAGGAGGTCCGACTG; R 5'GCGAAGTGCAGCTGCGTGATC; 25 cycles of 95°C for 20 s, 60°C for 20s and 72°C for 45 s), SCA8 (F 5'TTTGAGAAAGGCTTGTGAGGACTGAGAATG; R 5'GGTCCTTCATGTTAGAAAACCTGGCT; 25 cycles of 95°C for 20 s, 53°C for 20s and 72°C for 45 s).

Real-time RT-PCR was performed using the SYBR Green PCR Master Mix kit (Applied Biosystems), with 2 µl cDNA, which corresponds to 20 ng of total RNA in a total volume of 20 µl and 0.2 µM of each primer (for Dicer F 5'TTAACCAGCTGTGGGGAGAGGGCTG; R 5'AGCCAGCGATGCAAAGATGGTGTTG). Quantitative RT-PCR was performed in a Rotor-Gene 3000 (Corbett Research) at 95°C for 30 s, 61°C for 30s and 72°C for 45 s Allele-specific real-time RT-PCR was performed using 0.2 µM of each allele-specific primer (HD 5'GAGCTTCTGCAAACCCTGATC, 5'GAGCTTCTGCAAACCCTGATT; SCA1 5'GAGGCCACCAAGAAAGCTT, 5'GAGGCCACCAAGAAAGCCT), and the SYBR Green PCR Master Mix kit (Applied Biosystems) with 2 µl cDNA, which corresponds to 20 ng of total RNA.

siRNA and transfection conditions

Human fibroblasts were transfected with siRNA (specific for Dicer: sense strand 5'GGCACCCAUCUCUAAUUAUUt or for Ago2: sense strand 5'GCACGGAAGUCCAUCUGAAUt) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were cultured in 24-well plates to 80% confluence. Each well was supplemented with 30 nM Dicer- or Ago2-specific siRNA as well as control siRNA (random 21 nucleotide oligomers; Invitrogen) mixed

with 1 µl of Lipofectamine 2000 in 100 µl Opti-MEM. The mixture was added to the cells and incubated for 4 hours. After 24 hours a second transfection was performed in the same way. Total RNA was isolated after 48 hours from the second transfection and used in RT-PCR and real-time RT-PCRas well as Northern blotting. The RNA used for transfection, containing a 5'monophosphate group, with a sequence of: (CAG)7 5'AGCAGCAGCAGCAGCAGCAGC, and (CUG)7: 5'UGCUGCUGCUGCUGCUGCUGC or 5'CUGCUGCUGCUGCUGCUGCUG as well as (CUG)6, (CUG)8 and (CUG)9 with appropriate numbers of CUG repeats were synthesized by CureVac (Germany) .

Western blotting

48 hours following the second transfection, the cells were suspended in 1X PBS with protease inhibitors (Protease Inhibitor Cocktail; Roche) and the protein was isolated using cycles of freezing/thawing. 20 µg of lysate proteins were separated using SDS-PAGE, and then electrotransferred onto a nitrocellulose membrane (Sigma). After blocking with 5% milk, the membrane was incubated for 1 hour with one of the primary antibodies: polyclonal antibody against human Dicer (Imgenex; diluted 1:500), monoclonal antibody against Ago2 (Abnova; diluted1:500), a monoclonal antibody against GAPDH (Chemicon; diluted 1:500). Following the reaction the membranes were incubated in a secondary anti-rabbit or anti-mouse antibody conjugated with biotin. The chromogenic reaction for the detection of specific was performed using the Fast BCIP/NBT kit (Sigma).

Northern hybridization

To analyze DMPK mRNA, 10 µg of total RNA was separated on a 1.2 % agarose gel with formaldehyde. The RNA was electrotransferred onto a Hybond-N^+ membrane (Amersham Pharmacia Biotech). The membranes were then hybridized using a 5' end labeled probe, specific for DMPK or GAPDH transcripts in a buffer containing 200 mM Na_2PO_4 pH 7.0, 5% SDS and 50 µg/ml denatured herring sperm DNA at 40°C overnight. To amplify the DMPK and GAPDH fragments to be used as probes, using the following primers and PCR conditions: DMPK (F 5'AGGCTTAAGGAGGTCCGACTG; R 5'GCGAAGTGCAGCT GCGTGATC; 35 cycles of 95°C for 5 s, 60°C for 5s and 72°C for 30 s), GAPDH (F 5'GAAGGTGAAGGTCGGAGT; R 5'GAAGATGGTGATGGGATTTC; 30 cycles of 95°C for 15 s, 60°C for 15s and 72°C for 20 s). The membranes were washed with 2x

SSC and 0.5% SDS and exposed to X-ray film overnight. The detection of short RNA was performed using 30 μg total RNA isolated from healthy, HD, SCA1 and DM1 fibroblasts. Electrophoresis was performed in 10% polyacrylamide gels with 7 M urea in 1X TBE. The RNA was then electrotransferred onto a Hybond-N^+ membrane and hybridized with a specific probe in a buffer containing 5X SSPE, 5X Denhardt's solution, 0,2% SDS, 25% formamide and 50 μg/ml denatured herring sperm DNA at 37°C overnight. 5' end-labeled RNA and DNA probes were used to detect siCNG and miRNA respectively.

Patent Claims

1. A siRNA sequence, characterised in that it is composed of the repeats of one type of trinucleotide motif, either CUG, CCG, CGG or CAG, occurring in the form of a single strand, a hairpin structure or an unstable duplex with periodically occurring unpaired bases, wherein the siRNA is directed against a complementary sequence occurring in the transcripts of mutated genes which cause inheritable neurological diseases of the TRED group.
2. A sequence according to Claim 1, characterised in that its length is equal to or longer than the length of repeated sequence fragments generated by the Dicer ribonuclease from mutated transcripts.
3. A sequence according to Claim 1 or 2, characterised in that it contains chemical modifications in the form of: 2'-O-methyl, 2'-deoxy-2'-fluoro, oxyethane or LNA.
4. A vector, characterised in that it releases a siRNA sequence into a cell which is composed of the repeats of one type of trinucleotide motif, either CUG, CCG, CGG or CAG, occurring in the form of a single strand, a hairpin structure or an unstable duplex with periodically occurring unpaired bases, wherein the siRNA is directed against a complementary repeat sequence occurring in the transcripts of mutated genes which cause inheritable neurological diseases of the TRED group.
5. A vector according to Claim 4, characterised in that the length of the siRNA fragments it releases into a cell is equal to or longer than the length of repeated sequence fragments generated by the Dicer ribonuclease from mutated transcripts.
6. A vector according to Claim 4 or 5, characterised in that it is a viral or plasmid vector.

7. A molecular target for siRNA reagents and vectors introduced into cells and tissues, characterised in that it is a characteristic hairpin structure formed by a region of trinucleotide repeats, one of CUG, CCG, CGG or CAG, in the transcripts of genes undergoing mutations leading to inheritable neurological diseases of the TRED group.

8. A method of evaluating the specificity of the silencing of a mutated transcript, characterised in that in addition to the level of the silenced gene the analysis encompasses the level of the healthy allele as well as the levels of a series of other transcripts containing regions of identical trinucleotide motif repeats, either CUG, CCG, CGG or CAG, by amplifying repeat sequence regions from cDNA using PCR and allelospecific PCR in real time.

9. A method of evaluating the interactions of enzymes of the RNA interference pathway, characterised in that the interactions of Dicer and Ago2, enzymes of the RNA interference pathway, with transcripts containing repeat sequences composed of the repeats of one type of trinucleotide motif, either CUG, CCG, CGG or CAG, using trancripts generated *in vitro* as well as recombinant nucleases, in cell extracts and in the cell, are examined, as well as methods of identifying and characterising the products of said interactions.

10. Use of a siRNA, composed of the repeats of one type of trinucleotide motif, either CUG, CCG, CGG or CAG, occurring in the form of a single strand, a hairpin structure or an unstable duplex with periodically occurring unpaired bases, wherein the siRNA is directed against a complementary repeat sequence occurring in the transcripts of mutated genes which cause inheritable neurological diseases of the TRED group, in the treatment of diseases caused by trinucleotide repeat expansion (TREDs).

11. Use according to Claim 10, characterised in that the length of the sequence used is equal to or longer than the length of repeated sequence repeat fragments generated by the Dicer ribonuclease from mutated transcripts associated with TREDs.

12. Use according to Claim 10 or 11, characterised in that the sequence used contains chemical modifications in the form of: 2'-O-methyl, 2'-deoxy-2'-fluoro, oxyethane or LNA.

13. Use of a vector, which releases siRNA into cells which is composed of the repeats of one type of trinucleotide motif, either CUG, CCG, CGG or CAG, occurring in the form of a single strand, a hairpin structure or an unstable duplex with

periodically occurring unpaired bases, wherein the siRNA is directed against a complementary duplicated sequence occurring in the transcripts of mutated genes which cause inheritable neurological diseases of the TRED group, in the treatment of diseases caused by trinucleotide repeat expansion (TREDs).

14. Use according to Claim 13, characterised in that in that the length of the sequence released is equal to or longer than the length of repeated sequence fragments generated by the Dicer ribonuclease from mutated transcripts associated with TREDs.

15. Use according to Claim 13 or 14, characterised in that the vector is a viral or plasmid vector.

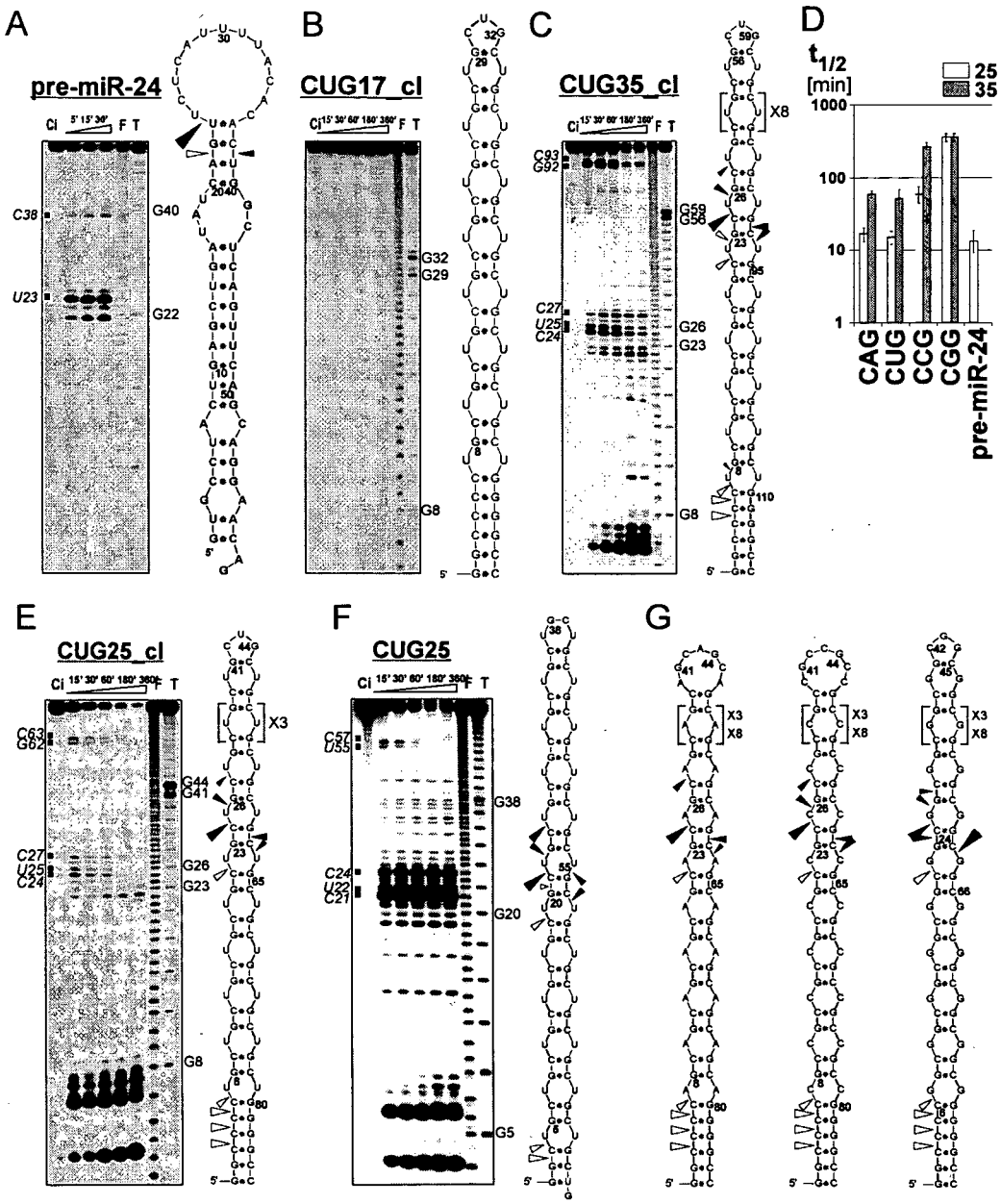

A
pre-miR-24
B
CUG17_cl
C
CUG35_cl
D
t1/2 [min]
25
35
CAG
CUG
CCG
CGG
pre-miR-24
E
CUG25_cl
F
CUG25
G

Fig. 1

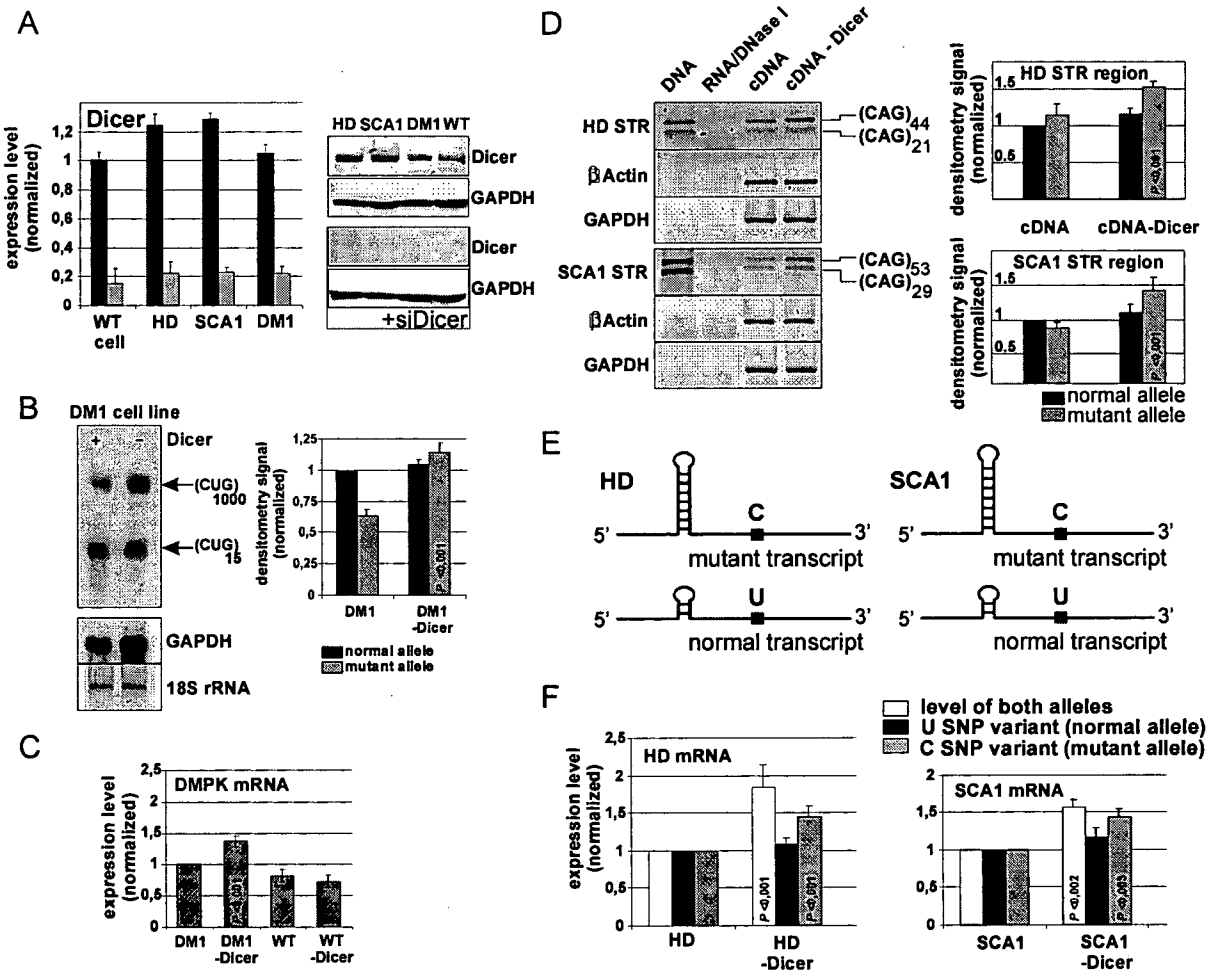

A
Dicer
expression level (normalized)
WT cell
HD
SCA1
DM1
HD SCA1 DM1 WT
Dicer
GAPDH
+siDicer
B
DM1 cell line
(CUG) 1000
(CUG) 15
GAPDH
18S rRNA
densitometry signal (normalized)
DM1
DM1 -Dicer
normal allele
mutant allele
C
DMPK mRNA
DM1
DM1 -Dicer
WT
WT -Dicer
D
DNA
RNA/DNase I
cDNA
cDNA - Dicer
HD STR
βActin
GAPDH
SCA1 STR
(CAG)44
(CAG)21
(CAG)53
(CAG)29
HD STR region
SCA1 STR region
cDNA
cDNA-Dicer
E
HD
SCA1
5'
3'
C
U
mutant transcript
normal transcript
F
level of both alleles
U SNP variant (normal allele)
C SNP variant (mutant allele)
HD mRNA
SCA1 mRNA
HD
HD -Dicer
SCA1
SCA1 -Dicer

Fig. 2

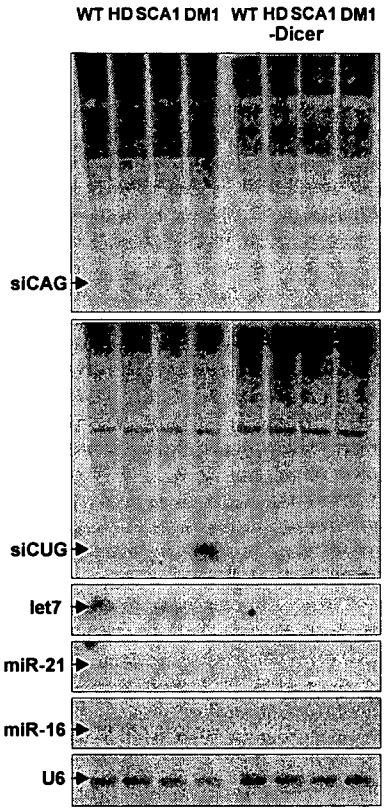

WT HD SCA1 DM1 WT HD SCA1 DM1
-Dicer
siCAG
siCUG
let7
miR-21
miR-16
U6

Fig. 3

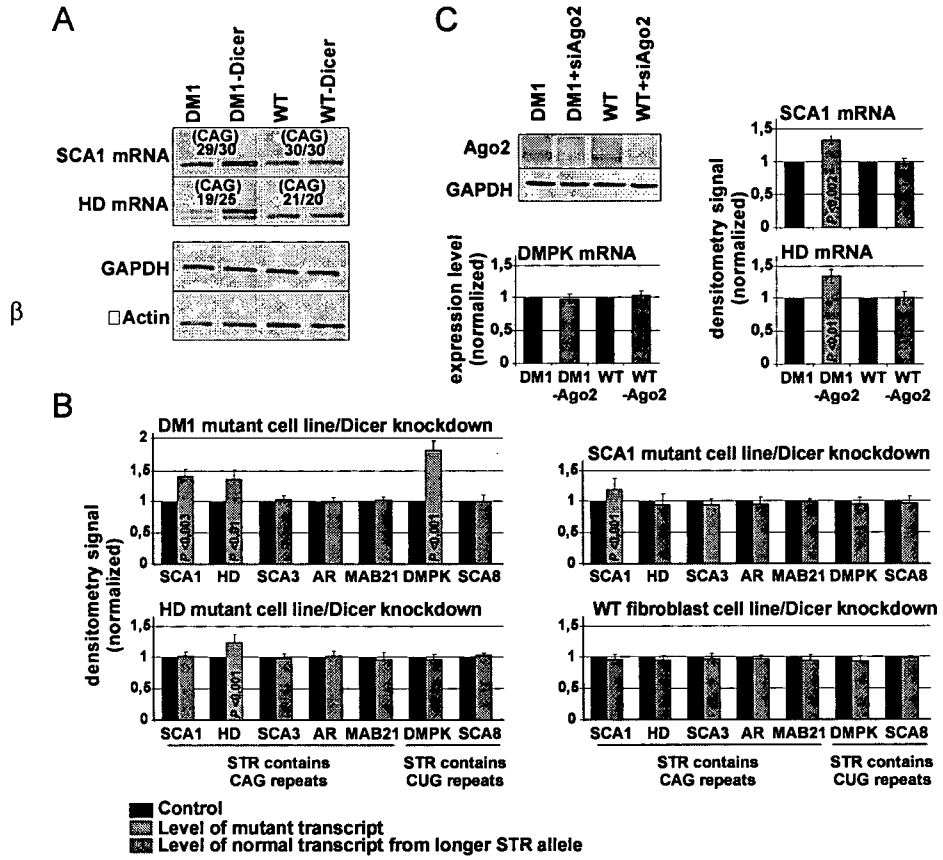
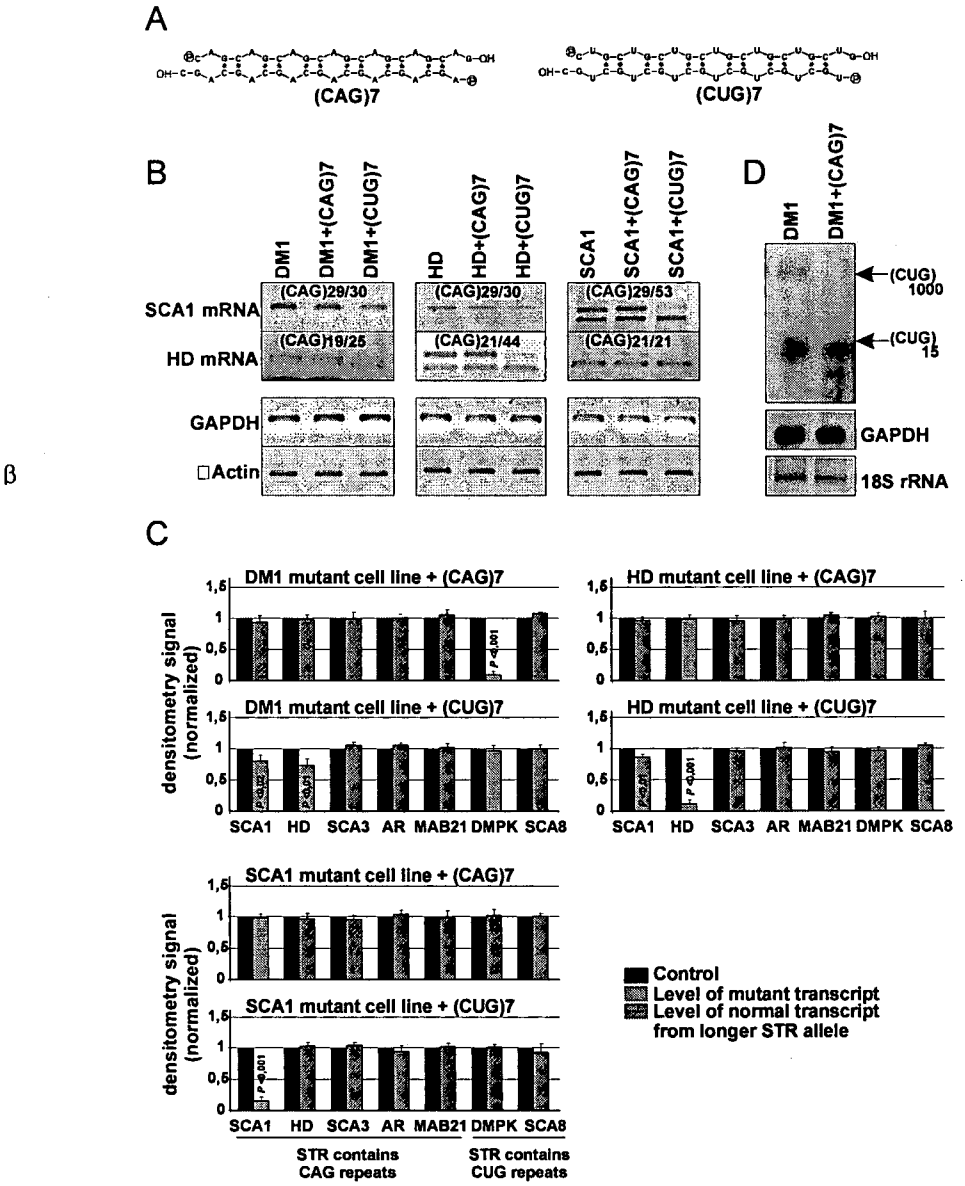

A
DM1
DM1-Dicer
WT
WT-Dicer
SCA1 mRNA
(CAG) 29/30
(CAG) 30/30
HD mRNA
(CAG) 19/25
(CAG) 21/20
GAPDH
□Actin
β
C
DM1
DM1+siAgo2
WT
WT+siAgo2
Ago2
GAPDH
DMPK mRNA
expression level (normalized)
DM1 DM1 -Ago2 WT WT -Ago2
SCA1 mRNA
HD mRNA
densitometry signal (normalized)
B
DM1 mutant cell line/Dicer knockdown
SCA1 mutant cell line/Dicer knockdown
HD mutant cell line/Dicer knockdown
WT fibroblast cell line/Dicer knockdown
densitometry signal (normalized)
SCA1 HD SCA3 AR MAB21 DMPK SCA8
STR contains CAG repeats
STR contains CUG repeats
Control
Level of mutant transcript
Level of normal transcript from longer STR allele

Fig. 4


A
(CAG)7
(CUG)7
B
DM1
DM1+(CAG)7
DM1+(CUG)7
HD
HD+(CAG)7
HD+(CUG)7
SCA1
SCA1+(CAG)7
SCA1+(CUG)7
SCA1 mRNA
HD mRNA
(CAG)29/30
(CAG)19/25
(CAG)29/30
(CAG)21/44
(CAG)29/53
(CAG)21/21
GAPDH
□Actin
D
DM1
DM1+(CAG)7
(CUG) 1000
(CUG) 15
GAPDH
18S rRNA
C
DM1 mutant cell line + (CAG)7
HD mutant cell line + (CAG)7
DM1 mutant cell line + (CUG)7
HD mutant cell line + (CUG)7
densitometry signal (normalized)
1,5
1
0,5
0
SCA1 HD SCA3 AR MAB21 DMPK SCA8
SCA1 mutant cell line + (CAG)7
SCA1 mutant cell line + (CUG)7
Control
Level of mutant transcript
Level of normal transcript from longer STR allele
STR contains CAG repeats
STR contains CUG repeats

β

Fig. 5

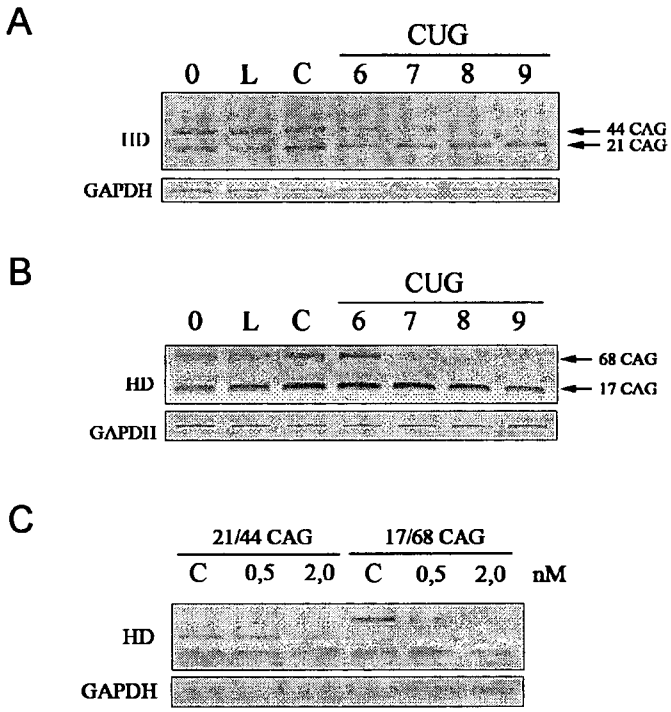

A
CUG
0 L C 6 7 8 9
HD
44 CAG
21 CAG
GAPDH
B
CUG
0 L C 6 7 8 9
HD
68 CAG
17 CAG
GAPDH
C
21/44 CAG
17/68 CAG
C 0,5 2,0 C 0,5 2,0 nM
HD
GAPDH

Fig. 6

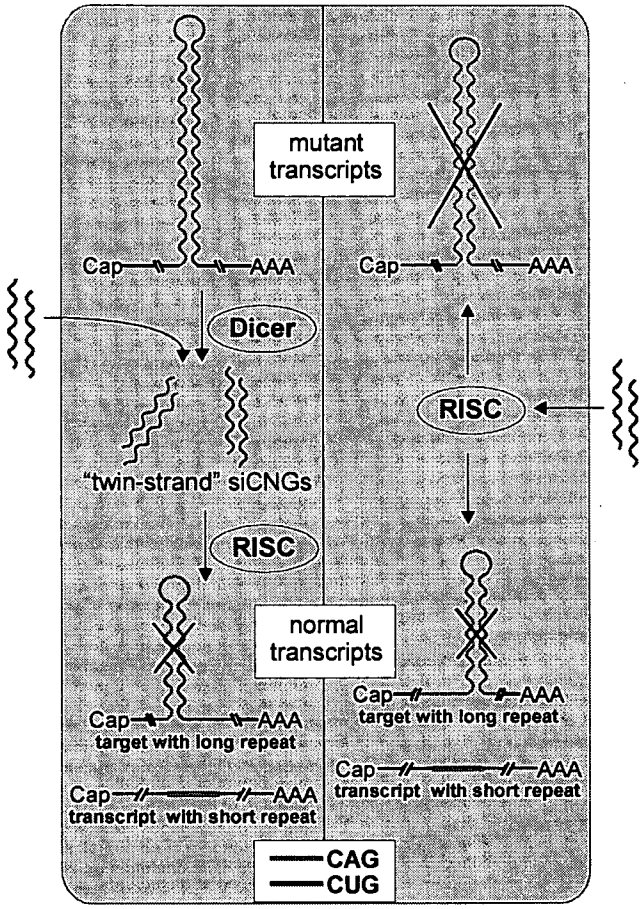

mutant transcripts
Cap
AAA
Dicer
"twin-strand" siCNGs
RISC
normal transcripts
target with long repeat
transcript with short repeat
CAG
CUG

Fig. 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/PL2008/000017

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/11 A61K31/713

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)
C12N A61K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FURLING D. ET AL.: "Viral vector producing antisense RNA restores myotonic dystrophy myoblast functions" GENE THERAPY, vol. 10, no. 9, May 2003 (2003-05), pages 795-802, XP002484831 the whole document	
A	XIA H. ET AL.: "RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia" NATURE MEDICINE, vol. 10, no. 8, August 2004 (2004-08), pages 816-820, XP002484832 plus five pages of Supplementary Figures and Legends	

-/--

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document 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
18 June 2008	04/07/2008
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Authorized officer Macchia, Giovanni

INTERNATIONAL SEARCH REPORT

International application No
PCT/PL2008/000017

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HARPER S.Q. ET AL.: "RNA interference improves motor and neuropathological abnormalities in a Huntington's disease mouse model" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 102, no. 16, 19 April 2005 (2005-04-19), pages 5820-5825, XP002484833	
A	CAPLEN N.J.: "RNAi quashes polyQ" NATURE MEDICINE, vol. 10, no. 8, August 2004 (2004-08), pages 775-776, XP002484834	
A	BONINI N.M. ET AL.: "Silencing polyglutamine degeneration with RNAi" NEURON, vol. 48, 8 December 2005 (2005-12-08), pages 715-718, XP002484835	
X	LI Y. ET AL.: "Sequence-dependent and independent inhibition specific for mutant Ataxin-3 by small interfering RNA" ANNALS OF NEUROLOGY, vol. 56, 2004, pages 124-129, XP002484836 the whole document	8
X	CAPLEN N.J. ET AL.: "Rescue of polyglutamine-mediated cytotoxicity by double-stranded RNA-mediated RNA interference" HUMAN MOLECULAR GENETICS, vol. 11, no. 2, 2002, pages 175-184, XP002484837 the whole document	1-6,8
X	MILLER V.M. ET AL.: "Allele-specific silencing of dominant disease genes" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 100, no. 12, 10 June 2003 (2003-06-10), pages 7195-7200, XP002484838 plus one page of Data Supplement the whole document	8
A	MALININA L.: "Possible involvement of the RNAi pathway in trinucleotide repeat expansion diseases" JOURNAL OF BIOMOLECULAR STRUCTURE AND DYNAMICS, vol. 23, no. 3, 2005, pages 233-235, XP002484839 the whole document	

-/--

INTERNATIONAL SEARCH REPORT

International application No
PCT/PL2008/000017

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SASAGAWA N. ET AL.: "An expanded CTG trinucleotide repeat causes trans RNA interference: a new hypothesis for the pathogenesis of Myotonic Dystrophy" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 264, 1999, pages 76-80, XP002484840 the whole document	1,2,4-6
X	JASINSKA A. ET AL.: "Structures of trinucleotide repeats in human transcripts and their functional implications" NUCLEIC ACIDS RESEARCH, vol. 31, no. 19, 2003, pages 5463-5468, XP002484841 the whole document	7
X	MITAS M.: "Trinucleotide repeats associated with human disease" NUCLEIC ACIDS RESEARCH, vol. 25, no. 12, 1997, pages 2245-2253, XP002484842 plus 26 paqges of Supplementary Material the whole document	7
P,X	KROL J. ET AL.: "Ribonuclease Dicer cleaves triplet repeat hairpins into shorter repeats that silence specific targets" MOLECULAR CELL, vol. 25, 22 February 2007 (2007-02-22), pages 575-586, XP002484844 the whole document	1-15
A	WO 2005/027980 A (UNIVERSITY OF MASSACHUSETTS [US]; ARONIN N.; ZAMORE P.D.) 31 March 2005 (2005-03-31) page 2 - page 3 the whole document	
X	SOBCZAK K. ET AL.: "RNA structure of trinucleotide repeats associated with human neurological diseases" NUCLEIC ACIDS RESEARCH, vol. 31, no. 19, 2003, pages 5469-5482, XP002484843 the whole document	7
A	AU 2004 239 114 A1 (JAPAN SCIENCE AND TECHNOLOGY AGENCY [JP]) 25 November 2004 (2004-11-25) the whole document page 19	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/PL2008/000017

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. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 10-15 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

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:

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

2. [ ] As all searchable claims could be searched without effort justifying an 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 reportcovers 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.:

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

Information on patent family members

International application No
PCT/PL2008/000017

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2005027980 A	31-03-2005	CA 2579638 A1	31-03-2005
		EP 1670518 A1	21-06-2006
AU 2004239114 A1	25-11-2004	CA 2526893 A1	25-11-2004
		WO 2004101787 A1	25-11-2004
		US 2008015158 A1	17-01-2008